FACTORS INFLUENCING THE THROMBOGENICITY OF INJURED RABBIT AORTAE

BY

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ABSTRACT

The interaction of platelets with damaged vessel walls plays a role in the development of atherosclerosis and its thromboembolic complications. The platelets that adhere to injured vessels release a growth factor that promotes the migration of medial smooth muscle cells through the internal elastic lamina into the subendothelial connective tissue where they proliferate and form a neointima. If a vessel is exposed to repeated or continuous injury the process continues and ultimately a frank atherosclerotic plaque develops. A plaque contains not only smooth muscle cells, but large quantities of intracellular and extracellular lipid and cholesterol. These plaques can lead to disturbed flow, and the development of thrombi, particularly in regions where abnormal flow patterns caused further endothelial cell injury or desquamation. The purpose of the work reported in this thesis was to examine the factors influencing the response of vessels to injury, since our understanding of these should contribute to our knowledge concerning the processes involved in atherosclerosis and its complications. Previous studies have examined the responsiveness of normal vessels of young animals to a single injury and may therefore have little relevance to injury of previously damaged or diseased vessels. The present experiments were therefore designed to

produce conditions likely to be found in older vessels that have been exposed to previous injury. In these experiments rabbit aortae were de-endothelialized with a balloon catheter and a smooth muscle cell-rich neointima was allowed to develop over a 7 day period. The response of this surface to a second injury with a balloon catheter was examined and compared with the response of normal vessels to a single injury. The initial response to a second injury was very different from the response to a single injury. Whereas a monolayer of platelets accumulates after de-endothelialization, following injury of a smooth muscle cell-rich neointima, large platelet-fibrin thrombi form on the injured surface. The platelet-fibrin thrombi are largely oriented in the direction of blood flow. Despite the fact that coagulation is activated (the thrombin that is generated leads to fibrin formation) the number of platelets (measured using platelets prelabelled with ^{51}Chromium) that accumulates on the injured aortic surface is similar to the number that accumulates on a de-endothelialized aorta, and the surface that is initially very reactive to circulating platelets rapidly loses its ability to attract fresh platelets. The reasons for the non-reactivity of normal endothelium and loss of reactivity of both de-endothelialized vessels and vessels subjected to a second injury were explored. Although it has been speculated that the PGI_2 produced by normal vessels or

injured vessels could account for the lack of interaction of platelets with these surfaces, this did not seem to be so, since inhibition of PGI_2 production by treatment of vessels in vitro or in vivo with aspirin did not lead to platelet accumulation on undamaged vessels or enhance platelet accumulation on injured vessels. Similarily, products of the lipoxygenase pathway that have been implicated as responsible for the non-thrombogenicity of vessels did not appear to be responsible for the lack of accumulation of platelets on these surfaces, since inhibitors of lipoxygenase such as ETYA(15-hydroxy-5,8,11,13-eicososatetraenoic acid) or NDGA(nordihydroguiaretic acid) did not influence the number of platelets that accumulated on the vessels under the conditions tested. To determine if factors other than platelets could account for the lack of vessel wall reactivity, platelet accumulation in vivo on de-endothelialized vessels or on vessels with an injured neointima was prevented by treating animals with injections of dipyridamole or with constant infusions of PGI_2. It was observed that 6 to 8 hours of these treatments are required for the vessels to become non-reactive; shorter durations of treatment do not allow the surface to develop its non-thrombogenic properties. Thus, the loss of reactivity of injured vessels do not require platelet adherence to the injured surface. In addition, it can be inferred that plasma factors or material from circu-

lating red blood cells does not contribute to this effect. It may be that the loss of reactivity that develops over a 6 to 8 hour period may be attributable to a factor(s) elaborated by injured vessels and this is an avenue worthy of exploration.

Several general conclusions can be reached based on the observations in this study. First, since injured vessels rapidly lose their reactivity to circulating platelets, repeated or continuous vessel injury is probably required for the development of severe arterial disease. Second, since activation of coagulation plays a major role in the response to injury of previously injured vessels, treatment with anticoagulants could be useful therapy in some forms of arterial thrombosis. Finally, since injured vessels lose their reactivity to platelets even when the initial interaction of platelets with the injury site is prevented, it may prove useful to administer drugs that inhibit platelet adherence to damaged vessels for short periods only, for example, after coronary bypass surgery or transluminal angioplasty. This would allow "passivation" of the surface, prevent the effects on smooth muscle cell proliferation of growth factors released from adherent platelets, and reduce the vessel wall thickening that could compromise flow to the tissues.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The endothelial cells lining vessel walls form a non-thrombogenic surface. Platelets do not adhere to normal, intact endothelium (Baumgartner et al., 1971; Stemerman et al., 1971), and coagulation factors are not activated when they come into contact with the surface of endothelial cells. However, the subendothelium exposed by vascular injury is highly reactive to circulating platelets which rapidly adhere to and spread on the subendothelial connective tissue. The adherent platelets form a non-reactive surface on the vessel and there is little further platelet accumulation. Although the surface exposed by the loss of many of the adherent platelets by 2 days following the removal of the endothelium appears morphologically similar to the subendothelium, this surface is non-reactive to platelets.

Among the granule contents released by the platelets that adhere to a damaged vessel is a mitogen that stimulates vascular smooth muscle cells to migrate and proliferate above the internal elastic lamina and form a thickened neointima (Ross and Glomset, 1976). The intimal thickening decreases lumen size and reduces blood flow through a vessel.

Repeated injury to the surface of large vessels leads to the development of atherosclerotic plaques (Moore, 1975). The surface of a diseased vessel can be highly thrombogenic. Mural thrombi composed of platelets and fibrin form on the

surface of diseased vessels, particularly on the surface of ruptured plaques and in areas of disturbed flow, such as at sites of stenosis. These thrombi can become dislodged and embolize in the distal circulation producing tissue ischemia and infarction. Thus, platelet response to vascular injury contributes to intimal thickening and the development of atherosclerosis, and platelet interaction with diseased vessels is involved in the formation of mural thrombi and the resulting thromboembolic complications of atherosclerosis including myocardial infarction and strokes, major causes of morbidity and mortality.

Since fibrin forms on the surface of reinjured or diseased vessels, it appears that the properties of this surface must be different from those of the exposed subendothelium where there is little evidence of fibrin formation (Stemerman, 1973; Groves et al., 1979). Since platelets are stimulated by thrombin that is generated following the activation of coagulation and interact with polymerizing fibrin, it appears likely that the activation of coagulation following injury of a previously injured or diseased vessel would enhance platelet accumulation on the vessel surface. If coagulation continued to be activated by the injured vessel, the surface would likely remain reactive to platelets. Since I observed in previous studies that normal vessels lose their reactivity to platelets following a single injury under conditions where there is little evidence of fibrin formation

(Groves et al., 1979), and since the activation of coagulation at the surface of injured vessels might increase platelet accumulation on the surface and maintain the reactivity of the surface for platelets, in the present studies the response of platelets to injury of vessels that had been previously injured was examined. In addition, some of the factors that might modify or limit platelet interaction with the surface of a damaged or previously injured vessel are investigated.

In this introductory chapter I shall discuss the structure and properties of undamaged arterial walls and consider factors that could be responsible for their non-thrombogenic properties; the factors that can produce vascular injury and expose a surface that is reactive to platelets; the factors involved in platelet interaction with injured or diseased vessels; the interaction of platelets with activated components of coagulation; the response of vessels to injury; the development of atherosclerosis; and the properties of the atherosclerotic vessel wall. During this discussion, I shall consider factors that might decrease or inhibit platelet interaction with a vessel wall, and factors that might enhance the reactivity of a vessel and promote platelet accumulation on its surface. Since platelets respond to stimuli through several pathways, and since these pathways can be inhibited with drugs, the use of antiplatelet drugs to determine the role of pathways of

platelet activation in the response of platelets to vessel injury will also be discussed. Finally, the hypothesis of this thesis and the specific aims of these studies will be presented.

A. Vessel Wall

The arterial wall consists of three morphologically distinct layers (Cowdry, 1933). The intima, the innermost layer of the arterial wall, consists of endothelial cells lying on a basement membrane, subendothelial connective tissue composed of collagen fibers and elastin, and an internal elastic lamina composed of a fenestrated sheet of elastin fibers. Only occasional smooth muscle cells are present in the intima in young children, but the number of intimal smooth muscle cells gradually increases with age (Ross and Glomset, 1973). The middle layer of the arterial wall, the media, consists of smooth muscle cells and varying amounts of collagen, small elastin fibers and proteoglycans. Unlike the intima, the morphology of the media does not generally change significantly with age. The adventitia, or outer layer of the vessel wall is composed of fibroblasts, smooth muscle cells, collagen and proteoglycans, and the external elastic lamina, a sheet of elastic tissue that lies adjacent to the media.

1. Intima

The vascular endothelial cells form a non-thrombogenic lining and provide a barrier that restricts the penetration of blood constituents into the arterial wall. The normal aortic endothelium, viewed with the scanning electron microscope, has a smooth cell surface with slightly elevated perinuclear zones and barely visible cell borders (Haudenschild, 1980). Although some early studies reported that cytoplasmic bulging and villi could be observed on the surface of normal endothelial cells, these are now thought to be artifacts resulting from poor preservation during the preparation and fixation of the specimens (Clark and Glagov, 1976). Several investigators observed that vascular endothelial cells are elongated in the direction of blood flow (Silkworth and Stehbens, 1975; Langille and Adamson, 1981). When examined with the transmission electron microscope, aortic endothelial cells appear tightly adherent to a 'reticular' basement membrane. The cytoplasm of endothelial cells is rich in mitochondria and pinocytic vesicles, and contains a Golgi apparatus, many free ribosomes (Haudenschild, 1980), bundles of actin fibers that tend to become aligned in the direction of stress and blood flow (Wong and Pollard, 1983), and Weibel-Palade bodies, that are specific for endothelial cells (Weibel and Palade, 1964). Gap junctions that are implicated in cell-cell communication, are present between adjacent endothelial cells in normal

blood vessels (Simionescu et al., 1976; Huttner and Peters, 1978). The theory that communication between adjacent cells through gap junctions might be important in limiting endothelial cell proliferation (Spagnoli et al., 1982) is supported by the the observation that the gap junctions are lost during growth and repair following endothelial injury (Schwartz et al., 1975; Schwartz et al., 1978).

Although the tightly interdigitating junctions that link arterial endothelial cells preclude the penetration of molecules as small as horse radish peroxidase with a diameter of 40-50 nm (Schwartz and Benditt, 1972; Huttner et al., 1973), pinocytic vesicles can transport lipoproteins through endothelial cells (Stein and Stein, 1973; Stein and et al., 1973), or fuse to form transient channels that allow proteins to penetrate through the endothelium (Simionescu et al., 1975a; Simionescu et al., 1975b).

In certain areas such as at arterial branch points or in the aortic arch, the permeability of the endothelium to radiolabelled cholesterol or albumin-bound dyes is increased (Duncan et al., 1963; Packham et al., 1967; Bjorkerud and Bondjers, 1971; Somer and Schwartz, 1971; Fry, 1973; Jørgensen et al., 1974). The increased permeability in these areas could be due to the increased hemodynamic stress that might stimulate endothelial cells to enhance transendothelial transport, induce focal endothelial cell damage, or produce desquamation of the endothelial cells. Atherosclerotic

plaques tend to develop in areas of a vessel where endothelial permeability is increased. It appears likely that the increased permeability that facilitates the penetration and accumulation in the vessel wall of substances such as cholesterol, low density lipoproteins, or growth factor released from platelets or monocytes adherent to injured areas of a vessel, contributes to the development of atherosclerosis in these regions.

a) Thromboresistance of endothelium

The formation of thrombi on a vessel wall can narrow or occlude a vessel lumen. Thus, for blood to flow unobstructed through the vasculature, the endothelium must remain non-reactive to blood components.

i) Resistance of endothelium to the interaction of platelets

Although platelets do not accumulate on the normal, undamaged endothelium of intact blood vessels (Baumgartner et al.,1971; Stemerman et al., 1971), isolated segments of vessel with intact endothelium (Baumgartner, 1973; Packham et al., 1978) or cultured endothelial cells (Booyse et al., 1975; Weckezak et al., 1975; Czervionke et al., 1978; Zetter et al., 1978), the mechanisms responsible for preventing platelet interaction with endothelial cells are unclear.

It has been suggested that the non-thrombogenic

properties of the normal vessel wall might be dependent on the production by the vessel wall of metabolites of the arachidonic acid pathway including PGI_2 (Moncada and Vane, 1979a), and products of the lipoxygenase pathway (Buchanan et al., 1983a).

PGI_2

Endothelial cells produce PGI_2 (prostacyclin) (Gimbrone and Alexander, 1975; Moncada et al., 1976; Weksler et al., 1977; MacIntyre et al., 1978; Nordoy et al., 1978; Baenziger et al., 1979). Production of PGI_2 by stimulated endothelial cells (approximately 20 ng/mg dry weight) is much greater per mg of tissue than the production by the remainder of the vessel under similar conditions (approximately 2 ng/mg of dry weight). Since PGI_2 is an extremely potent inhibitor of platelet function, Moncada and Vane (1979b) proposed that PGI_2 produced by endothelial cells is responsible for the non-thrombogenicity of the undamaged vascular surface.

For this to be so, one would have to assume that the normal endothelium was continually stimulated since the stimulation of cells is required to activate the phospholipases to free arachidonic acid for the generation of PGI_2. Under the conditions of laminar flow that are found in normal arteries, the concentration of agents that are required to stimulate endothelial cells would not likely be available, therefore, it appears that pulsatile flow would be the prim-

ary source of stimulation for the endothelium. Since pulsatile flow would not appear to be a potent stimulus for endothelial cells, it is unlikely that PGI_2 could be generated by the endothelial cells in sufficient amounts to inhibit platelet interaction with the vessel wall in vivo. In addition, several investigators have shown that the concentration of PGI_2 in the circulating blood is much less than that required to inhibit platelet function (Haslam and McClenaghan, 1981; Dollery et al., 1983). Therefore, it appears unlikely that PGI_2 produced by the vessel wall is responsible for preventing the accumulation of platelets on normal vascular endothelium. However, if PGI_2 production by the vessel wall is not responsible for the non-thrombogenic properties of endothelium, then the inhibition of PGI_2 production should not allow platelet accumulation on the surface of the endothelial cells.

The effects of inhibiting vascular PGI_2 production on platelet accumulation or thrombus formation on endothelium both in vitro and in vivo has been investigated in a number of studies. Curwen and her colleagues (1980) examined the effect of endothelial cell PGI_2 production on platelet adhesion to cultured endothelial cells. Although they observed that platelet adhesion to virally-transformed endothelial cells, which had lost their capacity to generate PGI_2, was enhanced when compared to adhesion to normal endothelial cells, the adherence of platelets to the normal endothelial

cells was not enhanced by pretreatment of the cells with aspirin or indomethacin. Czervionke and his colleagues (1979) also observed that treatment with aspirin did not enhance platelet accumulation on endothelial cells in culture. Since cells must be stimulated to generate PGI_2, it appears unlikely that endothelial cells lining the normal vessel walls would continually be exposed to the levels of stimulation required to maintain the generation of sufficient PGI_2 to inhibit platelet accumulation on the endothelium. Thus, it appears unlikely that the resistance of non-transformed, cultured endothelial cells to the accumulation of platelets on their surface is due to PGI_2 generated by the cells.

Since, thrombin is capable of stimulating endothelial cells to generate PGI_2 (Weksler et al., 1978), and thrombin binds to endothelial cells (Hatton et al., 1980; Lollar and Owen, 1980; Shuman et al., 1980), it seemed reasonable to speculate that the binding of thrombin generated at sites of vascular injury to the surface of adjacent endothelial cells might stimulate these cells to generate PGI_2. Exposure of the aortic endothelium to thrombin in vivo results in damage to the endothelium and the formation of thrombi on the surface of the vessel (Lough and Moore, 1975). Since PGI_2 is a potent inhibitor of thrombin stimulated platelet aggregation and release (Moncada et al., 1976b), it is possible that the PGI_2 produced by stimulated endothelial cells with

thrombin bound to their surface might be important in preventing extensive platelet interaction at sites of injury, and limiting the extension of thrombi formed in response to local injury.

Products of the lipoxygenase pathway

Arachidonic acid freed from membrane phospholipids by the action of phospholipases can also be metabolized by the lipoxygenase pathway. Products of this pathway have been reported to inhibit thromboxane generation (Aharony et al., 1981) by inhibiting either cyclooxygenase (Siegel et al., 1979a) or thromboxane synthetase (Hammarstrom and Falardeau, 1977).

Buchanan and coworkers (1983b) reported that when PGI_2 generation is inhibited, the inhibition of lipoxygenase by ETYA (4,7,10,13-eicosatetraenoic acid) results in increased platelet accumulation on the surface of cultured endothelial cells. They suggest that a product of the lipoxygenase pathway, generated by endothelial cells, is responsible for limiting platelet accumulation on the endothelial surface.

If products of the lipoxygenase pathway are responsible for preventing platelet interaction with the normal vascular endothelium or for limiting platelet interaction with the surface of injured vessels, then inhibiting the generation of products of the lipoxygenase pathway should result in the accumulation of platelets on the vessel.

Other factors

It is possible that other factors are responsible for the non-thrombogenicity of normal vascular endothelium. The glycoproteins that cover the surface of the endothelial cells cells might be responsible for the non-thrombogenicity of normal endothelium. It is also possible that the endothelial surface is inert to platelet interaction, as suggested by several investigators (Ashford, 1969; Mills, 1972; Salzman, 1976). If this were so, then it would not be necessary for normal endothelium to generate inhibitors to prevent platelet accumulation on its surface.

ii) Resistance of endothelium to the activation of coagulation

Since platelets are activated by thrombin and interact with polymerizing fibrin, it appears likely that the activation of coagulation on a vessel wall would promote platelet accumulation. However, the surface of normal endothelium is resistant, not only to platelet accumulation, but also to the activation of coagulation and the accumulation of fibrin on its surface. A number of factors might contribute to the resistance of the endothelium to the activation of coagulation. a) Although there is experimental evidence that thrombin binds to endothelial cells (Hatton et al., 1980; Lollar and Owen, 1980), the cell surface contains heparan

sulfate which interacts with antithrombin III so that the antithrombin III can rapidly neutralize thrombin (Buonassisi, 1973; Busch et al., 1980). b) Thrombomodulin on the surface of endothelial cells binds to thrombin and enhances the thrombin activation of protein C (Owen and Esmon, 1981). Protein C inactivates coagulation factors Va (activated factor V) (Kisiel, et al., 1977; Walker, 1979) and VIIIa (activated factor VIII) (Vehar and Davie, 1980). c).A number of years ago it was reported that plasma stimulates endothelial cells to release a fibrin-dependent plasminogen activator (Astrup and Permin, 1947). More recently, other investigators have shown that protein C stimulates endothelial cells to release plasminogen activator (Radcliff and Heinz, 1978; Collen, 1980). Thus, the resistance of endothelium to the activation of coagulation and the formation of fibrin on its surface is dependent on the presence of glycosaminoglycans and thrombomodulin on the cell surface, and on the release of plasminogen activator by stimulated cells. These factors might also limit the extension onto the surface of adjacent endothelium of thrombi that form at sites of vascular injury.

B. Vessel Wall Injury

When vascular injury results in the loss of endothelium, platelets rapidly accumulate on the exposed subendothelial structures (Baumgartner et al., 1971; Mustard et al.,

1978; Groves et al., 1979). Adherent platelets release a mitogen that stimulates vascular smooth muscle cells to form a thickened neointima (Ross et al., 1974). Atherosclerotic plaques tend to develop in regions of stenosis or disturbed flow since, in these regions, there appears to be increased endothelial damage, which together with the altered flow conditions, promotes the accumulation of platelets on the vessel wall. When platelet interaction with the damaged vessels is inhibited by inducing thrombocytopenia before vascular injury, smooth muscle cell proliferation does not develop (Moore et al., 1976; Friedman, 1978). Thus, the interaction of platelets with damaged vessel walls contributes to the development of atherosclerosis.

The extent of intimal thickening that develops in response to endothelial injury has been examined by a number of investigators. Removal of the endothelium by a single injury with a balloon catheter leads to the development of fibro-musculo-elastic intimal plaques (Baumgartner, 1963; Stemerman and Ross, 1972). Other investigators report that only transient intimal thickening develops in response to mild injury that results in the loss of endothelium (Bondjers and Bjornheden, 1970). Repeated endothelial injury produced by injections of serum that is cytotoxic to rabbit lymphocytes results in the development of raised lipid-containing plaques that undergo rapid regression (Friedman et al., 1976). Similarily, lesions that form when catheters are

introduced into the aorta of rabbits rapidly regress when the catheters are removed (Friedman et al., 1976).

1. Risk factors for the development of atherosclerosis

The development of atherosclerosis in man is associated with a number of risk factor such as hypercholesterolemia, smoking, hypertension and diabetes. When these factors occur in combination, the risk of developing coronary heart disease is several fold greater than the sum of the individual risk factors (Keyes, 1975). Since vascular injury leads to the development of atherosclerosis, a number of these risk factors have been investigated to determine their potential for inducing endothelial injury. Trauma produced during vascular surgery or the insertion of indwelling catheters can also result in neointimal thickening and the formation of atherosclerotic plaques. The effects of these factors on endothelium have been investigated in a number of studies. In addition, several experimental methods have been used in animals to produce endothelial injury and expose the subendothelium in order to study platelet interaction with damaged vessels, or the vessel wall changes that occur in response to vascular injury.

a) Hyperlipidemia

Hypercholesterolemia is a well established risk factor for the development of atherosclerosis (Dawber, 1957;

Kritchevsky et al., 1971; Wissler and Vesselinovitch, 1973; Mahley et al., 1975). A number of other studies which showed that hypercholesterolemia accelerates the development of atherosclerosis have been reviewed by Vesselinovitch and Fischer-Dzoga (1981), and by Buchwald and his colleagues (1982). In 1965, Keyes and his colleagues demonstrated a highly significant relationship between high dietary lipid intake, a high level of serum cholesterol, and an increased risk of atherosclerosis.

Under experimental conditions, hypercholesterolemia has been shown to produce endothelial injury in several species. Dietary-induced hypercholesterolemia causes endothelial injury and loss (Harker et al., 1976; Ross and Harker, 1976) and intimal thickening in non-human primates (Armstrong et al., 1974), and produces atherosclerosis in swine (Rowsell et al., 1958) and monkeys (Malmaros 1969), and enhances the development of atherosclerosis in monkeys whose aortae are injured with a balloon catheter (Ross and Harker, 1976). Signs of endothelial injury and loss have been reported in swine fed diets supplemented with egg yolk which is a rich source of cholesterol (Nelson et al., 1976). Evidence of endothelial injury is observed as early as 2 to 3 weeks after the beginning of diets enriched with cholesterol (Shimamoto et al., 1971; Weber et al., 1974; Goode et al., 1977).

The relationship between high plasma levels of low

density lipoproteins and the development of atherosclerosis has been demonstrated in a number of studies (for review see Stamler, 1983). Since approximately 70 percent of circulating cholesterol is bound to low density lipoproteins, diets that lower plasma cholesterol also produce a corresponding decrease in low density lipoprotein and decrease the risk of coronary heart disease (Multiple Risk Factor Intervention Trial) (Caggiula et al., 1981).

b) Smoking

Smoking is a well established risk factor in coronary heart disease. Fuster and his colleagues (1979) demonstrated that smoking is associated with the development of coronary heart disease and a decrease in platelet survival. The observation that smoking is associated with a decrease in platelet survival confirms the results of earlier studies (Mustard and Murphy, 1963).

Ten years after the Surgeon General of the United States Public Health Service warned of the hazards of cigarette smoking (Report of the Advisory Committee to the Surgeon General of the Public Health Service, 1964), tobacco consumption had decreased by 22 per cent with a corresponding decrease in mortality associated with vascular disease (Vital Statistics of the United States, 1975). As a result of the report of the American Heart Association, there was also a decrease in the dietary consumption of eggs and animal fat,

and an increase in the consumption of poly-unsaturated fats. Therefore, it is difficult to determine the contribution of the individual factors to the decline in mortality (Walker, 1977).

Although cigarette smoking is reported to alter vascular endothelium, and is associated with platelet adherence to the aorta in areas of disturbed flow around the orifices of branch vessels (Pittilo et al., 1982), the agents responsible for these changes are not fully understood. It is possible that nicotine (Booyse et al., 1981), carbon monoxide (Hugod et al., 1978), tobacco antigen (Becker et al., 1976), or a combination of these factors is responsible for the endothelial injury associated with cigarette smoking.

c) Diabetes

The development of atherosclerosis is accelerated in diabetics. Platelets from diabetic patients are more sensitive to aggregation by various stimuli (Heath et al., 1971; O'Malley et al., 1975; Colwell et al., 1976; Halushka et al., 1977), and large thrombi are formed more quickly in blood from diabetics than in the blood of non-diabetic controls (Rathbone et al., 1970). It has been pointed out that the hypersensitivity of platelets to aggregating agents is associated with a number of conditions involved in vascular disease (Mustard and Packham, 1977). Halushka and his colleagues (1977) observed that thromboxane production is en-

hanced in platelets from diabetics. The amount of arachidonic acid in the membrane phospholipids of platelets from diabetic patients is increased (Morita et al., 1983). Gerrard and his colleagues (1980) demonstrated that the phospholipase activity of stimulated platelets from diabetics is increased when compared to the activity in controls, and when arachidonic acid is added to platelets from diabetic patients, they generate more thromboxane B_2 than the control platelets. These results indicate that the enhanced thromboxane production by diabetic platelets is not due solely to the increased activity of the membrane phospholipases.

The observation that the hypersensitivity of platelets from diabetics is lost when the blood glucose returns to normal (Brownlee and Cerami, 1981; McDonald et al., 1982) raises the possibility that the high concentrations of blood glucose are responsible for the increased sensitivity of the platelets. However, insulin therapy that reduces the concentration of blood glucose also normalizes the high plasma concentrations of lipids and cholesterol that are frequently associated with the high concentrations of blood glucose (McDonald et al., 1982; Tamborlane et al., 1979). Since the incorporation of cholesterol into platelet membranes increases platelet hypersensitivity (Shattil et al., 1975), it is possible that the effects on platelet membranes of increased concentrations of plasma cholesterol is responsible for the hypersensitivity of platelets from diabetics. Since

the hypersensitivity of platelets from diabetics is abolished by regulating blood sugar to within normal values, it appears that the increased thromboxane production by platelets from diabetics is due to the high concentration of plasma glucose or lipids, rather than secondary to vessel wall changes (Mustard and Packham, 1984).

Several changes in the properties of the vessel wall also occur in diabetics. Vessels from diabetic patients and rats generate less PGI_2 than vessels from normal controls (Gerrard et al., 1980; Colwell et al., 1983). Since injured endothelial cells release von Willebrand factor into the blood, the presence of increased amounts of von Willebrand factor in the blood of diabetics (Colwell et al., 1983) could indicate that the endothelium is exposed to repeated stimulation. It is possible that either high levels of blood glucose or the associated high levels of cholesterol could be responsible for stimulating the endothelial cells. Since vascular endothelial cells also lose their ability to generate PGI_2 when they are exposed to repeated stimulation (Weksler et al., 1978), it is possible that the continuous stimulation of the endothelial cells in diabetics could decrease the capacity of the cells to generate PGI_2. Since PGI_2 generated by the vessel wall might have a role in limiting platelet interaction with damaged or diseased vessels, a decrease in the capacity of the endothelial cells to generate PGI_2 could result in increased platelet accumu-

lation on injured vessels in diabetic patients. This could account in part for the increased thrombotic tendency in diabetics.

Since glycosylation of collagen occurs in diabetics, and glycosylated collagen is a more potent stimulator of platelet aggregation (Le Pape et al., 1983), the glycosylated collagen in the vessel wall could enhance platelet adhesion at sites of vessel wall injury, and increase the amount of platelet derived growth factor made available to stimulate smooth muscle cell proliferation and the development of atherosclerosis in these patients. Thus, in diabetics, it appears that the increased sensitivity of platelets results from changes in the concentrations of plasma glucose or lipids, whereas the development of atherosclerosis is enhanced by continuous endothelial injury and platelet interaction with damaged vessel walls that leads to enhanced thrombogenicity.

d) Immunological injury

Evidence presented by a number of investigators indicates that immunological endothelial injury could contribute to the development of atherosclerosis (Minick et al., 1966; Hardin et al., 1973; Minick and Murphy, 1973; Poston and Davies, 1974; Friedman et al., 1975; Cerilli et al., 1977). Antibodies to food or tobacco glycoprotein have been implicated in the development of atherosclerosis (Becker et

al., 1976). Atherosclerotic lesions have been produced in rabbit carotid arteries in response to repeated injections of serum that is cytotoxic to rabbit lymphocytes (Friedman et al., 1975). Platelets are reported to interact with antigen-antibody complexes (Humphrey and Jaques, 1955; Movat et al., 1965), and circulating IgG is bound to the vascular endothelium in individuals with endothelial injury in transplanted organs (Ibels et al., 1974; Minick et al., 1974; Porter et al., 1976). Knicker and Cochrane (1968) reported that, in rabbits with serum sickness, platelet aggregates produced by immune complexes accumulate in regions of disturbed flow. They showed that substances such as serotonin released from the platelets might contribute to the development of focal vasculitis; when severe this causes aortic endothelial injury and loss.

Recently Parbtani and his colleagues (1984) reported that when purified bovine serum albumin is injected into rabbits to produce serum sickness, circulating immune complexes form but these complexes do not appear to promote platelet accumulation on the vascular endothelium. They also found that many preparations of bovine serum albumin are contaminated with endotoxin, and raised the possibility that reports of endothelial injury following repeated injections of bovine serum albumin to produce serum sickness, might not be a result of immune-complex formation, but might result from the injury of the endothelium by the endotoxin itself.

e) Activated leukocytes

The development of atherosclerosis is rapidly accelerated in patients undergoing repeated hemodialysis (Linder et al., 1974; Linder and Curtis, 1974). The dialyzer membrane is reported to activate C5 (the fifth component of complement) which induces the formation of aggregates of leukocytes that embolize in the pulmonary vascular bed (Sacks et al., 1978). Oxygen radicals generated by the activated granulocytes appear to be responsible for the endothelial cell damage that occurs throughout the systemic circulation. They observed that the extent of endothelial injury is greatest when there is close approximation of the leukocytes. These events have not been seen in individuals who are deficient in C5 when they are subjected to repeated hemodialysis (Sacks et al., 1978; Skubitz and Craddock, 1981). Thus, the interaction of activated leukocytes might be responsible for inducing endothelial injury under some conditions.

f) Mechanical injury

Endothelial cells can be injured mechanically both in clinical as well as experimental settings. A balloon catheter, developed by Fogarty and his colleagues (1963) for removing thrombi or arterial emboli, has been used by a number of investigators to remove the vascular endothelium (Baumgartner and Spaet, 1970; Stemerman et al., 1971;

Christensen and Garbash, 1973; Schwartz et al.,1975; Christensen et al., 1979; Groves et al., 1979; Reidy et al., 1982). The subendothelium exposed by a balloon catheter has been used to study the kinetics of platelet interaction with damaged vessel walls (Stemerman et al., 1972; Baumgartner, 1973; Groves et al., 1979; for review see Mustard et al., 1978). Cazenave studied platelet adherence to the subendothelium of rabbit aortae everted on a rotating probe (Cazenave et al., 1975; Cazenave et al., 1978), whereas Baumgartner studied platelet adherence to damaged vessel walls in an annular perfusion chamber (Baumgartner et al., 1976). The morphometric quantitation of platelet accumulation on damaged vessel walls in vitro and in vivo is both time consuming and dependent on observations made on areas that might not be representative of accumulation on the entire vessel. However, the use of radiolabelled platelets facilitates the rapid quantitation of platelet accumulation on damaged vessels both in vitro and in vivo (Cazenave et al., 1978; Groves et al., 1979).

Indwelling aortic catheters have been used experimentally to produce continuous endothelial injury (Friedman and Byers, 1961; Moore et al., 1976; Buchanan et al., 1979; Meuleman et al., 1980). Continuous or repeated endothelial injury resulted in the development of thrombo-atherosclerotic lesions (Moore et al., 1976). Several clinical investigators have also reported that thrombo-

atherosclerotic lesions develop in neonates following the introduction of a catheter into the umbilical artery to obtain specimens for the measurement of blood gases (deSa and Moore, 1975; Tyson et al., 1976).

There are a number of reports of endothelial injury and arterial intimal thickening following vascular surgery (Bonchek 1980; Haudenschild et al., 1981). Vascular injury induced during surgery could expose subendothelial structures to circulating platelets. Growth factor released by adherent platelets could stimulate intimal hyperplasia. This might contribute to the high incidence of intimal thickening and restenosis of vessels following vascular surgery (Chesbro et al., 1982). Haudenschild (1981) recently demonstrated that the integrity of the endothelium of vessels used in vascular grafts could be maintained if the vessels are handled gently to avoid mechanical injury to the endothelium, and maintained in a suitable medium to avoid physiological injury to the endothelial cells.

g) Other factors producing endothelial injury

A number of other factors can produce endothelial injury. Continuous infusion of homocysteine has been shown to increase endothelial injury in vivo (Harker et al., 1976) and in vitro (Wall et al., 1980), and the development of atherosclerosis is greatly accelerated in individuals with homocystinemia (Harker et al., 1976; McCully, 1983). Other

factors reported to produce endothelial damage include: epinephrine (Shimamoto, 1963; Hoff and Gotlob, 1967), increased levels of bile salts in plasma (Gutstein and Parl, 1973), cadmium poisoning (Gabbiani et al., 1974), ascorbic acid deficiency (Gore et al., 1975), anoxia (Morrison et al., 1977), and radiation (DeGowen et al., 1974).

C. Platelet Interaction with Damaged Vessels

Warton-Jones (1851) recorded one of the earliest observations of platelet response to injury of a vessel wall. He observed that following injury to the vessels in the web of a frog's foot there appeared a "conglomeration of colourless corpuscles...held together, apparently, by coagulated fibrin". Following endothelial injury platelets rapidly adhere to several subendothelial connective tissue components including collagen (Kjaerheim and Hovig, 1962), basement membrane (Baumgartner et al., 1967; Tranzer et al., 1976) and the microfibrils around elastin (Stemerman et al., 1971). Collagen induces activation of the arachidonic acid pathway, release of granule contents, and platelet aggregation (Bounameaux, 1959; Hugues, 1960; Kjaerheim and Hovig, 1962; Zucker and Borelli, 1962; Hovig, 1963) and release of the platelet granule contents (Holmsen, 1965). In the presence of von Willebrand Factor, a microfibrillar extract of aorta also induces platelet aggregation (Fauvel et al., 1983), indicating that microfibrils might be involved in

producing platelet aggregates on exposed subendothelium. The results of the studies by Fauvel and colleagues (1983) also indicate that platelet contact might be reversible; platelets that have contacted but not spread on the surface may be dislodged and return to the circulation to be replaced by new platelets.

1. Types of collagen

There are at least five types of collagen, four of which are found in vascular tissue (Packham and Mustard, 1984a). Type I is found mainly in the adventitia, although lesser amounts are present in the media and subendothelium. Type III collagen occurs mainly in the media with lesser amounts in the subendothelium and adventitia. Type III collagen occurs in relatively large amounts in vessel walls and is associated with the elastic laminae of the media, while type I collagen is located in the spaces between laminae (Gay et al., 1975; McCullagh et al., 1980). Type III collagen, but type I is not present in the intima between the basement membrane and the internal elastic lamina in normal arteries (Gay et al., 1975). However, in diseased vessels considerable type I collagen is present in the thickened intima (Barnes, 1981) and atherosclerotic plaques (McCullagh and Balain, 1975).

Although platelets adhere to both type I and type III collagens, they do not adhere to type IV and type V collagens

found in basement membrane (Barnes, 1981), or to type V collagen, that occurs in media, subendothelium and adventitia (McCullagh et al., 1980; Packham and Mustard, 1984a). Type III collagen is reported to be considerably more active than type I in stimulating platelets (Balleisen et al., 1975; Barnes et al., 1976; Hugues et al., 1976; Santoro and Cunningham, 1977). However, the aggregatory activity of type I collagen is greatly enhanced when it is incubated under conditions that promote collagen fibrillogenesis (Barnes et al., 1976; Barnes 1981). A number of investigators have reported that only fibrillar collagen can induce platelet aggregation (Muggli and Baumgartner, 1973; Brass and Bensusan, 1974; Jaffe and Deykin, 1974). Since type I collagen in the fibrillar form was reported to be as reactive as type III, it appears that the fibrillar or quarternary structure of collagen determines its ability to activate platelets. Although it has been reported that preparations of type IV and type V collagens do not stimulate platelets, when these collagens are treated so that they form fibrils with a periodicity of 67 nm, (the same as the periodicity of type III collagen), both type IV and V collagen induce platelet aggregation (Barnes, 1981). It is not known whether this is the native form of these collagens. These findings indicate that the quarternary structure of collagen determines its ability to stimulate platelets. It is possible that the procedures used to extract collagen from tissue may

alter the properties of the collagen and its ability to stimulate platelets. Although platelets adhere to and spread on native collagen in subendothelial basement membrane of the vessel wall, they do not release their granule contents (Huang and Benditt, 1978). It has been suggested that platelets might adhere to 'short chain' collagen in basement membrane, but this collagen might not be capable of stimulating platelets to undergo the release of granule contents (Balleisen and Rauterberg, 1980).

Wilner and his colleagues (1971) showed that blocking the epsilon amino groups of lysine completely abolishes the capacity of collagen to stimulate platelet aggregation, indicating that lysine may be directly involved in the reactivity of collagen for platelets. In 1979 Bentz and his colleagues identified an amino acid sequence in type III collagen that interacts with a platelet receptor. This nine amino acid sequence contains three polar amino acids (2 lysine and 1 glutamic acid residues) (Fauvel et al., 1979). Thus, it appears that platelets interact with a specific region of the collagen molecule.

2. Platelet interaction with collagen

a) Identity of receptor

Although Jamieson and his colleagues suggested that platelet glycosyl transferase mediates platelet-collagen

interaction (Jamieson et al., 1971), Cazenave and his colleagues (1974a) demonstrated that glucosamine, UDP and UDPG have no effect on platelet adhesion to collagen-coated surfaces or on collagen-induced aggregation, indicating that glycosyl transferase is not the platelet receptor for collagen. Michaeli and Orloff (1976) subsequently proposed that the galactosyl residues are required for the formation of the quarternary structure of collagen that is required for platelets to interact with the collagen fibril.

In 1975 Nurden and Caen described a specific abnormality in one of the membrane glycoproteins of platelets from patients with Bernard-Soulier syndrome, an autosomal recessive disorder, characterized by moderate to severe thrombocytopenia, large platelets, a prolonged bleeding time (Bernard and Soulier, 1948; Hardisty 1977), and markedly decreased platelet adherence to the subendothelium (Weiss et al., 1974; Caen et al., 1976). Other investigators confirmed that a marked decrease in the concentration of glycoprotein Ib in the platelet membrane is characteristic of Bernard-Soulier disease (Jenkins et al., 1975; Jenkins et al., 1976; Jamieson et al., 1979; Nurden et al., 1981).

In several studies it was demonstrated that hydrolysis of glycoprotein Ib (Jenkins et al., 1976; Okamura and Jamieson, 1976; Nachman et al., 1977), or the proteolytic cleavage of glycoprotein Ib (Camberg and Hakomori, 1973) results in inhibition of ristocetin-induced agglutination.

Purified membrane glycoproteins (Okamura and Jamieson, 1976), or antibodies against glycoprotein Ib (Nachman et al., 1977) also inhibit ristocetin-induced agglutination. In 1981 Ruan and his colleagues demonstrated that the adhesion of normal platelets to subendothelium was prevented by monoclonal antibody to glycoprotein Ib.

The adherence to the subendothelium of Bernard-Soulier platelets, which lack glycoprotein Ib, is defective (Weiss et al., 1974; Nurden and Caen, 1975), whereas platelets from patients with Glanzmann's thrombasthenia, which have normal glycoprotein Ib, but abnormal glycoproteins IIb and IIIa, adhere normally (Weiss et al., 1974; Tschopp et al.,1975). It has also been shown that von Willebrand factor binds to glycoprotein Ib on the platelet membrane. These findings indicate that glycoprotein Ib might be involved in the adhesion of platelets to collagen. The adhesion of platelets at high shear rates is impaired when the platelets lack glycoprotein Ib, or when von Willebrand factor is defective or absent. Since von Willebrand factor binds to glycoprotien Ib on the platelets membrane, it appears that the binding of von Willebrand factor to glycoprotein Ib is involved in platelet adhesion to collagen under conditions of high shear stress. However, since Bernard-Soulier platelets aggregate normally in response to collagen (Howard et al., 1973; Triplett, 1978; Hardisty and Caen, 1981), it appears unlikely that glycoprotein Ib is the receptor for collagen on platelet membranes.

b) Role of cytoskeleton and contractile elements

When platelets adhere to collagen and other subendothelial constituents they change shape and extend pseudopodia or spread on the subendothelial surface. This process involves the cytoskeleton composed of microtubules, microfilaments and submembranous filaments (White et al., 1981). Microtubules are involved in maintaining the disc shape of platelets (Hovig, et al.,1968; Benhke, 1970; White, 1971) and the centralization of platelet organelles (White, 1968a). The submembranous filaments help to maintain platelet shape and play a role in pseudopod formation and platelet adhesion (Haslam, 1975). The contractile force is provided by the contraction of the microfilaments formed by actin and myosin (White, 1971).

The disruption of the cytoskeleton or the inhibition of actin-myosin interaction blocks platelet shape change, platelet adhesion and clot retraction. The cytochalasins B,D and E inhibit the polymerization of platelet actin (Fox and Phillips, 1981), prevent fibrinogen binding (Harfenist et al., 1981), inhibit release of granule contents (Haslam et al., 1975), inhibit platelet shape change (Shepro et al., 1970; White, 1971a; White, 1971b; Haslam, 1972) and inhibit platelet adherence to collagen-coated glass (Cazenave et al., 1974b). Although it has been reported that colchicine, which disrupts platelet microtubules, also inhibits platelet adher-

ence (Soppit and Mitchell, 1969; Cazenave et al., 1974b), platelet aggregation (Soppitt and Mitchell, 1969; White, 1969) and clot retraction (White, 1969; Chao et al., 1976), several investigators observed indications of platelet damage that might reduce the capacity of platelet treated with colchicine to respond to stimuli (White, 1969; Cazenave et al., 1974b). Thus, the ability of platelets to respond to stimuli, change shape, expose receptors, and adhere to surfaces is dependent on an intact cytoskeleton and functional contractile elements.

The contractile processes in platelets are dependent on the internal concentration of ionic calcium (Ca^{++}) required for the phosphorylation of myosin light chain (Feinstein and Walenga, 1981). A Calcium-dependent myosin light chain kinase is responsible for the transfer of the terminal phosphate of ATP to myosin light chain (Adelstein and Conti, 1975). The phosphorylation of the myosin light chain results in activation of the actin-stimulated ATPase activity and the development of tension between the filaments (Lebowitz and Cooke, 1978). An increase in platelet cAMP (cyclic AMP) promotes the sequestration of internal calcium, and thereby inhibits the interaction of the contractile proteins actin and myosin. Thus, agents that increase levels of cAMP by stimulating adenylate cyclase to increase synthesis, or agents that prevent the break down of cAMP by inhibiting cAMP phosphodiesterase should inhibit those processes

that are dependent on the availability of calcium including platelet adhesion to surfaces, platelet aggregation, and platelet release of granule contents.

Platelet adhesion is inhibited by agents that stimulate adenylate cyclase, such as PGE_1, PGI_2 (Bergstrom, 1967; Wolfe and Schulman, 1969; Cazenave et al., 1979a; Higgs et al., 1979) and adenosine (Cazenave et al., 1974b), and by phosphodiesterase inhibitors, such as the pyridimo-pyrimidine compounds (Mills and Smith, 1971; Vigdahl et al., 1971), and caffeine (Cazenave et al., 1974b). Since these and other agents that increase cAMP inhibit platelet adhesion to surfaces and platelet aggregation in vitro, they could be useful for preventing platelet accumulation on damaged vessels in vivo, provided it was possible to achieve sufficiently high concentrations that were without deleterious side effects.

3. Steps involved in platelet interaction with injured vessels

The interaction of platelets with damaged vessels has been divided into several stages including: platelet contact, platelet spreading, and thrombus formation (Turitto and Baumgartner, 1979). The rate of platelet accumulation on an injured vascular surface is dependent on the rate of platelet arrival at the vessel surface, and the rate of platelet-vessel wall interaction (Turitto and Baumgartner, 1979). In

blood, the rate of arrival of platelets at the surface of a vessel is dependent on the rate of flow (Tutitto et al., 1977). Turitto and his colleagues (1977) also demonstrated that, under the conditions of their experiments, thrombus formation was substantially dependent on the shear rate; no thrombi formed at shear rates below 200 sec^{-1} (reciprocal seconds), and transient thrombi formed at shear rates between 200-1,000 sec^{-1}. At extremely high shear rates (>1000 sec^{-1}), substantially greater than those that occur in large arteries, the size and rate of thrombus formation increases sharply with an increase in shear rates.

4. Factors involved in platelet adhesion

a) von Willebrand factor

Although the mechanisms involved in platelet adhesion to subendothelium are not clearly understood, it has been suggested that von Willebrand factor may have a role in this interaction. Von Willebrand factor is normally present in plasma (Cornu et al., 1963; Howard et al., 1971; Zimmerman et al., 1971), platelets (Howard et al., 1974), endothelium (Bloom et al., 1973) and exposed subendothelium (Sakariassen et al., 1979; Rand et al., 1980).

Von Willebrand factor, is a part of the factor VIII complex. VIII:vWf designates the component required for normal bleeding time, and VIII:Rcof designates ristocetin

cofactor activity. The other functionally distinct components of the factor VIII system are: factor VIII clot-promoting activity (VIII:C), and factor VIII antigen (VIIIR:AG) (Nilsson, 1978).

Nilsson and her colleagues (1959) were the first to identify the factor that was decreased or abnormal in von Willebrand disease. Patients with this disease have a prolonged bleeding time, and their platelets exhibit decreased ristocetin-induced platelet aggregation (Bloom, 1980), decreased adhesion to subendothelium under conditions of high shear (Tschopp et al., 1974; Baumgartner et al., 1977; Lusher and Barnhart, 1977; Barnhart, 1978; Barnhart and Chen, 1978; Wilkins, 1978; Sakariassen et al., 1979; Fuster et al., 1983), and decreased adhesion to glass bead columns (Borchgrevink, 1960; Salzman, 1963). Both defective adhesion and aggregation can be corrected by normal plasma.

i) Role of von Willebrand factor in platelet adhesion under conditions of high or low shear forces

Although a number of investigators have reported that von Willebrand factor is required for platelet adhesion to vessels, it is only required for normal platelet adhesion under conditions of high shear, in the range found in the micro circulation (>1000 sec^{-1}) (Turitto et al., 1982). This is compatible with the observation that the abnormal bleeding in von Willebrand's disease is associated with

injury to the microvascular bed which is exposed to conditions of high shear. In vitro, under conditions of high shear, antibody against von Willebrand factor decreases platelet adhesion to segments of vessel wall (Meyer and Baumgartner, 1983). Since von Willebrand factor enhances platelet adhesion to fibrillar collagen (Sakariassen et al., 1986) and platelet spreading on vessel walls (Bolhuis et al., 1981; Barnhart, 1978), it is possible that the decrease in platelet adhesion associated with a lack of von Willebrand factor might result from the loss of platelets that contact the surface but are more likely to be washed away under conditions of high shear stress because they are not spread.

Under conditions of lower shear stress (500-1000 sec^{-1}), such as those that occur in large arteries, platelet adhesion is not dependent on von Willebrand factor. In pigs with von Willebrand's disease, platelet adherence to the arterial subendothelium exposed by air drying (Fuster et al., 1983), or mechanical denudation (Reddick et al., 1982), is similar to the adherence in normal animals.

Although von Willebrand factor is present in vessel walls, and platelet adhesion is reported to be proportional to the binding of von Willebrand factor (Sakariassen et al., 1979), the relative importance of vessel wall bound and plasma von Willebrand factor in platelet adhesion to large vessels is unclear.

ii) Role of von Willebrand factor in platelet interaction with components of vessel walls

The role of von Willebrand factor in platelet interaction with the components of vessel walls were investigated in a number of studies. Von Willebrand factor adsorbs to collagen fibers (Santoro, 1983), particularly type III (Legrand et al., 1978). Bernard Soulier platelets, which lack glycoprotein Ib (Jenkins et al., 1975; Jenkins et al., 1976; Jamieson et al., 1979; Nurden et al., 1981) fail to bind von Willebrand factor (Kao et al., 1979), do not aggregate to ristocetin (Howard et al., 1973), and fail to adhere in normal numbers to surfaces (Weiss et al., 1974). Therefore, it appeared that glycoprotein Ib is a receptor for von Willebrand factor and is required for normal platelet adhesion under conditions of high shear.

When platelets from patients with Glanzmann's thrombasthenia, that lack the glycoprotein IIb/IIIa complex, are stimulated with thrombin, they bind only 20 per cent as much von Willebrand factor as normal platelets (Ruggeri et al., 1982), indicating that glycoprotein IIb/IIIa might also act as a receptor for von Willebrand factor. However, since platelets from individuals with Glanzmann's thrombasthenia adhere to subendothelium in normal numbers (Baumgartner et al., 1977), and little von Willebrand factor binds to glycoprotein IIb/IIIa in the presence of physiological concentrations of fibrinogen (Gralnick et al., 1984; Pietu et al.,

1984), it appears unlikely that glycoprotein IIb/IIIa is of major importance in binding von Willebrand factor in vivo.

Fauvel and her colleagues (1983) demonstrated that platelet aggregation induced by a non-collagenous extract of aorta consisting mainly of microfibrills was decreased by a deficiency of von Willebrand factor or an antibody against glycoprotein Ib. These findings indicate that von Willebrand factor might be important in platelet interaction with subendothelial connective constituents other than collagen.

iii) Role of von Willebrand factor in the development of atherosclerosis

Fuster and his colleagues reported that pigs which lack von Willebrand factor are resistant to the development of spontaneous atherosclerosis (Fuster et al., 1978a), or atherosclerosis induced by diets enriched with cholesterol (Fuster et al., 1978b), whereas animals heterozygous for von Willebrand disease are less resistant to the development of atherosclerosis than homozygotes (Fuster et al., 1979). Fuster and his associates (1979) reported that there are few vessel wall changes in segments of normal aortae transplanted into pigs with von Willebrand disease, whereas atherosclerotic plaques develop in segments of vessels transplanted into normal pigs. Other investigators have observed that, although atherosclerotic changes occur in pigs with von Willebrand disease, these changes develop more slowly than in

normal pigs (Griggs et al., 1981).

If von Willebrand factor is required for platelet adhesion to large arteries, it might be expected that a deficiency in von Willebrand factor would reduce platelet adhesion at sites of injury, and would likely decrease the amount of platelet derived growth factor made available to stimulate smooth muscle cell proliferation and the development of atherosclerotic plaques. However, as indicated previously it appears that pathways that do not require von Willebrand factor are responsible for platelet adhesion under conditions of low shear (Meyer and Baumgartner, 1983: Packham and Mustard, 1984). Since experimental studies show that normal numbers of platelets adhere to damaged walls of large arteries in vivo in animals with von Willebrand's disease (Reddick et al., 1982; Fuster et al., 1983), it is not surprising that individuals with von Willebrand's disease develop atherosclerosis and thrombosis in large arteries (Silwer et al., 1966; Kernoff et al., 1981). Thus, although von Willebrand factor may be involved in platelet adhesion to damaged vessels of small diameter such as those of the microcircualtion, it is not important in platelet adhesion to the damaged walls of large arteries.

b) Fibronectin

Other factors also appear to be involved in mediating platelet adhesion to collagen. Fibronectin, an adhesive

protein that binds to a number of substances including collagen, appears to have a role in cellular adhesion to collagen and cell spreading (Klebe, 1974). Although there is little fibronectin on the surface of unstimulated platelets (Ginsberg et al., 1980), fibronectin binds to the surface of platelets stimulated with thrombin (Ginsberg et al., 1979; Ginsberg et al., 1980). Fibronectin from several sources is available to platelets following vessel wall injury: alpha-granules of platelets contain fibronectin that can be released when platelets are stimulated (Zucker et al., 1979), plasma contains fibronectin (reviewed by Giddings et al., 1982), endothelial cells synthesize fibronectin (Hynes, 1979a), and fibronectin is present in basement membrane (Ginsberg et al., 1979). The properties and function of fibronectin are discussed in a review by Mosher (1980).

The observation that when washed platelets are allowed to interact with collagen and then sonicated, fibronectin remains associated with the collagen, led Bensusan and his colleagues (1978) to hypothesize that fibronectin is the receptor on the platelet membrane for collagen. However, this appears unlikely for the following reasons: 1) the region of the fibronectin molecule that binds to collagen is not involved in mediation of platelet adhesion (Hynes and Yamada, 1982), 2) since there is little fibronectin on the surface of unstimulated platelets, there would be few sites to interact with collagen during the initial interaction with

collagen (Hynes et al., 1978), and 3) antibodies against fibronectin do not inhibit platelet adhesion to collagen (Santoro and Cunningham, 1979; Sochynsky et al., 1980).

The effect of fibronectin on platelet adhesion to collagen has been examined in a number of studies. The observation that the preincubation of collagen with fibronectin inhibits platelet adhesion (Bensusan et al., 1978), could indicate either, that fibronectin is the receptor for collagen, or that fibronectin inhibits the interaction of platelets with collagen (Sochynsky et al., 1980; Moon and Kaplan, 1981). Other investigators reported that fibronectin inhibits platelet adhesion and spreading on collagen (Chazov et al., 1981). Thus, the role of fibronectin in platelet adhesion to collagen remains unclear. Although it appears unlikely that fibronectin is the receptor for collagen on the platelet membrane, it does appear to be involved in the spreading and attachment of platelets to collagen (Houdijk et al., 1985), and may have a role in platelet adhesion to other components of the vessel wall.

Since fibronectin binds to fibrinogen (Rouslahti and Vaheri, 1975), and is released from alpha granules (Zucker et al., 1979), and fibrinogen receptors appear on thrombin stimulated platelets (Bennett and Vilaire, 1979; Marguerie et al., 1979), it appears that fibronectin might be involved in binding of fibrinogen to platelets. However, fibronectin is reported to have no effect on ADP induced platelet aggrega-

tion (Zucker et al., 1979), or the binding of ^{125}I-fibrinogen to platelets stimulated with ADP, nor is there an increase in fibronectin binding to platelets following stimulation with ADP (Harfenist et al., 1980). Since fibronectin is released from platelets stimulated by thrombin, it is possible that fibronectin may be involved in platelet accumulation on diseased vessels where thrombin is generated and platelets accumulate in platelet-fibrin thrombi.

c) Thrombospondin

Thrombospondin is a lectin-like (Gartner et al., 1981) alpha-granule protein (Zucker et al., 1979) available on the surface of platelets that have undergone the release reaction (Gartner et al., 1980). Phillips and his colleagues (1980) demonstrated that thrombospondin is bound to the membrane surface of thrombin stimulated platelets and that its binding to the membrane is dependent on the presence of the divalent cations calcium and magnesium. Thrombospondin is also synthesized by endothelial cells (Mosher et al., 1982). Since thrombospondin binds to fibronectin (Lahav et al., 1982), it was suggested that it might be the receptor on the platelet surface for the fibronectin attached to collagen. However, since fibronectin binds in normal amounts to platelets from patients with gray platelet syndrome which lack both thrombospondin and fibrinogen (Ginsberg et al., 1983), it appears that thrombospondin is not essential for the binding of

fibronectin to platelets. Thus, the role of thrombospondin in platelet adhesion to collagen remains unclear.

It was demonstrated that thrombospondin, fibrinogen, and the glycoproteins IIb/IIIa co-localized on the surface of stimulated platelets (Plow et al., 1979; Asch et al., 1984). Asch and his colleagues suggested that the macromolecular assembly of these proteins plays an important role in platelet aggregation, and that thrombospondin may stabilize the binding of fibrinogen between the glycoprotein IIIa/IIb complex on adjacent platelets.

5. Conditions affecting platelet adhesion

A number of conditions that affect the rate and extent of platelet adhesion to surfaces have been studied in vitro.

a) Shear force

As previously mentioned when discussing the steps involved in platelet accumulation on injured vessels, platelet accumulation on surfaces increases with an increase in shear force (Turitto and Baumgartner, 1979). Cazenave and his colleagues (1973) studied the effect of shear force on platelet adherence to collagen-coated glass mounted on a rotating probe. In this system, flow conditions were described as laminar with circular streamlines (Couette flow) at rotations of 50 to 200 rpm, corresponding to shear rates of 10 to 40 sec^{-1} (Feuerstein et al., 1975). Following the

exposure of vessels mounted on a rotating probe to suspensions of radiolabelled platelets, the vessels were rinsed in Tyrode's solution containing EDTA to remove platelet aggregates associated with the surface. Therefore, the adherence of individual platelets and not the accumulation of platelet aggregates was measured. Under the conditions of this system, platelet adherence to surfaces increased with an increase in shear.

Goldsmith and Karino (1979) demonstrated that platelet accumulation on a vessel is influenced by the rate of transportation of the platelets to the vessel wall. The delivery of platelets to the wall is enhance along curved streamlines where there is flow separation. Consequently platelet deposition on a vessel wall is enhanced in regions of disturbed flow downstream from a stenosis, in curved segments of vessels, and at bifurcations. An increase in the shear rate at the vascular surface increases both contact and spreading of platelets on the subendothelium. They observed that, at very high shear rates, greater than 10,000 sec^{-1}, the number of platelets that were in contact with the surface of the vessel but not spread was decreased. However, the spreading of those platelets that were in contact with the surface was unaffected. A possible explanation for these observations could be that, platelets that were in contact with the surface but not spread were more readily dislodged by the high shear forces at the vessel surface, whereas spread

platelets were more firmly attached and therefore less vulnerable to being dislodged.

b) Hematocrit

The extent of platelet adherence to collagen-coated glass or to exposed subendothelium mounted on a rotating probe increases with an increase in hematocrit (Cazenave et al., 1973). Others have also reported that red blood cells increase platelet diffusion to the surface in a flowing system (Goldsmith, 1972; Feuerstein et al., 1975; Turitto and Baumgartner, 1975: Goldsmith and Karino, 1982). Goldsmith (1972, 1974) demonstrated that the red blood cells in a flowing system collide with and displace other red blood cells and platelets, thereby increasing the delivery of platelets to the wall and increasing the opportunity for them to interact with the vascular surface.

c) Protein concentration

The effect of albumin concentration on platelet adherence was also examined by Cazenave and his colleagues, (1973). When platelets are suspended in a medium containing 0.35 per cent albumin, adherence is greater than when they are suspended in a medium containing 4 per cent albumin. Several investigators reported that albumin decreases adhesion to artificial surfaces by coating the surfaces to which platelets adhere (Packham et al., 1969; Lyman et al.,

1971; Mason, 1972). It is also possible that the increased viscosity of the suspension with the higher concentration of albumin decreases the delivery of platelets to the surface. An increase in viscosity also increases the shear force which tends to displace particles from the surface of the vessel (Turitto, 1982).

d) Other factors affecting platelet adhesion

Cazenave and his colleagues (1978) also demonstrated that platelet adhesion increases with an increase in platelet count, or an increase in speed of rotation of the probe. Either of these factors increase the delivery of platelets to the surface, thereby increasing the frequency of platelet interaction with the vessel wall. Platelet adherence to collagen-coated glass or aortae mounted on a rotating probe is markedly decreased when citrate, EDTA or EGTA is added to, or calcium and magnesium are omitted from the suspending medium, demonstrating that platelet adhesion is dependent on the presence of the divalent cations, calcium and magnesium.

6. Release of granule contents from adherent platelets

a) The platelet release reaction

Platelets release ADP from their granules (Hovig et al., 1963) and generate thromboxane A_2 when they are exposed to connective tissue components of injured vessel walls

(Smith et al., 1974; Hamberg et al., 1975), or to suspensions of collagen (Bell et al., 1979; Rittenhouse-Simmons, 1979). However, since inhibition of the arachidonic acid pathway with aspirin does not inhibit the release of granule contents by platelets adherent to collagen, the release reaction in these platelets is not dependent on arachidonate metabolism (Kinlough-Rathbone et al., 1980).

The release of granule contents depends on an increase in the concentration of calcium ions in the platelet cytosol. It is possible that calcium is mobilized from either the dense tubular system or the surface connecting cannilicular system (Cutler et al., 1978; reviewed by Gerrard et al., 1981). When platelets are exposed to collagen, calcium-dependent protein kinases are activated (Haslam and Lyman, 1977; Haslam et al., 1979). One of the proteins phosphorylated is believed to be the myosin light chain. The phosphorylation of this myosin light chain is required so that actin can induce the activation of myosin ATPase activity (Hathaway and Adelstein, 1979). The phosphorylation and dephosphorylation of the myosin light chain are believed to regulate the interaction of the contractile proteins actin and myosin; consequently a decrease in the concentration of ionic calcium in the cytosol decreases the phosphorylation of the myosin light chain (Haslam et al., 1978).

Since an increase platelet cyclic AMP concentration promotes the sequestration of internal calcium, it is not

surprising that agents that increase cyclic AMP inhibit the platelet release reaction. Therefore, it is apparent that an increase in the concentration of ionic calcium in the cytosol is required for the release of platelet granule contents.

An intact cytoskeleton is also required for platelets to undergo the release reaction; the disruption of microtubules with colchicine inhibits calcium ionophore (A23187) induced release of granule contents from platelets in suspension, whereas stabilizing microtubules with D_2O overcomes the effects of colchicine (Israel and Karpatkin, 1980).

b) Types of platelet granules

There are at least four types of platelet storage granules including: dense granules, alpha-granules, and two types of lysosomal granules (Fukami and Salganicoff, 1977). The results of studies on the release kinetics of platelet granule contents suggest that release from lysosomes containing acid hydrolase and from other granules may be governed by different factors (Holmsen et al., 1979).

The constituents of the different platelet granules are discussed in an article by Kaplan (1981). Among the constituents of the amine storage granules are serotonin, histamine, calcium, ATP, ADP, and pyrophosphate. Platelet proteins that include platelet factor 4, beta-thromboglobulin and a growth factor that is mitogenic for smooth muscle

cells, as well as cationic proteins, fibrinogen, factor VIII-von Willebrand factor, fibronectin, and albumin are contained in the alpha-granules. Acid hydrolases stored in platelet lysosomal granules include beta-N-acetyl-glucosaminidase, beta-N-acetyl-galactosaminidase, beta-glucuronidase and beta-galactosidase. Several substances released during the platelet release reaction, have not been localized to specific granules. These include collagenase, proelastase and elastase, alpha 1-antitrypsin, alpha 2-macroglobulin, a bacteriacidal protein, factor V and antiurokinase. In 1971, Joist and his colleagues reported that antiplasmin was released from platelets during thrombin or collagen induced aggregation. Plow and Collen (1981) subsequently reported that the antiplasmin released from platelets was alpha-2-antiplasmin. Thus, substances released from platelet granules could stimulate other platelets, contribute to or inhibit the activation of coagulation, stimulate smooth muscle cell proliferation, or digest components of the vessel wall and alter its properties.

c) Platelet-specific granule proteins in the circulation

Platelets release the contents of their granules when they are stimulated by interaction with damaged vessel walls or by thrombin generated following the activation of coagulation. Therefore, it was considered possible that circulating blood levels of platelet granule proteins such as

platelet factor 4 or beta-thromboglobulin might be an index of platelet activation and release in vivo. Since platelet factor 4 is rapidly cleared from the circulation by binding to the endothelial cells (Busch et al., 1980), levels of beta-thromboglobulin provide a more reliable index of the release of platelet granule contents in vivo. Although the stimulation of platelets during sampling procedures could increase the levels of platelet factor 4 or beta-thromboglobulin, the simultaneous measurement of both of these platelet proteins could provide a means of differentiating between in vivo release and release that occurred due to the activation of platelets during sampling (Kaplan and Owen, 1981). The ratio of platelet factor 4 to beta-thromboglobulin would be lower in the plasma than in the platelets when platelets release the contents of their granules in vivo, whereas the ratio of platelet factor 4 to beta-thromboglobulin in the plasma would be similar to the ratio of these proteins in the platelets if release occurred during blood sampling.

Although the results of some studies suggest that circulating levels of alpha-granule proteins are an indication of platelet activation in vivo in association with atherosclerotic disease, other studies show no correlation. A number of these studies are discussed in a paper by Stratton and his colleagues (1982), in which they report that there is no relationship between levels of either platelet

factor 4 or beta-thromboglobulin and myocardial ischemia during exercise.

Several of the substances released by stimulated platelets could affect the properties of the vessel wall. Enzymes could alter the surface of a damaged vessel, thromboxane A_2 could cause vasoconstriction, and platelet derived growth factor could stimulate smooth muscle cell proliferation. Evidence that materials released from platelets permeate the vessel wall is provided by a study of the penetration of platelet factor 4 into a de-endothelialized arterial wall (Goldberg and Stemerman, 1980). Although platelet factor 4 was present in the arterial wall 10 and 30 minutes following injury, by 4 hours following injury, platelet factor 4 could no longer be detected. Since platelet derived growth factor, as well as other platelet specific proteins are stored within the same granule as platelet factor 4, it appears likely that when these proteins are released from platelets, they also penetrate the vessel wall.

D. The Response of Vessels to Injury

1. Reactivity of injured vessel walls to platelets

Although platelets rapidly accumulate on the exposed subendothelium, few additional platelets accumulate on the injured vessel after the formation of the initial layer of adherent platelets (Groves et al., 1977; Groves et al.,

1979), indicating that the platelet-covered vascular surface rapidly loses its ability to interact with additional platelets. Two days following de-endothelialization, many of the adherent platelets are lost from the injured vessel leading to the exposure of a surface that is morphologically similar to the subendothelium; this surface is also non-reactive to circulating platelets. The smooth muscle cell-rich neointima that forms by 4 to 7 days following de-endothelialization is also non-reactive to platelets. Peipgras and his colleagues (1976) observed that following the inhibition of coagulation for 4 hours after carotid artery injury in cats, the vessels become non-reactive for circulating platelets, and fibrin does not accumulate on this surface. Other investigators demonstrated that platelet deposition on de-endothelialized rabbit aortae reaches an equilibrium by 2 hours following injury, and subsequently the number of platelets associated with the vessels is diminished (Spaet, et al., 1978; Drouet, et al., 1978). Thus, although the time required for the damaged vessels to lose their reactivity to circulating platelets varies when different procedures are used for inducing vascular injury, several investigators agree that the reactivity of an injured vascular surface to platelet accumulation decreases soon after mechanical removal of the endothelium.

However, the mechanisms responsible for this loss of reactivity are not known. Substances produced by the vessel

wall, the interaction of formed elements of the blood, or the interaction of components of the plasma could alter the properties of the surface so that its reactivity to platelets is lost.

a) Platelets

The layer of platelets that adheres to a damaged vessel forms a surface that is non-reactive to further platelet accumulation. The adherent platelets release the contents of their granules including proteolytic enzymes such as elastase (Kaplan, 1981); when these proteins are released from adherent platelets they could digest components at the surface of the vessel or penetrate the vessel and alter the properties of deeper structures. In addition, remnants of the platelet membranes might remain on the injured vessel when the adherent platelets are lost from the surface. Therefore, platelet interaction with damaged vessels could be responsible for the loss of reactivity of the surface soon after injury, and for the non-reactivity of the surface exposed by the loss of adherent platelets.

b) Leukocytes

Chemotactic factors generated following tissue injury and the activation of coagulation recruit leukocytes to sites of tissue injury (Snyderman et al., 1981). In monkeys fed cholesterol-enriched diets, macrophages accumulate on the

aorta, migrate between endothelial cells, and accumulate on or penetrate the vessel wall in the region of developing lesions (Gerrity et al., 1979; Gerrity, 1981a; Ross et al., 1984). Leukocytes contain numerous proteolytic enzymes and growth factors that could affect the properties of damaged vessel walls. Thus, the presence of leukocytes on the surface in some areas of injured vessels, and the potential of these cells to affect adjacent tissue suggests that leukocytes might be important in altering the properties of injured vessels, particularly in some regions. Since several aspects of leukocyte interaction with injured and diseased vessels, including quantity, localization, and duration of interaction, and chemical alterations in the vessel surface produced by leukocyte interaction, need to be investigated to determine the role of leukocytes in the loss of reactivity of injured vessels, this area of investigation was considered to be beyond the scope of this thesis. However, there is no doubt that this will be a fruitful area for future studies.

c) Red Blood Cells

The surface membranes of circulating red cells appear to be non-thrombogenic, since they do not activate coagulation and platelets in the circulation do not accumulate on their surface. The surface of the red blood cell membrane is composed of the exposed carbohydrate chains of glycoproteins and glycolipids (Gahmberg and Hakamori, 1973); the major

surface glycoprotein being glycophorin (Marchesi et al., 1972). The oligosaccharide chains of the glycoproteins and glycolipids contain numerous sialic acid residues (Zahler, 1968). Aminoff and his colleagues reported in 1976 that the sialic acid content of erythrocyte membranes decreases with age, and in 1978 they observed that a decrease in sialic acid content is associated with decreased erythrocyte survival; they concluded that sialic acid is lost from the surface of red cells as they age in the circulation. Since sialic acid-rich carbohydrates are exposed on the surface of red cell membranes, it appears possible that some surface components of the red cell membrane might be lost during their contact with the vascular surface. Thus, the accumulation of materials from red cell membranes might mask reactive sites on the damaged vessel wall and be responsible for its loss of reactivity.

d) Plasma Factors

A number of studies have been conducted over the past 20 years to examine the effect of plasma proteins on platelet adherence to surfaces. Most of these studies have been done in vitro and in general have shown that the reactivity of a variety of natural and prosthetic materials can be modified by plasma proteins (Salzman, 1971; Cazenave et al., 1977). The most common finding has been that albumin diminishes the ability of surfaces to attract platelets and it seems likely

that this plasma protein might also influence the reactivity of injured vessels in vivo. To determine whether the loss of reactivity of an injured vessels resulted from the adsorption of plasma proteins onto their surface, de-endothelialized vessels could be everted on a probe using the method developed by Cazenave and his colleagues (1977), and rotated in a medium containing plasma proteins before the adherence of platelets to their surface is tested.

e) Digestion by Proteolytic Enzymes Derived from Other Sources

Since platelets adhere to several components of subendothelial connective tissue including collagen (Baumgartner et al., 1977), basement membrane (Baumgartner et al., 1971), and the microfibrils around elastin (Stemerman et al., 1971), it appears likely that the digestion of these substances would decrease the reactivity of the surface. It is possible that proteolytic enzymes derived from sources other than platelets could alter the platelet-reactive sites of an injured vascular surface. Leukocytes contain numerous proteolytic enzymes that might alter the properties of an injured vessel in regions of leukocyte accumulation. In addition, the activation of plasminogen results in the formation of the proteolytic enzyme plasmin. Plasminogen can be activated 1) through the intrinsic pathway following its activation by factors derived from plasma including factor

XII, prekallikrein, or high molecular weight kininogen (Ratnoff, 1977; Ogston and Bennett, 1978), or 2) by tissue plasminogen activator derived from a number of different tissues including damaged endothelial cells (Astrup, 1966). There have been no systematic studies of the contribution of enzymes released or formed by the vessel wall on the reactivity of injured vessels.

f) Substances produced by the vessel wall

Although it appears unlikely that PGI_2 production by the endothelium is responsible for preventing platelet accumulation on undamaged vessel walls, and the capacity of vessel walls to generate PGI_2 is decreased following the removal of the endothelium, mechanical stimulation of the vascular cells during the passage of a balloon catheter, for example, could result in increased PGI_2 production by the vessel. However, whether PGI_2 production by vessel walls is sufficient to affect platelet accumulation on the surface of injured vessels is not clear.

Since substances such as glycosaminoglycans that are produced by cells of the vessel wall, inhibit platelet function (Kirk, 1975; Murata et al., 1975; Ts'ao et al., 1977) it is possible that the decrease in reactivity of an injured vessel might depend on substances produced by vascular cells in response to injury, or on substances present in the vessel wall that diffuse towards the injured surface and alter its

its properties. If substances generated by vessel walls are responsible for the loss of reactivity of an injured vessel, then such a vessel should gradually become less reactive to platelets and this could be tested in a number of ways.

2. Function of endothelial cells following vascular injury

Endothelial cells adjacent to sites of vascular injury generate substances that could affect platelet accumulation, thrombus formation, and the proliferative response of the injured vessel. PGI_2 produced by endothelial cells stimulated by injury or by thrombin generated at injury sites, might be capable of limiting platelet accumulation at the site of vascular injury. Plasminogen activator generated by endothelial cells could cause the formation of plasmin from its inactive precursor and initiate the lysis of fibrin that forms on an injured vessel. Thus, endothelial cells could be involved in limiting the extension of a thrombus, or in lysing thrombi that form in response to vascular injury.

Several investigators have reported that endothelial cells produce substances that affect the proliferation of vascular smooth muscle cells. Although it has been reported that endothelial cells produce a mitogen for smooth muscle cells (Gajdusek et al., 1980; Castellot et al., 1981), Karnovsky (1981) showed that endothelial cells also produce a heparin-like substance that inhibits smooth muscle cell growth. He speculated that the heparin-like compound or

similar compounds might be involved in the regulation of vascular smooth muscle cell growth in uninjured vessel walls. Thus, it appears that endothelial cells produce factors that could stimulate smooth muscle cell proliferation following vascular injury, and factors that could restrict smooth muscle cell growth in undamaged vessels.

3. Formation of neointima

As indicated previously, platelets release a factor from their alpha granules that is mitogenic and chemotactic for vascular smooth muscle cells (Ross et al., 1974; Ross and Vogel, 1978; Gerrard et al., 1980). The platelet-derived growth factor (PDGF), consists of two polypeptide chains joined by disulfide bonds and appears to exist in multiple forms (Raines and Ross, 1982). Binding of ^{125}I-labelled PDGF to arterial smooth muscle cells in culture is specific, reaches a peak at 30 to 60 minutes, and then declines, coincident with the appearence of trichloracetic acid-soluble ^{125}I in the medium, indicating that PDGF may be internalized and degraded by the cells (Bowen-Pope and Ross, 1982).

The observations by Moore and Friedman (1976) that smooth muscle cell proliferation in response to removal of the aortic endothelium in rabbits is inhibited by inducing thrombocytopenia before injury, whereas inducing thrombocytopenia 3 hours following injury fails to prevent intimal thickening (Friedman, 1978), indicates that the platelet

interaction with a damaged vessel wall that occurs soon after injury is important in the development of intimal hyperplasia. These results are confirmed by the findings that: a) released platelet granule proteins rapidly penetrate the vessel wall following injury, but are not found in the wall 2 to 4 hours following injury (Goldberg and Stemerman, 1980), b) PDGF is rapidly bound to vascular smooth muscle cells (Bowen-Pope and Ross, 1982), and c) exposure to PDGF for 60 minutes is sufficient to recruit 25 percent of smooth muscle cells in culture to synthesize DNA and undergo cell division (Ross et al., 1978).

Stemerman (1981) demonstrated that the incorporation of ^{3}H-thymidine into aortic smooth muscle cells is increased at sites of injury. The incorporation of thymidine into smooth muscle cells begins 24 hours following injury, reaches a peak by 2 to 4 days and returns to a base-line value by 4 weeks. He also found that ^{3}H-thymidine incorporation appears to be increased in other vessels that are remote from the injury site. For example, injury to the abdominal aorta results in increased DNA synthesis in smooth muscle cells in the uninjured thoracic aorta. These findings raise the possibility that some of the PDGF released at a site of injury escapes into the circulation and is capable of stimulating smooth muscle cell proliferation in regions remote from the site of release of platelet granule contents.

In damaged regions of a vessel, smooth muscle cells migrate through the internal elastic lamina and into the intima by 5 to 7 days following removal of the endothelium with a balloon catheter (Stemerman and Ross, 1972; Ross and Glomset, 1973; Groves et al., 1979). However, little intimal thickening occurs in regions of a vessel where injury produces medial necrosis (Moore et al., 1982). One to 3 months following injury, the intima reaches a maximum thickness of 5 to 15 cell layers. The number of intimal cell layers subsequently decreases until only 2 to 3 layers remains several months after injury (Ross and Glomset, 1973). Although a number of investigators have reported regression of atherosclerotic lesions induced by diets (Stary, 1979; Wagner et al., 1980; Clarkson et al., 1981; Manilow, 1981), or the combination of mechanical injury and diet (Daoud et al., 1981), the mechanisms responsible for the regression of the thickened neointima are poorly understood.

4. Development of atherosclerosis

There is considerable evidence that atherosclerosis can develop in response to vessel injury. This hypothesis, first proposed by von Rokitansky (1852), is supported by the results of a number of subsequent studies (Duguid and Robertson, 1957; Mustard, 1963: French, 1966; Mustard and Packham, 1975; Ross and Glomset, 1976). Smooth muscle cell-rich lesions with increased extracellular connective tissue, form in

response to several types of experimental injury induced by: indwelling cannulae in rabbits (Moore et al., 1975), other forms of mechanical injury (Bojkerud and Bondjers, 1971; Fishman et al., 1975; Mustard and Packham, 1975), hypercholesterolemia in rabbits (Kritchevsky et al., 1981), monkeys (Vesselinovitch et al., 1974), rats (Scott et al., 1964), and baboons (Harker et al., 1974), and by immune injury (Hardin, 1973; Minick and Murphy, 1973). In many cases these lesions are similar to the early lesions that develop in man. Repeated or continuous vessel injury results in repeated platelet interaction with damaged vessel walls and release of growth factor that stimulates further smooth muscle cell proliferation. The absence of endothelium in regions of vascular injury increases vessel wall permeability and allows substances such as plasma proteins and cholesterol to penetrate the vessel more easily than in areas with an intact endothelium.

The importance of platelet interaction in inducing smooth muscle cell proliferation and atherosclerotic plaque formation is illustrated in studies by Moore and his colleagues (1976). As previously discussed, they showed that the induction of thrombocytopenia in rabbits before introducing indwelling aortic cannulae prevented the development of lesions in response to injury. However, if thrombocytopenia was induced 3 hours after injury, the development of lesion was not inhibited (Friedman, 1978).

Atherosclerotic lesions primarily involve the intimal

layer (Ross and Glomset, 1976). The atherosclerotic lesions contain smooth muscle cells, some macrophages, lipids, and extracellular connective tissue components including collagen, elastin and proteoglycans. Fatty streaks are believed to represent the earliest stage of lesion development. They are ubiquitous in the population by the age of 10 years, and may be found in much younger children. By the age of 25 years, approximately 25 per cent of the aortic surface is covered with fatty streaks. Fibrous plaques are raised lesions of lipid-laden smooth muscle cells, collagen, elastic fibers and proteoglycans forming a fibrous cap overlying free extracellular lipid. Complicated lesions contain areas of calcification, necrotic cells and evidence of haemorrhage.

The formation of mural platelet-fibrin thrombi on the surface of ruptured atherosclerotic plaques, indicates that this surface is highly thrombogenic. Mural thrombi can narrow the lumen and reduce or obstruct blood flow, and fragments can be dislodged from the thrombus and embolize in the distal circulation. Thus, platelet interaction with damaged or diseased vessels is not only potentially important in the initiation and development of atherosclerotic lesions, but is also involved in the thromboembolic complications of atherosclerotic disease.

E. Platelets and Coagulation

As previously discussed, when platelets interact with

freshly injured vessels there is little or no evidence of fibrin formation (Stemerman et al., 1971; Clowes and Karnovsky, 1977; Groves et al., 1978; Groves et al., 1979). However, thrombi composed of platelets and fibrin form on the injured surface of a previously damaged or atherosclerotic vessels (Jørgensen et al., 1967; Woolf et al., 1968; Stemerman, 1973). The activation of coagulation on a reinjured or diseased vessel indicates that the surface of these vessels is more thrombogenic than the freshly exposed subendothelium. It is also possible that disturbed flow produced by vessel narrowing and raised lesions of an atherosclerotic vessel could promote the accumulation of activated coagulation factors; this could enhance the generation of thrombin, the formation of fibrin, and the incorporation of platelets into thrombi.

1. Activation of coagulation in response to vessel injury

Coagulation can be activated through two pathways. The exposure of factor XII (Hageman factor) to collagen activates the intrinsic pathway of coagulation (Ratnoff and Rosenblum, 1957), whereas tissue factor (tissue thromboplastin) activates the extrinsic pathway of coagulation (Howell, 1912; Nemerson and Bach, 1982).

It appears that thrombus formation on a damaged vessel can involve two pathways: a) platelet adherence to connective tissue components, or b) the activation of coagulation by the

injured surface. Since fibrin forms on reinjured or diseased vessels (Stemerman, 1973), and platelets are stimulated by thrombin and interact with fibrin during its polymerization (Niewarowski et al., 1972a), some of the platelet accumulation on the injured neointima might be dependent on the activation of coagulation. Therefore, it is important to determine a) the role that thrombin generation and fibrin formation play in the accumulation of platelets on an injured vessel, and b) the pathway of coagulation responsible for the formation of fibrin on a reinjured vessel.

a) Activation of the intrinsic pathway

Plasma factor XII is activated when it is exposed to collagen or other negatively charged surfaces (Ratnoff and Rosenblum, 1957). Since the neointima is rich in type III collagen produced by the vascular smooth muscle cells (Barnes et al., 1981), it is possible that collagen exposed by injury to the neointima might be responsible for activating factor XII of the intrinsic pathway of coagulation. Walsh and Griffin (1981) showed that platelets stimulated with collagen could promote the activation of factor XII in the presence of kallekrein and high molecular weight kininogen (HMWK). However, although the clotting time of blood from individuals deficient in factor XII, prekallikrein, or HMWK is prolonged, these patients do not have an increased tendency to bleed, (Nemerson and Bach, 1982). These findings indicate that

activation of the intrinsic pathway by activated factor XII might not be important in activation of coagulation in vivo.

b) Activation of the extrinsic pathway

The extrinsic pathway of coagulation is activated by a complex formed by tissue factor and factor VII. This complex catalyzes the activation of factor X (Williams and Norris, 1966). The tissue factor activity is localized to a polypeptide chain which must be complexed to a phospholipid in order for procoagulant activity to be expressed (Nemerson and Bach, 1982). In 1977, Osterud and Rapaport demonstrated that tissue factor-factor VII complex, in the presence of calcium, also activates factor IX. Activated factor IX (IXa), in the presence of factor VIII, activates factor X (Hougie et al., 1967; Macfarlane et al., 1964).

Although undamaged endothelial and vascular smooth muscle cells do not express tissue factor activity on their surface (Rodger, 1983), even mild injury stimulates vascular cells to make tissue factor activity available to activate the extrinsic pathway of coagulation (Nemerson and Bach, 1982). A number of vascular cells, including endothelial cells, smooth muscle cells, and fibroblasts express tissue factor activity when they are perturbed or injured (Maynard et al., 1977). Mild proteolytic digestion of the cell surface glycocalyx that does not kill the cell or produce leakage of the cytoplasmic constituents, results in the

expression of procoagulant activity by the cells (Maynard et al., 1975). Although these results indicate that tissue factor activity on the surface of these cells is present in a protected or cryptic form, it is also possible that the treatment might have stimulate the cells to produce tissue factor. The expression of procoagulant tissue factor activity by tumor cells in culture is reported to be associated with the production of plasma membrane vesicles (Dvorak et al., 1982). Although Neimetz (1972) reported that leukocytes stimulated with endotoxin release tissue factor into the suspending fluid, other investigators have since reported that monocytes only express tissue factor activity following disruption of the cells, indicating that the activity is masked within the cells (Levy et al., 1981). Therefore, damaged smooth muscle cells, as well as other vascular cells, might make tissue factor available to initiate coagulation on the surface of an injured vessel.

Since plasma is reported to contain a specific inhibitor of tissue factor activity (Nemerson and Bach, 1982), it is possible that this inhibitor might have a role in modifying the activation of coagulation by tissue factor made available by damaged cells.

2. Interaction of Platelets with Coagulation Factors

Thrombin, generated following the activation of coagulation, stimulates platelets to generate thromboxane A_2

(Holmsen, 1977; Bills et al., 1977) and release the contents of their granules (Grette, 1962; Davey and Luscher, 1968; Mustard and Packham, 1970; Kinlough-Rathbone et al., 1977; Packham et al., 1977). Thromboxane A_2 generated following activation of the arachidonic acid pathway, and ADP and serotonin released from platelet granules, could stimulate other platelets to release their granule contents.

Platelets also contribute to the activation of coagulation through several mechanisms (Reviewed by Walsh, 1981). a) Platelet factor 3, a phosphatidyl serine located on the inner surface of the membrane in unstimulated platelets but exposed on the membrane surface of stimulated platelets (Zwaal, 1978), is a negatively charged phospholipid that contributes to the activation of factor XII, factor XI (Walsh, 1972; Walsh and Griffin, 1981), factor X (Lundblad and Davie, 1964; Walsh and Briggs, 1972), and prothrombin (Miletich et al., 1974) (Reviewed by Hemker et al., 1983). Therefore, platelet factor 3 made available by platelets that have undergone the release reaction could further enhance the activation of coagulation and the generation of thrombin (Spaet and Cintron, 1965; Hardisty and Hutton 1966; Joist et al., 1974). b) Platelets release the coagulation proteins factor V, factor VII and fibrinogen (Kaplan et al., 1979); factor V released from platelet granules binds to the platelet membrane and acts as the platelet receptor for factor Xa (Miletich et al., 1977; Miletich et al., 1978a;

Miletich et al., 1978b). c) The platelet membrane provides a surface for the activation of factor XII, factor XI (Walsh and Griffin, 1972), factor X (Lundblad and Davie, 1964; Walsh and Briggs, 1972) and prothrombin (Miletich et al., 1977). d) The platelet membrane also affords protection to activated coagulation factor XIa (Walsh and Briggs, 1972) and factor Xa (Walsh and Briggs, 1972; Marciniak, 1973; Miletich et al., 1978a) against inactivation by plasma inhibitors.

Fibrin formed by the action of thrombin stabilizes platelet aggregates (Hovig, 1968). In the presence of thrombin platelets also interact with polymerizing fibrin; stimulation of the platelets that are adherent to fibrin results in clot retraction (Niewarowski, 1972b). Thus, platelets are not only affected directly by thrombin, but also contribute to the activation of coagulation.

Since platelets appear to accumulate on injured vessels in association with fibrin, the activation of coagulation on the surface of an injured vessel might be responsible for some of the platelet accumulation. If the injured surface continued to activate coagulation, the reactivity of the surface for platelets might be maintained. Thus, platelet accumulation on the injured neointima might be enhanced and the accumulation of platelets might continue for longer than on the subendothelium exposed by a single injury.

3. Effects of thrombosis on platelets

The exposure of platelets to substances generated at sites of thrombus formation could alter the properties of platelets and affect their function in several ways.

a) Platelet survival

Where there is intravascular thrombosis in diseased vessels, many of the circulating platelets can encounter factors that could alter their properties and increase their rate of clearance from the circulation by the reticuloendothelial system. The incorporation of platelets into thrombi can also decrease platelet survival.

The surface sialic acid of membrane glycoproteins is decreased as platelets age in vivo (Bocci, 1976; George et al., 1978; Rand et al., 1981; Rand et al., 1983). The removal of platelet membrane sialic acid in vitro by proteolytic enzymes such as neuraminidase, trypsin, chymotrypsin or plasmin increases their rate of clearance from the circulation and shortens the survival of platelets following their infusion into experimental animals (Greenberg et al., 1975). Therefore, it is possible that following in vivo exposure to proteolytic enzymes such as plasmin (Greenberg et al., 1979), which may be generated in regions of thrombus formation, the platelets are recognized as "foreign" by the reticuloendothelial system and are cleared more rapidly from the circulation than normal platelets.

Since the number of platelets that adhere to the subendothelium exposed following injury of the aorta with a balloon catheter represents less that one per cent of the circulation platelet population, and there is little further platelet interaction with the vessel following the initial platelet adhesion, (Groves et al., 1979), platelet survival is not shortened following a single injury to a blood vessel. However, Harker and his colleagues (1976) demonstrated that in experimentally-induced homocystinemia the extent to which platelet survival is decreased closely correlates with the extent of endothelial cell loss.

Platelet survival is decreased by indwelling aortic catheters in rats or rabbits. (Meuleman et al., 1980; Winocour et al.,1982; Winocour et al.,1983: Somers et al., 1983). Since greater numbers of platelets were incorporated into the thrombi that form in association with indwelling aortic catheters in rabbits than on the subendothelium exposed by a single injury with a balloon catheter (Groves et al., 1979; Somers et al., 1980), it is not surprising that platelet survival is shortened by indwelling catheters Buchanan et al. 1979; Somers et al., 1983; Meuleman et al., 1980; Winocour et al., 1982; Winocour et al., 1983). However, the incorporation of platelets into thrombi does not appear to be solely responsible for the shortened platelet survival under these conditions.

Since thrombi do not form in association with indwel-

ling aortic catheters in rats, it appears that the decrease in platelet survival in these animals is caused by repeated injury of the vessel wall caused by the catheter (Winocour et al., 1983). Thus, platelet survival can be decreased by the incorporation of platelets into thrombi or as a result of the increased turnover of platelets on the surface of a repeatedly injured vessel wall; the platelets that escape from the injured vessel wall are altered, recognized as 'foreign', and cleared from the circulation.

It is possible that the platelet membrane might be altered by exposure to substances such as plasmin encountered during formation of platelet-fibrin thrombi. Changes in the surface properties of the platelet membrane can increase the rate of platelet clearance from the circulation. The alteration of the platelet surface by removal of sialic acid by treatment with neuraminidase, or removal of sialoglycopeptides by treatment with plasmin accelerates the clearance of platelets from the circulation and shortens platelet survival (Greenberg, et al., 1979). However, platelets that have been exposed to repeated stimulation with thrombin to induce degranulation in vitro, survive normally when they are reinjected into rabbits (Reimers et al., 1976). Therefore, the exposure of platelets to thrombin does not produce membrane changes that accelerate their clearence from the circulation.

Although several investigators have observed that vascular disease is associated with a decrease in platelet

survival (Abrahamsen, 1968: Steele et al., 1978a; Steele et al., 1978b: Fuster et al., 1981), others have reported that platelet survival is unchanged in patients with atherosclerotic disease (Doyle et al., 1980). There are several possible explanations for the differences in findings. In patients with atherosclerosis, platelet survival may be shortened only during episodes when the surface is reactive to platelets (with or without thrombosis), whereas during periods when the surface of the vessel is relatively quiescent, platelet survival may be normal (Abrahamsen, 1968). This may explain Abrahamsen's observation that platelet survival is decreased shortly after myocardial infarction and returns to normal during the later stages of recovery. Thus, the results obtained could depend upon when platelet survival is measured in relation to the atherosclerotic vascular disease process. It is also posssible that other factors, such as smoking which can decrease platelet survival, could also contribute to shortened platelet survival in patients.

The values for platelet survival depend on the mathematical model used in their calculation, and on other factors such as the times at which samples were obtained. The earlier samples obtained for the determination of platelet survival may have a greater effect on the calculated values for survival. In 1977 the International Committee for Standardization in Hematology made a number of recommendations for standardizing platelet survival studies, including

the recommendation that the 'multiple hit model of gamma function' be used to calculate platelet survival (Murphy et al., 1973; Murphy and Bolling, 1978). Since the 'gamma function' is a computer analysis of a semicontinuous variable, and is sensitive to variations between logarithmic and linear curves for platelet survival, this method is more sensitive than either linear or logarithmic methods of calculating platelet survival. This point should be considered when comparing the values for platelet survival obtained by different investigators.

b) Platelet density and size

The normal circulating platelet population is heterogeneous with respect to size and density. Although some investigators conclude that the heterogeneity of the circulating platelets results from a decrease in platelet size and density as the platelets age (Karpatkin, 1978; Charmatz and Karpatkin, 1974; Corash et al., 1977; Corash et al., 1978; Rand et al., 1981), others propose that the heterogeneity results from the production by megakaryocytes of platelets of different size and density (Boneu et al., 1973; Paulus, 1975; Pennington et al., 1976; Boneu et al., 1977; Paulus et al., 1979).

Rand and her colleagues (1981) demonstrated that the size of the most dense rabbit platelets separated on Stractan density gradients is greater than the size of the least dense

platelets. Following the injection of ^{35}S into rabbits, the relative specific activity of the most dense platelets increases more rapidly than the specific activity of the least dense platelets. Rand and her colleagues also demonstrated that, when the platelets are returned to the circulation, the survival of the most dense platelets is greater than the survival of the least dense platelets. These results indicate that in rabbits the young platelets are the most dense.

The density of platelets is decreased following exposure to ADP (Packham et al., 1978), thrombin (Cieslar et al., 1979), or plasmin (Guccione et al., 1985), substances to which platelets may be exposed during the formation of thrombi. Several investigators have reported that a loss of alpha-granule contents is related to a decrease in platelet density (Vicic and Weiss, 1983; van Oost et al., 1984). Experimentally-induced thrombosis results in a shift of platelets from the most dense to the least dense fraction of the gradient (Somers et al., 1983), indicating that circulating platelets are altered by the effects of intravascular thrombosis. However, some of the agents that decrease platelet density [eg. thrombin (Cieslar et al., 1979) and ADP (Packham et al., 1985)] do not shorten platelet survival. Thus, although platelet density could be decreased by exposure to thrombotic events, under some conditions platelet survival may not be shortened.

c) Platelet function

The increase in platelet consumption in response to acute thrombosis may stimulate platelet production and an increase the numbers of young platelets in the circulation (Mustard, et al., 1966; Harker and Slichter, 1970). Since young platelets are reported to be more metabolically active (Detwiller et al., 1962; Hirsh et al., 1976) and more haemostatically effective than old platelets (Harker and Slichter, 1972), Buchanan and his colleagues (1979) speculated that an increase in the proportion of young platelets in the circulation might be associated with an increase in the ability of the circulating platelets to participate in thrombosis. However, although platelet turnover is increased in rabbits with indwelling aortic catheters, Buchanan and his colleagues (1979) observed that the thrombogenicity of the platelet population was decreased. They concluded that platelets that are stimulated by factors associated with thrombosis become less reactive to subsequent stimuli. Reimers and his colleagues (1976) observed that thrombin degranulated platelets are less haemostatically effective, an observation that might support the findings of Buchanan and his coworkers.

A number of factors are capable of stimulating platelets. Several of the platelet membrane glycoproteins have been identified as specific receptors for some of these stimuli. Thrombin binds to glycoprotein V and glycoprotein

Ib, although it is not known whether binding to either of these glycoproteins is responsible for inducing the activation of platelets (Phillips, 1980); glycoprotein Ib appears to be the receptor for von Willebrand factor on platelets (Phillips, 1982); the glycoprotein IIb/IIIa complex is involved in fibrinogen binding (Bennett and Vilaire, 1979), and in some cases von Willebrand factor-binding; fibronectin on the platelet membrane binds to collagen (Bensusan, et al., 1978) (although fibronectin does not appear to be the receptor for the stimulation of platelets by collagen); and the receptor for the Fc fragment of IgG appears to be a glycoprotein (Pfueller and Luscher, 1972). Since membrane glycoproteins are receptors for substances that stimulate platelets, alteration of the membrane glycoproteins by exposure to substances generated during thrombosis could be a factor in decreasing the responsiveness of platelets.

F. Effects of Drugs on Thrombosis

As previously discussed, several pathways are involved in the formation of thrombi on arterial walls. Thrombus formation is initiated by the interaction of platelets with components of the subendothelial connective tissue exposed by injury including: collagen, microfibrils, and basement membrane (Baumgartner et al., 1971). Collagen stimulates platelets to release ADP (Hovig, 1963) and generate thromboxane A_2 (Hamberg et al., 1975; Bell et al., 1979) which

stimulates platelets to aggregate. Thrombin generated following the activation of coagulation by exposure of factor XII to collagen (Niewiarowski et al., 1965), or by tissue factor made available by damaged cells (Nemerson and Pitlick, 1972; Nemerson and Pitlick, 1974; Zwaal, 1978) causes the release of platelet granule contents and the activation of the arachidonic acid pathway (Holmsen, 1977). Thrombin can also cause platelet aggregation and release by mechanisms that are independent of the release of ADP or the activation of the arachidonic acid pathway with the formation of thromboxane A_2 (Kinlough-Rathbone et al., 1977). Platelet factor 3 made available on the surface of stimulated platelets accelerates the activation of coagulation (Joist et al. 1974; Hardisty and Hutton, 1965; Spaet and Citron, 1965; Zwaal, 1978). Since platelets adhere to polymerizing fibrin, thrombin also has a role in stabilizing platelet aggregates (Niewiarowski et al., 1972b). Therefore, drugs interfere with different pathways and thereby have different effects on thrombosis.

1. Drugs that inhibit platelet function

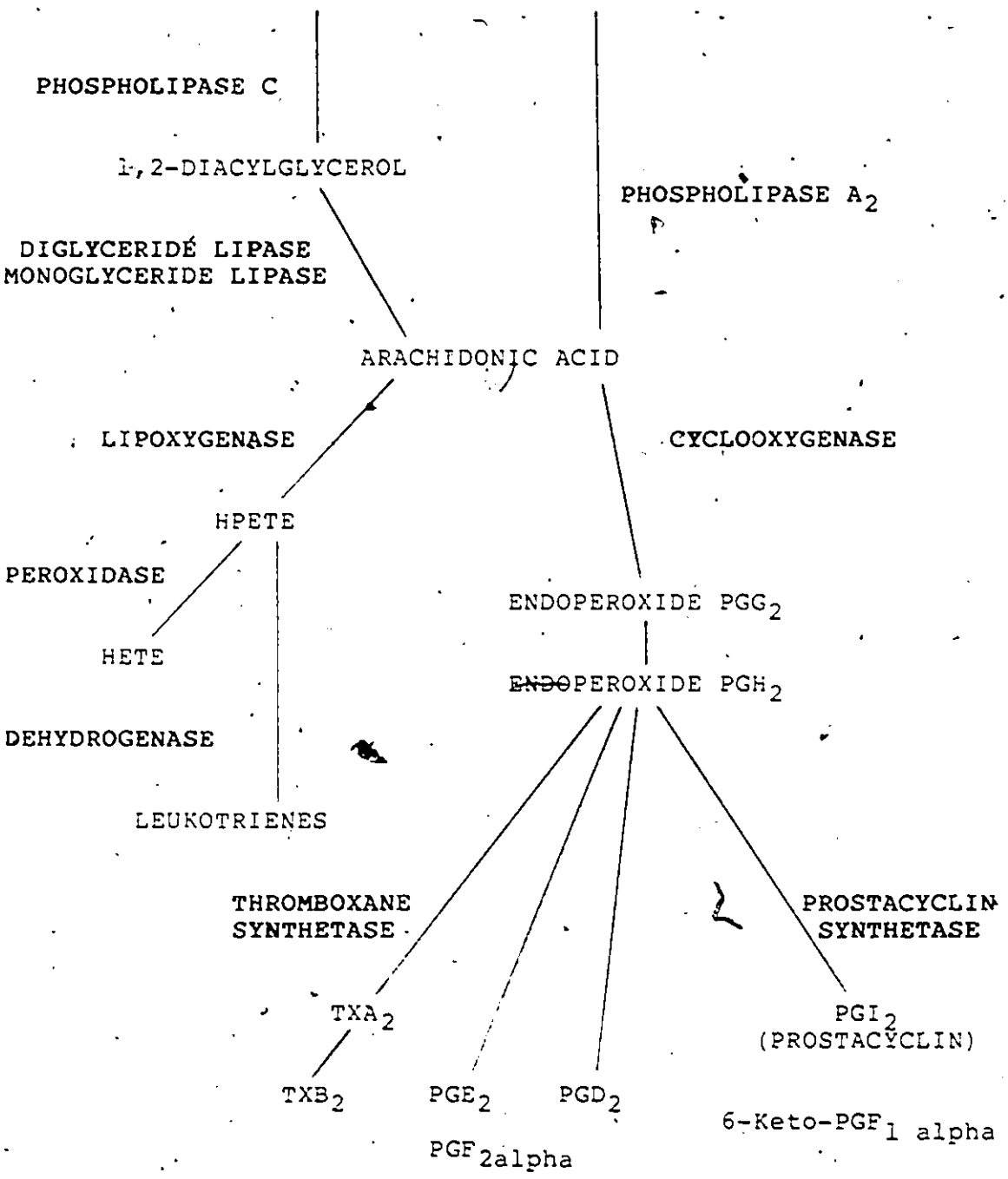
Since platelets respond to stimuli through the activation of several pathways, agents that inhibit one of the pathways of activation, or inhibit the generation by other cells of substances that stimulate platelets, would likely only inhibit the response of platelets to particular stimuli.

At present drugs that inhibit platelet function can be classified as agents that: 1) inhibit the arachidonic acid pathway, 2) increase levels of cyclic AMP, 3) inhibit thrombin, or 4) act through other mechanisms. In addition, anticoagulants prevent the generation of thrombin which can stimulate platelets.

a) Drugs that inhibit the arachidonic acid pathway

Arachidonic acid is released from membrane phospholipids by the action of phospholipase A_2 (Broekman et al., 1980), phospholipase C and diglycerol lipase (Bell et al., 1979). The arachidonic acid can be metabolized by either the cyclooxygenase or lipoxygenase pathways (outlined on the following page) to form several substances that have potent effects on platelet function. PGG_2, PGH_2 and thromboxane A_2 stimulate platelets (Smith et al., 1974a; Hamberg et al., 1974; Hamberg et al., 1975; Malmsten et al., 1975), whereas PGI_2 and PGD_2 are potent inhibitors of platelet function (Cazenave et al., 1974b; Smith et al., 1974; Higgs and Moncada, 1978; Cazenave et al., 1979a; Weiss and Turitto, 1979; Karniguian et al., 1982a; Karniguian et al., 1982b). Although platelets can generate the prostaglandin endoperoxides and thromboxane A_2, they can not produce PGI_2 since they lack prostacyclin synthetase (Weksler, 1982). On the other hand, since vascular endothelial cells and smooth muscle

ARACIDONIC ACID PATHWAY



cells have prostacyclin synthetase, they are capable of generating PGI_2 (Moncada and Vane, 1977; McIntyre et al., 1978; Baenziger et al., 1980).

Drugs can be used to inhibit several of the enzymes in the arachidonic acid pathway including: phospholipases that free arachidonic acid, cyclooxygenase that converts arachidonic acid to prostaglandin endoperoxides, and thromboxane synthetase that converts prostaglandin endoperoxides to thromboxane A_2. The inhibition of either the phospholipases or cyclooxygenase prevents the formation of both thromboxane A_2 and PGI_2.

i) Inhibitors of phospholipase

Drugs that prevent the release of arachidonic acid from membrane phospholipids include mepacrine which inhibits phospholipase A_2 (Winocour et al., 1979), and the steroidal anti-inflammatory drugs which stabilize the platelet membrane (Flower, 1978). Methylprednisolone inhibits platelet adhesion to collagen-coated glass and to subendothelium (Cazenave et al., 1975). Other drugs that stabilize membranes and inhibit platelet adhesion to surfaces such as collagen-coated glass include: sodium pentobarbital (Joist et al., 1973), lidocaine, promethazine (Cazenave et al., 1974), reserpine (Cazenave et al., 1977) and propranolol (Weksler et al., 1977). The observation that corticosteroids reduce the increased platelet consumption associated with idiopathic

thrombocytopenia purpura (Neame et al., 1976), indicates that steroids might decrease platelet activation in vivo. However, it is also possible that methylprednisolone decreases platelet consumption by inhibiting the activity of the reticuloendothelial system which is responsible for clearing altered platelets form the circulation.

ii) Inhibitors of cyclooxygenase

Drugs that inhibit cyclooxygenase include the non-steroidal anti-inflammatory agents such as aspirin, indomethacin, ibuprofen, phenylbutazone and sulfinpyrazone (Packham and Mustard, 1980). Aspirin inhibits the activity of the enzyme by acetylating its serine residues (Roth et al., 1975). Although aspirin inhibits the platelet aggregation that is dependent on the generation of thromboxane A_2, it does not inhibit platelet adhesion to collagen-coated glass or subendothelium (Cazenave et al., 1978), or the release of granule contents from the adherent platelets (Kinlough-Rathbone, et al., 1980). Thus, it is not surprising that aspirin does not inhibit the smooth muscle cell proliferation that develops in response to vascular injury (Clowes and Karnovsky, 1977).

The effect of aspirin on experimental thrombosis depends on the conditions under which it is tested. It has been reported that aspirin inhibits, has no effect on, or enhances thrombus formation (Mustard and Packham, 1980). It

appears that under conditions where PGI_2 formed by the vessel wall has a role in limiting thrombus formation, treatment with aspirin may enhance thrombosis. On the other hand, when thromboxane formation has a major role in thrombus formation, treatment with aspirin decreases the extent of thrombus formation.

Platelet cyclooxygenase is reported to be more sensitive than vessel wall cyclooxygenase to inhibition by aspirin (Burch, 1978). In addition, since platelets are not capable of generating new cyclooxygenase, treatment with aspirin results in the acetylation and the permanent inhibition of the platelet cyclooxygenase. In contrast, endothelial and smooth muscle cells generate new enzyme and regain enzyme activity by several hours following treatment with aspirin ((Jaffe and Weksler, 1979). Therefore, it appears that the greatest antithrombotic effect would be achieved by administering low doses of aspirin to inhibit thromboxane formation by platelets without inhibiting PGI_2 formation by vascular cells. Although low dose aspirin (160 mg/day) decreases thrombosis in arteriovenous shunts (Harter et al., 1979), treatment of patients with high doses of aspirin over long periods of time does not increase the incidence of thrombosis (Linos et al., 1978).

Studies of the effect of aspirin in patients with coronary artery disease provide no clear cut evidence concerning a beneficial effect of this drug in this condition

(see Mustard et al , 1983 for review). However, when the data from all these studies are pooled (Peto, 1978), aspirin appears to have a beneficial effect. Although aspirin does not reduce the incidence of sudden death in patients who have suffered a myocardial infarction, it reduces the incidence of transient attacks of cerebral ischemia in patients (Fields et al., 1977; Canadian Cooperative Study Group, 1978; Barnett et al., 1979). These results indicate that thromboxane generation may be of major importance in the mechanisms involved in the generation of thrombi in transient cerebral ischemia, but not in the mechanisms involved in the development of myocardial infarction. Since aspirin inhibits the production of PGI_2 as well as thromboxane A_2, it is possible that the beneficial effects of inhibiting thromboxane generation might be outweighed by the adverse effects of preventing PGI_2 production. Although aspirin is reported to be of less benefit in preventing thromboembolism in women than in men (The Canadian Cooperative Study Group, 1978), the reason for the difference remains unclear. When used in combination with dipyridamole, aspirin is reported to prolong shortened platelet survival (Harker and Slichter, 1970), and reduce the incidence of myocardial infarction in patients with a previous myocardial infarction (The Persantine-Aspirin Reinfarction Study Research Group, 1980). Recently there have been two major clinical trials to determine the effect of aspirin in patients with unstable angina (Lewis, et al., 1983; Carins et

al., 1985), which indicate that aspirin decreases the incidence of myocardial infarction and death by approximately 50 per cent. Thus, although the effect of aspirin on intravascular thrombosis is not entirely clear, it is probably useful in selected circumstances.

Sulfinpyrazone, a weak cyclooxygenase inhibitor, is reported to normalize shortened platelet survival in patients with gout (Smythe et al., 1965), and vascular disease (Steele et al., 1973) and to decrease the incidence of sudden death in patients with a previous myocardial infarction (The Anturan Reinfarction Trial Group, 1980). However, it was not benificial in patients with transient cerebral ischemia (The Canadian Cooperative Study Research Group, 1978), or in patients with unstable angina (Carins et al., 1985).

Buchanan and his colleagues (1978) observed that sulfinpyrazone inhibited collagen-induced thrombocytopenia in a biphasic manner. The first phase of inhibition paralleled the plasma drug level, while the second phase of inhibition appeared after the drug was cleared from the plasma, indicating that a drug metabolite might be responsible for the second phase of inhibition (Butler et al., 1979). Subsequently, Pedersen and Jacobsen (1981) identified two metabolites of sulfinpyrazone that appeared in the plasma during the same period as the second phase of cyclooxygenase inhibition. The sulfite metabolite of sulfinpyrazone is a potent cyclooxygenase inhibitor. Thus, the antithrombotic effects

of sulfinpyrazone over long periods in vivo are probably largely dependent on a metabolite of the drug.

iii) Inhibitors of thromboxane synthetase

The thromboxane synthetase inhibitors prevent the conversion of PGH_2 to thromboxane A_2, thereby preventing the proaggregatory effects of thromboxane A_2. These drugs have no effect on the generation of PGH_2 from arachidonic acid so that the capacity to generate PGI_2 with its antiaggregatory and vasodilatory effects is preserved. Therefore, it was anticipated that these drugs might have potent antithrombotic effects. However, the prostaglandin endoperoxides themselves are capable of causing platelet aggregation and the release reaction. When dazoxiben, a selective inhibitor of thromboxane synthetase, was added to platelet rich plasma, arachidonic acid-induced aggregation was inhibited in specimens from some individuals but not from others (Bertele et al., 1981; Bertele et al., 1984). These results indicated that some individuals are responders while others are nonresponders. Since prostaglandin endoperoxides, PGE_2, and PGD_2 have been reported to accumulate in excess following inhibition of thromboxane synthetase, it was possible that the failure of dazoxiben to inhibit arachidonic acid induced platelet aggregation consistently, might depend on the balance between the generation of pro and antiaggregatory products of the arachidonic acid pathway. Dale and his colleagues (1983)

found that inhibition of thromboxane synthetase with dazoxiben has less effect on platelet aggregation than inhibition of cyclooxygenase with aspirin; they also suggested that weak inhibitory effects of dazoxiben could be due to the generation of prostaglandin endoperoxides. Although dazoxiben is reported to reduce experimental thrombosis in carotid arteries in vivo (Randall and Wilding, 1982), thrombus formation on dacron arterial grafts is not reduced (Hanson and Harker, 1983). These results suggest the possibility that dazoxiben might inhibit thrombus formation only on surfaces that are capable of generating PGI_2.

Thus, although drugs that inhibit the arachidonic acid pathway are likely to be of limited value as antithrombotic agents, under selected circumstances such as transient attacks of cerebral ischemia and unstable angina they are clinically useful.

iv) Inhibitors of the lipoxygenase pathway

Several steps in the lipoxygenase pathway can be inhibited with drugs. These include ETYA (15-Hydroxy-5,8,11,13-eicosatetraenoic acid) (Vanderhoek et al., 1980; Sun et al., 1981), and NDGA (nordihydroguiaretic acid) (Higgs and Vane, 1983) which inhibit lipoxygenase, and sodium salicylate which blocks the peroxidation of HPETE to HETE (Siegel et al., 1979b).

b) Agents that increase cyclic AMP

Increased levels of cyclic AMP result in the sequestration of internal calcium and thereby inhibit platelet functions that are dependent on the availability of calcium. Levels of cAMP are increased by agents that stimulate adenylate cyclase to increase synthesis of cAMP (Gorman et al., 1977)., or agents that inhibit phosphodiesterase to prevent the break down of cAMP.

i) Stimulators of adenylate cyclase

Agents that stimulate adenylate cyclase include PGI_2, PGD_2 and PGE_1 (Mustard and Packham, 1980). PGI_2 is formed by vascular cells (Moncada and Vane, 1979a), whereas PGD_2 is formed by platelets (Smith et al., 1974b).

In experimental studies PGI_2 inhibits platelet shape change, aggregation and release induced by all stimuli, and inhibits platelet adherence to surfaces (Cazenave et al., 1979; Higgs et al., 1979; Packham and Mustard, 1984a). PGI_2 inhibits the conformational changes in the platelet membrane and inhibits release that leads to the exposure of platelet factor 3 on the platelet membrane; platelet factor 3 is a phospholipid that contributes to the activation of coagulation (Erhman, 1979; Ehrman and Jaffe, 1980; Harsfalvi et al., 1980). Since, PGI_2 inhibits the contribution of platelets to the activation of coagulation, it appears likely that it would also reduce the coagulation-dependent accumulation

of platelets on the surface of injured neointima.

Evidence of the potent antithrombotic effects of treatment with PGI_2 in vivo includes: decreased platelet consumption and preservation of platelet function during cardiopulmonary bypass surgery (Plachetka et al., 1979; Longmore et al., 1981; Machin et al., 1981; Coppe et al., 1981; Walker et al., 1981), promotion of healing in peripheral vascular disease (Hossman et al., 1981; Szczeklik et al., 1980), decreased platelet accumulation on damaged vascular surfaces (Gryglewski et al., 1976; Higgs et al., 1978a; Higgs et al., 1978b; Higgs et al., 1979), inhibition of thrombosis in coronary arteries (Aiken et al., 1979), inhibition of thrombosis during hemodialysis (Woods et al., 1978; Weston et al., 1979), decreased platelet aggregation during hemoperfusion (Langley et al., 1979), and reversal of the lethal effects of endotoxemia, thought to result from the formation of microemboli and the activation of intravascular coagulation (Krausz et al., 1981). In addition, since PGI_2 is also a potent vasodilator, some of its antithrombotic effects might depend on its vasodilatory properties.

Although its potent anti-platelet effects make PGI_2 a potentially useful antithrombotic drug, problems are encountered during treatment with infusions of PGI_2 including hypotension, headaches, and nausea (Data et al., 1981). Sinzinger and his colleagues (1981) also reported that the sensitivity of platelets to PGI_2 is decreased during long

term infusions of the drug. The clinical usefulness of the drug is also limited by its very short half-life in the circulation (3 minutes) (Rosenkranz et al., 1980). Recently it was shown that the more stable proctacyclin analogue ZK36374 has potent antiplatelet effects in vivo (Belch et al., 1983).

ii) Inhibitors of phosphodiesterase

Although dipyridamole is only a weak inhibitor of platelet phosphodiesterase, the enzyme that breaks down cAMP (Mills and Smith, 1971; Vigdahl et al., 1971), it is reported to cause a substantial increase in cAMP when used in combination with agents that stimulate adenylate cyclase (DiMinno et al., 1978; Haslam, 1973; Haslam et al., 1978). Dipyridamole inhibits platelet aggregation (Emmons et al., 1965; Cucuianu et al., 1975; Mustard and Packham, 1978; Niewiarowski et al., 1975; Oblath et al., 1978; Philp, 1974; Rajah et al., 1977). Dipyridamole also inhibits platelet adherence to collagen (Cazenave et al., 1978) and to artificial surfaces (Harker et al., 1979), prolongs shortened platelet survival when used alone or with aspirin (Harker and Slichter, 1976; Mustard and Packham, 1978), reduces the extent of atherosclerosis that develops in baboons infused with homocysteine (Harker and Slichter, 1972), and decreases intimal thickening following coronary bypass surgery in dogs (Josa, et al., 1981). Since dipyridamole alone is only a weak phosphodiesterase inhibitor and was not used with drugs

that increase platelet cAMP, it appears that the major platelet inhibitory effects of dipyridamole are probably not dependent on its phosphodiesterase activity. Thus, the mechanisms responsible for the effects of the drug are not clearly understood. However, under some conditions treatment with persantine may be clinicially useful. In the Persantine Aspirin Reinfarction Study (1980), treatment with dipyridamole (persantine) in combination with aspirin decreased the incidence of reinfarction; the treatment was of greatest benefit to patients that entered into the study within 6 months of having had a myocardial infarction.

Since platelets rapidly accumulate on injured vessels and release PDGF to be taken up by smooth muscle cells of the vessel wall, it is not surprising that treatment with dipyridamole begun one to 4 days following vascular surgery, did not inhibit intimal thickening. In a recent study, Chesebro and his colleagues (1982) reported that when treatment with dipyridamole was begun 48 hours before coronary bypass surgery, and aspirin was added 7 hours after the operation, the incidence of graft occlusion was reduced during the 4 month period following surgery, indicating that early treatment with dipyridamole may have inhibited vessel wall thickening and thrombosis in response to the vessel wall injury associated with the grafts. In contrast to the results of the studies by Chesebro and his colleagues (1982), Pantely and his coworkers (1979) reported that when treatment with

dipyridamole was begun one to 4 days following surgery, intimal hyperplasia was not inhibited. These results are consistent with the hypothesis that the platelet-vessel wall interaction that occurs soon after injury is important in stimulating vessel wall thickening and thrombosis.

2. Drugs that inhibit coagulation

Agents that inhibit coagulation prevent the generation of thrombin and its subsequent effects on platelets. Heparin potentiates the effect of antithrombin III in inhibiting activated factors IX, X, XI, XII and thrombin (Rosenberg, 1977). Results of studies on the effect of heparin on platelets, indicate that heparin can inhibit or potentiate aggregation induced by other aggregating agents (Mustard and Packham, 1980). However, heparin has also been reported to induce thrombocytopenia in rabbits (Eika and Godal, 1971), and to stimulate platelet aggregation (Eika, 1972a; Eika, 1972b; Thompson et al., 1973; Salzman et al., 1980). The high molecular weight fraction of heparin has a greater platelet aggregatory effect than the low molecular weight fraction (Salzman et al., 1980). Since heparin-induced aggregation is inhibited by EDTA, adenosine, apyrase, and aspirin which inhibits the release of ADP, it appears that heparin may induce platelet aggregation through the release of ADP (Eika, 1972b). Heparin has also been reported to inhibit the activation of adenylate cyclase by PGI_2 (Saba et

al., 1979), or PGE_1 (Reches et al., 1979). Although heparin bound to prosthetic devices has been reported to be thrombogenic (Salzman, 1969), in more recent studies heparin bound to surfaces has been shown to be non-thrombogenic (Kwaan and Hotem, 1975; Menys and Davies, 1982). Thus, the effects of heparin on platelets are complex and variable.

Since one of the objectives of this study is to determine the effect of platelets on the properties of the damaged vessel wall, several of these drugs will be used to inhibit platelet accumulation on damaged vessels so that the changes in the vessel wall that occur independently of platelet accumulation can be determined.

G. Hypothesis

Although platelet interaction with injured aortae is involved in the initiation and development of atherosclerosis, and in the clinical complications associated with the disease, many of the factors influencing the reactivity of the vessel surface in relation to thrombosis have not been extensively studied.

Although I previously demonstrated that normal aortae exposed to a single injury with a balloon catheter lose their reactivity soon after injury, the factors responsible for this loss of reactivity are unclear. In these initial experiments the platelets that adhered to the vessel surface made the injury site non-reactive to platelets within 30

minutes.

Since the smooth muscle cell proliferation that occurs in response to a single injury is analogous to the smooth muscle cell proliferation that contributes to the formation of the early lesions of atherosclerosis, and since atherosclerotic lesions are thrombogenic it is hypothesized that injury to these smooth muscle cells would result in enhanced platelet accumulation and sustained reactivity of the injury site to constituents of the circulating blood.

H. Specific Objectives

The specific objectives therefore are as follows:

1. In vessels exposed to a single injury, to determine the factors influencing its reactivity including:
   a) platelet adherence
   b) adsorption of plasma proteins
   c) material made available by red blood cells
   d) products of arachidonic acid metabolism such as prostaglandin endoperoxides, PGI_2, or products of the lipoxygenase pathway.

2. In vessels that had been subjected to injury of a newly formed neointima to determine:
   a) factors contributing to the thrombogenicity of the surface such as the activation of coagulation

b) factors influencing the subsequent reactivity of these vessels;

i) the accumulation of platelets

ii) prostaglandin endoperoxides, PGI_2, and lipoxygenase products.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

A. MATERIALS

1. Tyrode's solutions

Stock solution I

NaCl	160 g	
KCl	4 g	per litre
$NaHCO_3$	20 g	
NaH_2PO_4	1 g	

Dissolve and make up to 1000 mL with distilled water.

Stock solution II

$MgCl_2.6H_2O$	20.33 g	per litre

Dissolve and make up to 1000 mL with distilled water.

Stock solution III

$CaCl_2.6H_2O$	21.91 g	per litre

Dissolve and make up to 1000 mL with distilled water.

a) **Calcium-free Tyrode's albumin (washing platelets)**

Stock solution I	50	mL
Stock solution II	20	mL
albumin	3.5	g
glucose	1.0	g

Make up to 1000 mL with distilled water. Adjust pH to 6.2. The osmolarity is 290.

b) Tyrode's albumin (resuspending rabbit platelets)

Stock solution I	50	mL	
Stock solution II	10	mL	
Stock solution III	20	mL	per litre
bovine albumin	3.5	g	
glucose	1.0	g	

Make up to 1000 mL with distilled water. Adjust pH to 7.35. The osmolarity is 290 milliosmols.

2. Sodium pentobarbital anaesthetic

sodium pentobarbital	6.0	g	per 100 mL
ethanol	2.0	mL	

Add 0.1 N NaOH if required to dissolve the sodium pentobarbital.

3. Eagle's medium (GIBCO, Grand Island, NY)

Add 2.2 g of $NaHCO_3$ and 3.5 g of bovine albumin to each litre of Eagle's medium. Add 5 mmol/L of Hepes buffer (N-2-hydroxyethyl-piperazine-N-2 ethansulfonic acid) (Sigma Chemical Company, St. Louis, MO) to maintain pH. Adjust pH to 7.35.

4. Dipyridamole for injection

Dissolve dipyridamole (Boehringer Ingelheim, Quebec) at a concentration of 5 mg/mL in 0.1 N HCl. Maintain pH at 2.8 to 3.0 to prevent the dipyridamole from precipitating out of solution.

5. Aspirin for injection

Solutions:

A 5 g Na_2CO_3 in 20 mL distilled water.

B 10 g acetylsalicylic acid (Matheson, Coleman and Bell, East Rutherford, NJ) in 80 mL distilled water.

Add solution 'A' dropwise to solution 'B'. Maintain pH at 6. Precipitate and froth will form and redissolve. Make up to 100 mL with distilled water. Store in plastic vials at -70°C for up to 10 days.

6. PGI_2 (prostacyclin) solution for infusion

Stock solution of PGI_2:

Dissolve 5 mg of PGI_2 in 5 mL of ethanol. Divide into aliquots and store at -70°C.

Tris buffer (0.1 M):

Dissolve 12.1 g of trisma base (tris[hydroxymethyl]aminomethane) in distilled water. Make up to 1 litre and adjust to pH 9 with HCl.

Solution for infusion:

Dilute stock solution of PGI_2 in ethanol with 0.1 M Tris buffer to obtain the required concentration of PGI_2. Keep on ice. Solution is stable for at least 6 hours when prepared and stored in this way.

7. Evan's Blue solution

Dissolve Evan's blue (Allied Chemical, New York, NY) 4.52 mg/ml in sterile isotonic saline. Buffer to pH 7.4 with 0.02 mol/L phosphate buffer, filter through Whatman #1 filter paper, and then filter through a 0.22 micron millipore filter.

Phosphate buffer (0.02 M)

Solutions:

A 0.07 mol/L potassium monophosphate

(0.08 g KH_2PO_4/litre)

B 0.07 mol/L disodium phosphate

(11.88 g $Na_2HPO_4.H_2O$/litre)

Add 19.6 mL of solution 'A' to 80.4 mL of solution 'B'.

8. Locke's-Ringer solution

NaCl	9.0 g	
KCl	0.42 g	
$CaCl_2$	0.24 g	per litre
$NaHCO_3$	0.15 g	
glucose	0.10 g	

Bubble with oxygen for one hour immediately before use.

9. 4 per cent gluteraldehyde in 0.1 mol/L cacodylate buffer

0.2 mol/L solution of cacodylate buffer contains:

Na cacodylate

$(CH_3)_2AsO_2Na.3H_2O$	42.8 g	per litre
distilled water	800 mL	

Adjust pH to 7.4 with 1.0 N HCl and make up to 1000 mL with distilled water.

4 per cent gluteraldehyde in cacodylate buffer contains:

0.2 mol/L sodium cacodylate	500 mL	per litre
gluteraldehyde	40 g	

Make up to 1000 mL with distilled water.

10. 4 per cent paraformaldehyde in 0.1 mol/L phosphate buffer

paraformaldehyde	40 g	
$NaH_2PO_4.H_2O$ (monobasic)	6.35 g	per litre
$NaHPO_4$ (dibasic)	21.87 g	

Dissolve paraformaldehyde in 200 mL distilled water at 82°C or less. Do not over heat as paraformaldehyde breaks down at higher temperatures. Filter and make up to approximately 800 mL with distilled water. Adjust pH to 7.4. Make up to 1000 mL and readjust pH to 7.4 if required.

B. METHODS

1. Preparation of Suspensions of Washed Platelets Labelled with Radioisotopes

a) Labelling with ^{51}Chromium

Suspensions of washed rabbit platelets were prepared as previously described (Ardlie et al., 1970) from blood collected into acid-citrate-dextrose anticoagulant (Aster and Jandl, 1964) through a polyethylene cannula placed in the carotid artery of rabbits anaesthetized with sodium pentobarbital. The red blood cells were separated from the platelet-rich plasma by centrifugation at 120 x g for 15 minutes at room temperature. The platelets were labelled in the first washing solution of calcium-free Tyrode's solution containing 0.35 per cent albumin and EGTA (2.6×10^{-4} mol/L) for 30-45 minutes at room temperature with $Na_2{}^{51}CrO_4$ (New England Nuclear, Canada Ltd., Lachine, Quebec; 200-500 uCi/ug Cr; 150 uCi of ^{51}Cr were used to label platelets obtained from the blood of each rabbit).

b) Labelling with ^{14}C-serotonin

In some in vitro adherence studies platelets were doubly-labelled in the first washing fluid with ^{14}C-serotonin (^{14}C-5HT)(5 hydroxytryptamine-3'-^{14}C-creatinine sulphate); 55 uCi per umole, Amersham/Searle, Arlington Heights, Illinois;

2uCi were used to label platelets obtained from the blood of each rabbit) for 10 minutes at 22°C. The platelets were separated by centrifugation at 1,100 x g for 10 minutes, resuspended in calcium-free Tyrode's albumin solution for 10 minutes, separated by centrifugation, and resuspended in Eagle's medium.

2. Animals

New Zealand White rabbits were used throughout. Male animals were used for all experiments. The weights of the animals were between 2.2 and 3.2 kg, and animals of approximately the same weight were used within an experiment.

Before surgery the animals were anaesthetized with sodium pentobarbital (approximately 40 mg/kg). When animals were to be prepared for perfusion of fixative they were pre-anaesthetized with ketamine (Ketaset; Rogar/STB, Div. of BTA Products, London, Ontario; 100 mg intramuscularly), and atropine (0.1 mg subcutaneously) 20 minutes before they were anaesthetized with sodium pentobarbital (approximately 40 mg per kg) given intravenously.

The procedures used in these studies comply with the institutions guidelines on animal care.

3. Removal of Aortic Endothelium with a Balloon Catheter

The procedure for removing the endothelium with a balloon catheter was a modification of the procedure

described by Baumgartner (1973), using arterial embolectomy catheters (size 4F) obtained from Edwards Laboratories, Santa Anna, California. In the present experiments the extent of injury was standardized throughout the length of the aorta. The balloon was inflated with the minimum pressure required to maintain contact of the balloon with the surface of the vessel and completely remove the endothelium, without causing extensive injury to the deeper layers of the vessel. To do this, a pressure reservoir was used to help maintain a constant pressure within the inflated balloon as the catheter was withdrawn down the gradually narrowing lumen of the aorta.

A pressure of 180 mm Hg above the pressure at which the balloon could be inflated outside the animal was used during the removal of the aortic endothelium. This inflation pressure was used to produce a pressure in the balloon of approximately 80 to 100 mm Hg above the systolic blood pressure of the animal. Even when an inflation pressure of 180 mm Hg was used, the blood pressure of the rabbit was occasionally sufficient to prevent inflation of the balloon within the aortic arch. However, under these conditions a slight increase in pressure inflated the balloon.

To confirm that the pressure was adequate to inflate the balloon within the aorta, a three-way valve attached to a syringe was inserted between the catheter and the pressure reservoir so that the air within the inflated balloon in the

aorta could be allowed to empty into the syringe. The volume of air contained within the inflated balloon in the aortae was estimated from the volume of air that emptied into the syringe at atmospheric pressure. The air in the syringe was injected back into the balloon, the catheter was reconnected to the pressure reservoir, and the inflated balloon withdrawn down the aorta.

Since the reservoir contained approximately 60 mL of air and the inflated balloon in the aortic arch contained approximately 1 mL of air, when the balloon was withdrawn down the narrowing lumen of the aorta until it contains only 0.5 mL of air, the 0.5 mL of air that was displaced from the balloon flowed into the reservoir, and the pressure within the system underwent little change.

For example:

If P_1= the pressure of the reservoir and the inflated balloon in the aortic arch,

P_2= the pressure of the reservoir and the inflated balloon in the abdominal aorta,

V_1= the volume of air in the reservoir and the inflated balloon in the aortic arch,

V_2= volume of air in the reservoir and the inflated balloon in abdominal aorta,

and $P_1V_1 = P_2V_2$,

then $P_2 = \frac{P_1V_1}{V_2} = \frac{P_1 \times 61\ mL}{60.5\ mL} = P1 \times 1.008$

or P_2 = 100.8 % of P_1

Therefore, the change in pressure was less than 1 per cent.

Without a pressure reservoir, the pressure within the balloon in the abdominal aorta would be twice the pressure within the balloon in the aortic arch.

For example:

If V_1= 1 mL,

and V_2= 0.5 mL,

then $P_2 = \frac{P_1 V_1}{} = \frac{P_1 \times 1 \text{ mL}}{} = P_1 \times 2$

or P_2 = 200% of P_1

Therefore, the change in pressure is 200 per cent.

To remove the endothelium from a rabbit aorta the right femoral artery of an anaesthetized rabbit was isolated and the catheter was introduced and passed up the aorta to the level of the aortic arch. The catheter was connected to the pressurized reservoir, the balloon was inflated, the inflation of the balloon was checked, and the catheter withdrawn into the femoral artery. The balloon was deflated, returned to the aortic arch, reinflated, and withdrawn a second time, usually within 30 seconds of the first passage. The femoral artery was then ligated and the incision closed with silk sutures.

To determine the extent to which the balloon catheter removed the endothelium from the aortae some of the animals were given an intravenous injection of Evans blue dye (4.5 mg per kg) 30 minutes before they were killed. Evans blue dye,

which binds to albumin, penetrated the vessels in the regions where the endothelium was removed. In previous studies, morphological examination of the vessels confirmed that the endothelium was removed by the passage of the balloon catheter, and there was little damaged to deeper layers of the vessel (Groves et al., 1979).

4. Injury to the Neointima of Rabbit Aortae with a Balloon Catheter

The right femoral artery (the same vessel through which the catheter was introduced for the initial injury with a balloon catheter 7 days to 4 weeks earlier) was isolated and a small incision was made in the vessel proximal to the first incision. Thrombus occluding the vessel was removed, and the catheter was inserted and passed to the level of the aortic arch. The procedure used to damage the neointimal surface was the same as that described for removing the endothelium.

5. Accumulation of Platelets on Damaged Aortae In Vivo

Platelets (8 mL of 3 x 10^9 platelets per mL) were labelled with ^{51}Chromium and injected into rabbits 18 hours before a balloon catheter was used to remove the aortic endothelium or damage the aortic neointima. Before surgery, the animals were preanaesthetized with ketamine (100 mg intramuscularly), and given atropine (1.0 mg subcutaneously)

20 minutes before they were anaesthetized with sodium pentobarbital (approximately 40 mg per kg intravenously). Shortly before the animals were perfused, cannulae (polyethylene tubing, PE-190, Clay Adams Division of Becton Dickinson and Company, Parsippany, New Jersey) were inserted into the carotid and both femoral arteries. Immediately before the animals were killed by perfusion-fixation at specific times following balloon injury, they were given an intravenous injection of 1,000 units of heparin (Hepalean, Harris Laboratories, Brantford, Ontario; or Hepalean, Organon, Toronto, Ontario). The animals were perfused through the carotid cannula at a pressure of approximately 80 to 100 mm Hg with Locke's-Ringer solution containing 1 unit per mL of heparin and maintained at a pH of 7.35 and a temperature of 37°C. When the solution draining from the cannulated femoral arteries was almost clear of blood, the perfusion fluid was replaced with 4 per cent gluteraldehyde in 0.1 mol/L cacodylate buffer or 4 per cent paraformaldehyde in 0.1 mol/L phosphate buffer. The perfusion pressure was maintained, and after 1 to 2 minutes the femoral arteries were clamped. The perfusion of fixative was continued for several minutes until the flow ceased. The carotid and femoral arteries were then clamped to maintain the pressure of the fixative within the vessels. The animals were left at 4°C for 16 to 18 hours. The aortae were then carefully dissected free of extraneous tissue in situ. The aortae were

removed from the animals, 15 cm of vessel from the aortic bifurcation to the thoracic aorta was cut into 2.5 cm segments, and the radioactivity associated with the segments was measured in a well type gamma scintillation counter. The number of adherent platelets per mm^2 of damaged aorta was calculated:

$$\frac{\text{cpm for vessel}}{\text{vessel area } mm^2} \times \frac{\text{platelets/mL blood}}{\text{cpm/mL blood}} = \text{platelets } mm^2 \text{ of aorta}$$

To determine if ^{51}Cr-labelled platelets or non-platelet bound radioactivity became associated with the undamaged vessel, ^{51}Cr-labelled platelets were injected into an animal whose aorta had not been damaged, and ^{51}chromium that became associated with the vessel was measured.

In previous studies, platelet adherence to the damaged vessel wall was measured morphometrically and with radioactive isotopes (Groves et al., 1979). It was shown that isotopic determinations of platelet adherence correlated with the morphometric observations.

6. Reactivity of the Vessel Wall for Platelets during Specific Intervals after Injury to the Neointima with a Balloon Catheter

To determine the ability of the vessel wall to react with circulating platelets after different intervals had elapsed following injury to the aortic neointima, platelets

labelled with ^{51}chromium (8 mL of 3 x 10^9 platelets per mL) were injected into rabbits at specific times following injury. Thirty minutes after the platelets were injected, the animals were given an injection of heparin (1000 units) and killed by perfusion-fixation. The aortae were removed, the radioactivity associated with the vessels measured, and the number of platelets associated with the aortae calculated as previously described (see previous page).

7. Adhesion of Platelets to Vessel Walls In Vitro

The animals were anaesthetized with intravenous sodium pentobarbital (approximately 40 mg/kg) given intraveously, and exsanguinated through a cannula placed in the carotid artery. The thorax and abdomen were opened to expose the aorta. To obtain normal vessels, an incision was made in the aortic arch and in the abdominal aorta and the vessel was rinsed once with Eagle's medium containing 0.35 per cent bovine albumin, and then with Eagle's medium containing 4 per cent albumin and supplemented with $NaHCO_3$ (2.2 g per litre) and Hepes buffer (5 mmol/L). The aortae were dissected free of extraneous connective tissue, removed and immersed in Eagle's medium containing 4 per cent albumin at 37°C.

To prepare vessels for use in studies of the adherence of platelets to the subendothelium or the injured neointima, the vessels were injured with a balloon catheter in situ. The vessels were rinsed with Eagle's medium before a balloon

catheter was introduced through an incision in the abdominal aorta and passed to the level of the aortic arch. The balloon was pressurized to 80 to 100 mm Hg above the inflation pressure of the balloon at atmospheric pressure. The balloon was withdrawn into the iliac artery, deflated, and the procedure repeated. The freshly ballooned aorta was rinsed with Eagle's medium containing 4 per cent albumin, dissected free of surrounding tissue, removed from the animal, and immersed in Eagle's medium containing 4 per cent albumin an 2.2 g per litre of $NaHCO_3$ at 37 ^{o}C and Hepes buffer (5 mmol/L).

For the in vitro studies of platelet adherence the platelets were labelled and washed as previously described, and finally suspended in Eagle's medium supplemented with 2.2 g sodium bicarbonate per litre and containing 4 per cent bovine albumin and apyrase (prepared from potatoes by the method of Molnar and Lorand, 1961) at a concentration capable of converting 0.25 umol/L ATP to AMP within 120 seconds at 37^{o}C. Five mmol/L Hepes buffer (Sigma Chemical Company, St. Louis, MO) was included in the final suspending medium to maintain the pH at 7.35. Red cells were washed twice in calcium-free modified Tyrode's solution containing 0.1 per cent glucose at pH 7.35, and resuspended in the same medium as platelets, except that the pH was adjusted to 9 before the addition of red blood cells since the red cells release acidic by-products that decrease the pH. The platelet counts were adjusted to 500,000 per mm^3 in the total volume of the

suspension including the volume occupied by the washed red cells that were added to produce a hematocrit of 40 per cent.

To prepare the vessels for adhesion studies they were immersed in Eagle's medium in a plastic petri dish and immobilized by pinning the ends to the dish with 18 gauge needles. Extraneous tissue was dissected from the surface of the vessel and the intercostal arteries were removed. The vessels were trimmed between the lowest intercostal arteries and the celiac trunk. A small hole was cut in the vessel approximately 1 mm from the caudal end. A small wire with a hook in the end and an applicator stick were placed inside an 8 cm segment of polyethylene tubing (PE-320) so that the hook extended approximately 1 mm beyond the end of the stick, and the applicator stick extended aproximately 5 mm beyond the end of the tubing. The hook was passed through the hole in the vessel and the wire withdrawn so that the end of the hook was embedded in the end of the applicator stick, thereby securing the end of the vessel. Forceps were used to evert the vessel over the end of the stick and approximately 5 mm of the tubing. The stick, wire, and 2 mm of attached vessel were withdrawn into the tubing so that the end of the tubing was covered with a smooth surface of everted vessel. The remainder of the vessel was then carefully everted onto the tubing. During eversion the vessel was kept immersed in Eagle's medium. Since the ends of the vessel were damaged

duringmanipulation, they were removed with a sharp scalpel blade leaving a 5.5 cm segment of everted thoracic aorta on the tubing.

The everted vessels on the tubing were mounted on metal probes, immersed in the platelet suspensions at 37°C, and rotated at 200 rpm for 10 minutes. The vessels were rinsed in Tyrode's solution containing 10 mmol/L EDTA, pH 7.35 at 200 rpm for 5 minutes to ensure that only single platelets and not platelet aggregates adhered to the surface. The cut ends of the aortae were removed (0.25 cm) and the vessels were cut into 5 segments each 1 cm in length. Each segment was placed in a test tube containing 1 mL of 4 per cent paraformaldehyde in 0.1 mol/L phosphate buffer. The radioactivity associated with the segments of aorta was measured in a well-type gamma scintillation counter and the number of platelets per mm^2 of vessel was calculated.

8. Association of Non-Platelet-Bound ^{51}Chromium with Aortae

Since there was some non-platelet-bound radioactivity in the supernatant of suspensions of ^{51}Cr-labelled platelets, and the vessels were exposed to this supernatant when platelet suspensions were infused into animals or the vessels were rotated in suspensions of labelled platelets in vitro, it was possible that some of the non-platelet-bound radioactivity might penetrate the vessels. Therefore, the non-platelet-bound radioactivity that became associated with the aortae

following the infusion of suspensions of ^{51}Cr-labelled platelets in vivo, or during rotation of aortae in suspensions of labelled platelets in vitro was determined.

a) In vitro

Methods table I shows that when platelets were rotated in a suspension of red cells in the supernatant obtained after centrifuging a suspension of ^{51}Cr-labelled platelets, the amount of radioactivity that became associated with the vessels represented approximately 5.9 per cent of the radioactivity that became associated with de-endothelialized vessels when they were exposed to suspensions of ^{51}Cr-labelled platelets. This was similar to the percentage of radioactivity that became associated with undamaged vessels exposed to suspensions of ^{51}Cr-labelled platelets. Thus, some of the radiocativity that became associated with the vessels rotated on the probe was derived from the supernatant that penetrated the vessel wall during the period of rotation and did not represent ^{51}Cr-platelets adherent to the vessel. Thus, when the values for adherence were low, most of the radioactivity associated with the vessels was derived from the supernatant and the actual platelet accumulation on the vessel was negligible. When platelet accumulation on injured vessels was high, the values for platelet adherence that were determined radiometrically were similar to the values quanti-

METHODS TABLE I

ASSOCIATION OF ^{51}CHROMIUM WITH UNDAMAGED AORTAE IN VIVO AND IN VITRO

TREATMENT OF AORTAE	VESSELS EXPOSED TO	APPARENT PLATELET ACCUMULATION (NO. PER SQ. MM.) CALCULATED FROM ^{51}Cr ASSOC. WITH AORTAE	^{51}Cr ASSOC. WITH AORTAE EXPRESSED AS % ^{51}Cr ASSOC WITH DEENDOTHELIALIZED AORTAE
IN VITRO			
REMOVAL OF ENDOTHELIUM	^{51}Cr-PL	32,800 ± 4,200	100
UNDAMAGED	^{51}Cr-PL	1,100 ± 200	3.4
UNDAMAGED	SUPERNAT. FROM SUSP. OF ^{51}Cr-PL	1,900	5.9
IN VIVO			
REMOVAL OF ENDOTHELIUM	^{51}Cr-PL	40,300 ± 6,700	100
UNDAMAGED	^{51}Cr-PL	1,600 ± 500	4.0

Mean values - SEM. In the in vitro studies, vessels on the rotating probe were exposed to a suspension of ^{51}Cr-platelets or the supernatant from the suspension for 10 min before rotation for 2 min in calcium free Tyrode's containing EDTA. In the in vivo studies rabbits were infused with ^{51}Cr-platelets. The endothelium was removed from the aortae with a balloon catheter 10 min before the animals in one group were killed by perfusion-fixation. Animals in the group that received no injury to the aorta were killed by perfusion-fixation 30 minutes following the infusion of ^{51}Cr-platelets. The radioactivity associated with the vessels was measured and the accumulation of ^{51}Cr-platelets calculated.

tated morphometrically (Groves et al., 1979). Thus, when the platelet adherence was high, the non-platelet-bound radioactivity represented only a small percentage of the radioactivity associated with the vessels.

b) In vivo

When suspensions of ^{51}Cr-labelled platelets were infused into rabbits, the radioactivity that became associated with the undamaged aortae was approximately 4 per cent of the radioactivity that became associated with the de-endothelialized vessels. Since no platelets were seen on the surface of undamaged vessels when they were examined morphologically, it appeared that the radioactivity associated with the undamaged vessels was not platelet-bound, or was radioactivity associated with platelets associated with the vasa vasorum or with adventitia remaining on the vessel. Therefore, when the levels of radioactivity associated with the vessels was low (calculated to be approximately 2,000 platelets per mm^2 or less), platelet accumulation on the vessels in vivo was negligible. When the number of ^{51}chromium-labelled platelets that accumulated on a vessel in vivo or in vitro were low, much of the associated radioactivity was not platelet bound, and the actual platelet accumulation was substantially less than the value indicated by the measurement of radioactivity.

9. Adhesion of Platelets to Collagen-Coated Glass

a) Preparation of acid-soluble collagen

Acid-soluble collagen was prepared as described by Cazenave and coworkers (1973). One gram of bovine tendon collagen (Sigma Chemical Company, St. Louis, MO) was mixed in 12 mL of glacial acetic acid in 188 mL of distilled water for 10 minutes at 22°C. The mixture was chilled on ice and homogenized for 10 minutes in the 250 mL container of a Sorval Omnimix. The entire homogenate was centrifuged at 2,500 x g for 15 minutes at 22°C and the white film on top was discarded. The supernatant and the gelatinous precipitate were mixed with 20 mL of water and centrifuged at 1000 x g for 10 minutes.

b) Coating Glass with Acid-Soluble Collagen

Glass tubing (external diametre 7 mm) was cut into 1 cm segments, washed in detergent and then soaked in ethanol for 10 minutes, rinsed in distilled water, and soaked in 95 percent ethanol for 10 minutes, rinsed in distilled water, and soaked in potassium hydroxide (1 N) for 10 minutes. The tubes were then washed in distilled water, soaked for 18 hours in hydrochloric acid (1 N), washed once more in glass-distilled water and dried at 100°C. Four segments of glass separated by rubber "O" rings were mounted on metal probes. The assembled segments were immersed in acid soluble collagen for 2 minutes and then rinsed in modified Tyrode's solution

(no calcium or glucose) for 30 seconds at room temperature. The segments on the probe were stored in a humid atmosphere for 15 minutes before use.

The segments of collagen-coated glass were rotated in a suspension of 300,000 platelets per mm^3, rinsed in calcium-free Tyrode's solution containing EDTA (10 mmol/L); the radoactivity associated with the segments was measured and adherence was calculated as described previously.

10. Release of ^{14}C-serotonin from Adherent Platelets

The extent of release of granule contents from platelets adherent to collagen-coated glass mounted on the rotating probe was determined as previously described (Cazenave et al., 1973). Platelets were doubly-labelled with ^{14}C-serotonin and ^{51}chromium. Following rotation of the collagen-coated glass segments in a platelet suspension containing 5 mmol/L imipramine (to prevent reuptake of released serotonin), and rinsing in Tyrode's solution containing 10 mmol/L EDTA as previously described, the segments of glass were incubated in a scintillation vial with 1 mL of tissue solubilizer (NCS; Amersham/Searle, Arlington Heights, IL). Scintillation fluid (10 mL) was added to the vial and the ^{14}C and the ^{51}Cr were determined by counting in a Phillips liquid scintillation analyzer. The radioactivity due to each isotope was measured by the method of Sheppard and Marlow (1971). The ^{14}C-serotonin released from the platelets

adherent to the surface was calculated by comparing the ratios of ^{14}carbon to ^{51}chromium in the platelets in suspension before rotation with the ^{14}carbon to ^{51}chromium ratio in the adherent platelets and expressed as a percentage.

11. Reactivity of Injured Aortae for Platelets following Incubation in Eagle's Medium, Plasma, or Serum

The effect of incubating vessels in tissue culture medium at 37°C on the reactivity of the damaged aortic surface was examined. A balloon catheter was used to remove the endothelium from the aortae in situ and the vessels were everted on segments of polyethylene tubing as previously described. The vessels were placed on the rotating probe and incubated overnight in flowing, oxygenated Eagle's medium, citrated platelet-poor plasma, or heat inactivated serum; 50 units of penicillin and 50 units of streptomycin per mL of medium were added to inhibit bacterial growth. As before, Eagle's medium was supplemented with 2.2 g sodium bicarbonate per litre and 4 per cent bovine albumin, and contained 5 mmol/L Hepes buffer to maintain pH at 7.35.

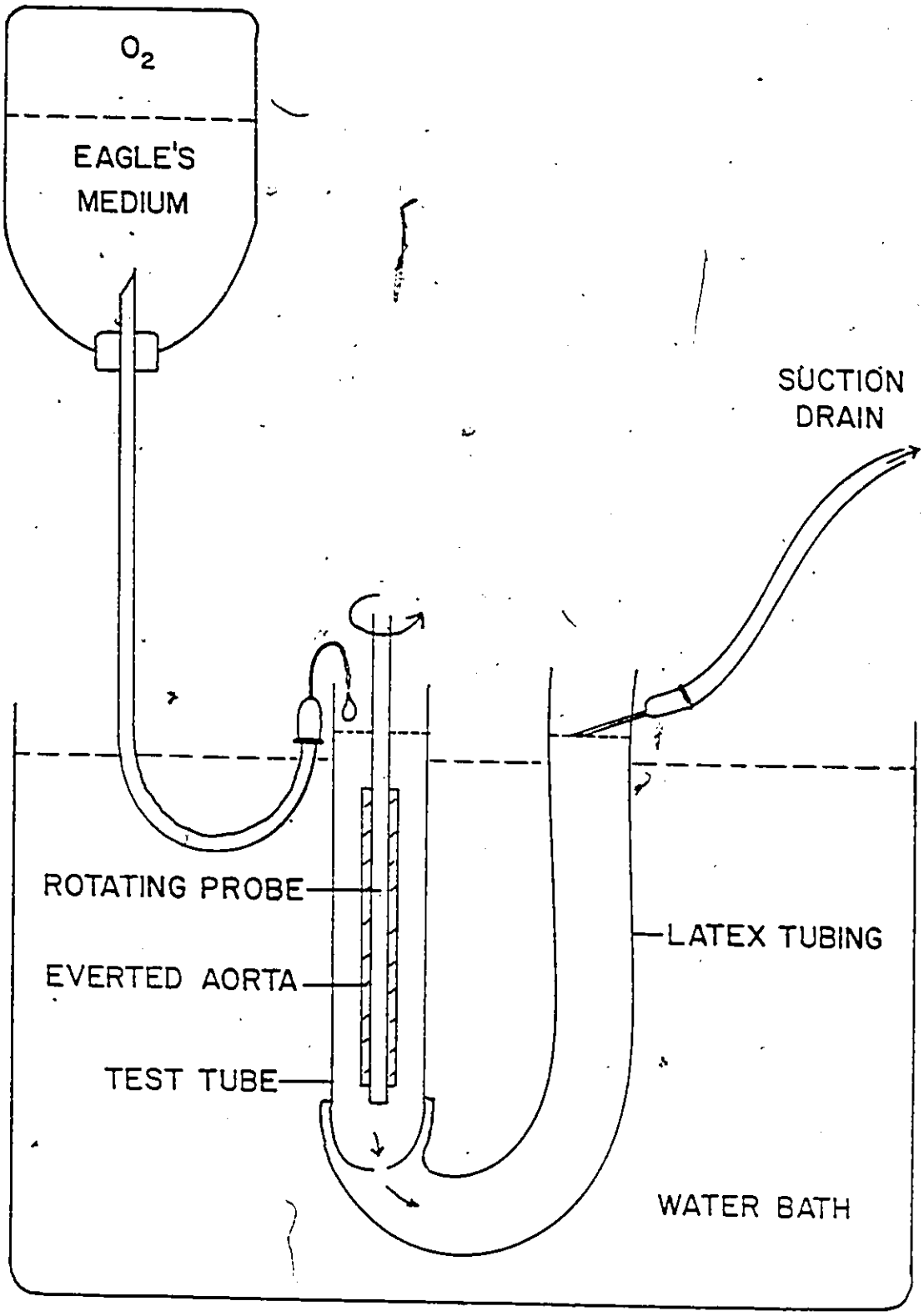
To ensure a continuous flow of fresh medium over the vessels, one end of a segment of latex tubing was secured over the bottom of a test tube containing the everted aorta, and the other end was secured at the same level as the top of the test tube (Figure 1). A small hole in the bottom of the

FIGURE 1

APPARATUS FOR INCUBATING AORTAE

This diagram illustrates the apparatus used for the incubation of everted rabbit aortae mounted on a rotating probe in Eagle's medium, plasma, or serum for eight hours. Fresh oxygenated medium was delivered from an intravenous bottle through tubing to the test tube containing the aorta mounted on the probe. Medium drained from the test tube through a small hole in the bottom of the tube, and was removed by suction from the top of the latex tubing. The tube containing the aorta and the medium, and the attached latex tubing was immersed in a water bath at 37°C. The apparatus is described in more detail in the text.

FIGURE 1


O_2
EAGLE'S
MEDIUM
SUCTION
DRAIN
ROTATING PROBE
EVERTED AORTA
TEST TUBE
LATEX TUBING
WATER BATH

tube permitted the medium to flow from the glass tube to the segment of tubing. A 25 gauge needle connected to a vacuum drainage system was placed through the rubber tubing just below the top of the test tube, so that, as fresh medium was added to the top of the glass tube, the medium at the bottom flowed through the hole in the bottom of the test tube and was withdrawn from the top of the rubber tubing. This provided a flow of fresh medium past the surface of the rotating vessel.

Oxygenated Eagle's medium, plasma, or serum was placed in an intraveous bottle and the air in the bottle was replaced with oxygen to maintain the oxygenation of the medium. The bottle of medium was suspended above the rotating probe system, and connected to the test tube containing the vessel by an intravenous administration set. The flow rate was adjusted to deliver 20 mL of medium per hour into the glass tube containing the aorta mounted on the rotating probe (140 rpm). At the end of the period of incubation in culture medium, the reactivity of the surface of the vessel was determined by exposing the vessels on a rotating probe to a suspension of ^{51}Cr-labelled platelets and quantitating platelet adhesion as previously described (see page 112).

12. Studies of Platelet Survival in Rabbits

^{51}Cr-labelled platelets (5 mL of 2.5×10^6 platelets per mm^3 suspended in plasma or Tyrode's-albumin solution)

were injected into rabbits 18 hours before the aorta was injured with a balloon catheter. Samples of blood (1.5 mL) were collected from the marginal ear vein immediately before the aortae were injured, and 10, 30 and 60 minutes, and daily for 5 days following injury. The radioactivity in the samples of whole blood was measured and the mean platelet survival was calculated using the gamma function described by Murphy and coworkers (Murphy et al., 1973; Scheffel et al., 1977; Murphy and Bolling, 1978). The computer program for these calculations was kindly supplied by Dr E.A. Murphy, Johns Hopkins Hospital School of Medicine, Baltimore, Maryland.

13. Studies of Platelet Aggregation

The procedure used for the study of platelet aggregation was previously described by Kinlough-Rathbone and her colleagues, 1983. Blood was collected through a polyethylene cannula in the carotid artery into 3.8 per cent trisodium citrate (1 part anticoagulant to 9 parts blood), transferred to a polycarbonate centrifuge tube and centrifuged at 190 x g for 15 minutes. The platelet-rich plasma (PRP) was transferred with a siliconized pipette to a siliconized glass tube and stored in a water bath at 37°C until used. The platelet count was adjusted to 500,000 per mm^3 with platelet-poor plasma. One mL of PRP was transferred to a glass cuvette that was placed in a turbidometric device

at 37°C (Payton Aggregation Module, Payton Associates, Scarborough, Ontario). An annealed nickel wire stir bar was added and the speed of the magnetic stirrer adjusted to 1,100 rpm. After 1 minute the aggregating agent was added to the PRP in the cuvette. Light transmission at 609 nm was recorded continuously with a potentiometric pen recorder. The extent of platelet aggregation was indicated by the maximum height of the aggregation curve.

14. Effect of Aspirin Treatment on Platelet Accumulation on Rabbit Aortae In Vivo

a) Accumulation of ^{51}Cr-platelets on damaged vessels

The effect of intravenous administration of acetylsalicylic acid (ASA; aspirin, 25 or 2.5 mg/kg) on platelet accumulation on the endothelium, subendothelium, or injured neointima of rabbit aortae in vivo was examined. Animals that had received an infusion of ^{51}Cr-labelled platelets 18 hours earlier were given an intravenous injection of aspirin 10 minutes before a balloon catheter was used to remove the endothelium from the aortae or to injure the neointima of a previously damaged vessel wall. Ten minutes following the injury the animals were killed by perfusion-fixation.

b) Association of ^{51}Cr- platelets with uninjured vessels

To examine the effect of aspirin on platelet accumulation on the endothelium of undamaged vessels, or on

aorta that were injured from 1 to 7 days previously, animals received an infusion of ^{51}Cr-labelled platelets and an injection of aspirin 30 minutes before they were killed by perfusion-fixation. The radioactivity associated with the vessels was measured, and platelet accumulation calculated as previously described (see page 109).

15. Effect of Aspirin Treatment of Aortae on Adherence of Platelets In Vitro

To determine the effect of incubating aortae with aspirin on platelet adhesion to the vessels, aortae were incubated in aspirin (2 mmol/L) or Tyrode's solution (control) in Eagle's medium containing 4 per cent albumin for 30 minutes. The vessels were placed on a probe and rotated in a suspension of ^{51}Cr-labelled platelets, and the platelet adherence was determined as previously described (see page 112).

16. Effect of Aspirin Treatment of Platelets on their Adherence to Damaged Vessel Walls

Platelets were labelled with ^{51}chromium in the first washing solution and incubated with 2 mmol/L aspirin for 30 minutes. The labelled, aspirin-treated platelets were then injected into rabbits. The endothelium was removed from the aorta with a balloon catheter, and 30 minutes following injury, the animals were killed by perfusion-fixation.

Platelet adherence was determined as previously described.

17. Bioassays for PGI_2-like Activity

PGI_2 was assayed by its ability to inhibit thrombin-induced release of ^{14}C-serotonin from prelabelled platelet suspensions. PGI_2-like activity was determined by a modification of the method of Baenziger et al., (1979). Suspensions of washed rabbit platelets were prelabelled in the first washing solution with ^{14}C-serotonin and were incubated with 1 mmol/L aspirin for 15 minutes at 22°C in the second washing solution. The platelets were treated with aspirin to prevent them from contributing prostaglandin endoperoxides or thromboxane A_2 that could act synergistically with thrombin and mask any inhibitory effects of low concentrations of PGI_2 during bioassay of the test samples. The platelets were finally suspended in Tyrode's solution containing 0.35 per cent albumin, apyrase and 5 mmol/L Hepes buffer.

Samples for assay of PGI_2-like activity were prepared in two ways. In the first method, samples were taken from the supernatants prepared from the platelet-red cell suspensions in which the everted aortae had been rotated for the determination of platelet adherence. In the second method, segments of vessel were mechanically stimulated in a small volume of fluid to maximize the concentration of PGI_2 in the fluid.

a) Method 1:

Immediately after the aortae were rotated in the platelet-red cell suspension for tests of platelet adherence, they were removed from the suspension and the platelet-red cell mixture was centrifuged at 12,000 x g for 1 minute in an Eppendorf Centrifuge (Brinkman, Rexdale, Ontario). The PGI_2 in the supernatant was measured approximately 3 minutes after completion of the platelet adherence study. In each test, 0.4 mL of the suspension of washed ^{14}C-serotonin labelled platelets (platelet count $10^6/mm^3$) was stirred at 1,100 rpm in a turbidometric device at 37°C; imipramine (at a final concentration of 2 umol/L) was added to the suspension to prevent reuptake of ^{14}C-serotonin by the platelets. Five seconds later, 0.6 mL of the supernatant test fluid or control solution was added to the aggregation cuvette and 30 seconds later thrombin was added. A concentration of thrombin was chosen that caused approximately 40 per cent release of granule contents at 60 seconds and 60 per cent at 120 seconds (suitable concentrations ranged from 0.015 to 0.025 U/mL). The extent of the release reaction was measured at 60 seconds or 120 seconds on samples prepared as follows; 0.1 mL of the mixture of platelet suspension and test material was taken directly into 0.1 mL of ice-cold 1.2 per cent paraformaldehyde, centrifuged for 60 seconds at 12,000 x g and a sample of the supernatant fluid taken for measurement of released ^{14}C-serotonin. The percentage inhibition of

thrombin-induced release caused by the test material was calculated and the amount of PGI_2 produced was determined using a dose-response curve that was constructed using the same concentration of thrombin and a range of concentrations of synthetic PGI_2 (The Upjohn Company, Kalamazoo, MI).

b) Method 2:

A segment of aorta (approximate surface area 56 mm^2) was removed from the everted aorta just before platelet adherence was to be measured. The segment was incubated in 0.15 mL of Eagle's medium containing 4 per cent bovine serum albumin and 5 mmol/L Hepes for 5 minutes at 37°C. In experiments in which the endothelium had been removed with a balloon catheter, the segment was mechanically stimulated with a Pasteur pipette for 30 seconds at the beginning of the 5 min incubation period. Samples (0.8 mL) of platelet suspension (500,000/mm^3) containing 2 umol imipramine were stirred in an aggregation cuvette to which 0.1 mL of test fluid was added followed in 30 seconds by thrombin. Samples for PGI_2 determination were taken and assayed as described in method 1. Although it was not possible to standardize the mechanical stimulation of the aortic segment, it was possible to ascertain whether aspirin treatment of the segment had abolished its ability to produce PGI_2. The dry weights of the segments of aortae were obtained after fixation in 2 mL of formalin overnight. The segments were removed from the

formalin solution and dried for 5 hours at 56°C before they were weighed. With the concentration of thrombin used, the assay could detect as little as 0.02 ng of PGI_2/mg dry weight of vessel wall.

18. Effect on Reactivity of Vessel Walls of Inhibiting Platelet Accumulation with Dipyridamole

The effect of a single dose of dipyridamole given 10 minutes before injury on the accumulation of ^{51}Cr-labelled platelets at several times following the removal of the endothelium with a balloon catheter was examined. In some studies, the initial injection of dipyridamole (12.5 mg/kg given 10 minutes before injury) was followed by hourly injections (5 mg/kg) for 4 or 8 hours to maintain a concentration of dipyridamole in the blood that inhibited platelet accumulation on the injured surface.

Dipyridamole was administered through intravenous cannulae in animals that were given repeated doses of the drug. Immediately before injury to the aorta with a balloon catheter, a cannula of PE 190 polyethylene tubing, filled with saline, was passed through a skin incision at the base of the skull, introduced into the right jugular vein, and secured with silk sutures. The incision was closed with silk sutures and the cannula was secured at the point of exit. Dipyridamole was administered through an 18 gauge needle attached to the PE 190 tubing. The accumulation of platelets

on the vessel surface immediately after the drug treatment, and 8 hours following the end of drug treatment was determined.

19. Infusion of PGI_2

a) Short term infusions (ten minutes)

A Harvard infusion pump (model 927) was used to provide a constant rate of infusion of PGI_2. For each animal, the pump was calibrated to infuse 850 ng of PGI_2 in 0.1 mL of solution per min per kg of body weight. A syringe and a length of polyethylene cannula tubing (PE 190) filled with a solution of PGI_2 was connected to the infusion pump. Animals that had received an infusion of ^{51}Cr-labelled platelets were preanaesthetized with ketamine (1 mL intramuscularly) and atropine (0.1 mg subcutaneously) 20 minutes before being anaesthetized with sodium pentobabital (40 mg/kg). A cannula was introduced through the left common carotid artery and passed into the ascending aorta to approximately 0.5 cm above the aortic valve. The infusion of PGI_2 was begun 30 seconds before the aorta was injured with a balloon catheter, and was continued for a 10 minute period after the aorta was de-endothelialized with a balloon catheter. Two minutes before the completion of the PGI_2 infusion, cannulae were introduced into the femoral arteries to prepare the animals for perfusion-fixation. Immediately before the end of the

PGI_2 infusion the animals received an intravenous injection of 1,000 units of heparin. Locke's-Ringer solution containing heparin (1 U/mL) was perfused through the carotid cannula immediately following the end of the PGI_2 infusion; this was followed by perfusion-fixation. The vessels were removed, the radioactivity associated with the vessels measured, and the number of platelets associated with the vessels calculated as previously described (see page 109).

b) Long term infusion of PGI_2 (up to eight hours)

Studies on the effect of inhibiting the interaction of platelets with the vessel walls with PGI_2 required that a constant infusion of the drug be maintained for periods of up to 8 hours. Although the rabbits were anaesthetized for the surgical procedures, they were allowed to recover from the anaesthetic and were conscious for the remainder of the infusion period.

A Harvard infusion pump was calibrated to deliver a constant amount of a solution of PGI_2 (850 ng/kg/min) from a 20 mL syringe connected to polyethylene tubing (PE 20); the infusion rate was 1 mL per hour per kg of body weight. A PE 20 cannula was used in these studies because, for a given rate of fluid infusion, the linear rate of flow through the cannula was much higher in the smaller diameter cannula (14.7 cm per min per kg of body weight for PE 20; compared to 1.5 cm per min per kg of body weight for PE 190 tubing). The

higher linear rate of flow in the PE 20 cannula reduced the possibility of a backflow of blood that occurred in the PE 190 cannula when the infusion rate was low. The PE 20 tubing was not required in the short term studies because a less concentrated solution of PGI_2 was infused at a higher rate of flow. (Since the pump could not provide the pressure required to produce the flow rate through a PE 20 cannula that would be required to deliver the larger volume of more dilute PGI_2 solution, PE 190 tubing was used only in the short term studies. A linear flow rate of 88.2 cm per min per kg of body weight would have been required for a PE 20 cannula.)

The syringe and polyethylene tubing were filled with a solution of PGI_2, and air bubbles were removed from the system to prevent fluctuations in infusion flow that could result from volume changes in the system produced by the compression of the gas due to changes in the animals' blood pressure, or pressure changes that occur during the cardiac cycle. The syringe attached to the pump was packed in ice bags to prevent the breakdown of PGI_2 during the period of infusion.

The infusion pump was turned on to establish the flow of the solution of PGI_2 and the end of the cannula for the infusion of PGI_2 was passed subcutaneously from an incision at the base of the scalp and introduced through the right common carotid artery to approximately 0.5 cm above the aortic valve. To ensure that a constant rate of infusion of

PGI_2 was established before the vessel wall was injured, the flow of PGI_2 was begun for 10 minutes before the endothelium was removed with a balloon catheter. The anaesthetized animals were placed in individual plastic baskets at the same level as the pump and allowed to recover in a quiet, darkened room. The animals were monitored continuously during the remainder of the infusion period to ensure that their activity during and following recovery did not interfere with the continuous infusion of PGI_2.

Shortly before the end of the infusion period, the animals were anaesthetized and prepared for perfusion-fixation through a PE 190 cannula introduced through the left common carotid artery. The animals were given an intravenous injection of heparin (1000 U) and the perfusion of Locke's-Ringer solution containing heparin (1 U/mL) was begun immediately following the end of the PGI_2 infusion.

20. Measurement of Blood Pressure during Infusion of PGI_2 or Treatment With Dipyridamole

A pressure-sensitive transducer was placed in the right carotid artery of the anaesthetized animals and connected to a blood pressure recorder (Mingograf 34, Elma Schonander, Stockholm, Sweden). The monitoring of blood pressure was begun before the administration of PGI_2 or dipyridamole and continued until the animal was prepared for perfusion of Locke's-Ringer solution and fixative.

21. Activation of Coagulation by Rabbit Aortae In Vitro

A coagulation chamber was used to determine the capacity of the luminal surface of the aorta to activate coagulation. The chamber was designed so that the luminal surface of the vessel formed the floor of the chamber, and the internal surface of a segment of siliconized glass tubing formed the walls. The glass was siliconized so that contact with the glass would not activate factor XII. Plasma was added to the chamber, recalcified, and the time required for a fibrin clot to form was measured.

a) Apparatus:

The coagulation chamber used in these studies is illustrated in Figures 2a and 2b. The plexiglass blocks forming the base and top of the apparatus were held together with bolts secured with wing nuts. A one cm segment of glass tubing (outside diametre 0.9 cm and inside diametre 0.6 cm) formed a liner that fit precisely into a central hole in the top block. The area of tissue exposed at the floor of the chamber was 0.28 cm^2. At the top of the glass liner the hole in the plexiglass top narrowed to form a shoulder that prevented the glass from shifting and kept the bottom of the glass level with the base of the upper plexiglass block. A removable siliconized glass liner was used to form the walls of the chamber so that it could be replaced quickly with a

FIGURE 2

AORTA-CHAMBER USED TO DETERMINE

THE PROCOAGULANT ACTIVITY OF A VESSEL

The parts of the apparatus are illustrated in the diagram. The plexiglass base and top were secured with wing nuts (W) and bolts (B) (Figure a). The siliconized glass liner (G) fit into the hole in the top block. The tissue (T) at the bottom of the glass liner, formed the floor of the chamber. The tissue was surrounded by the gasket (K).

Figure b shows a diagram of the assembled chamber. Plasma was added to the chamber, recalcified, and the time required for coagulation to occur was determined. The wire hook was passed through the plasma at 1 to 2 second intervals. The coagulation time was measured as the time when the plasma viscosity increased, or fibrin strands formed on the wire hook. The bend in the wire at the top of the chamber prevented the hook from coming in contact with and damaging the tissue.

FIGURE 2a

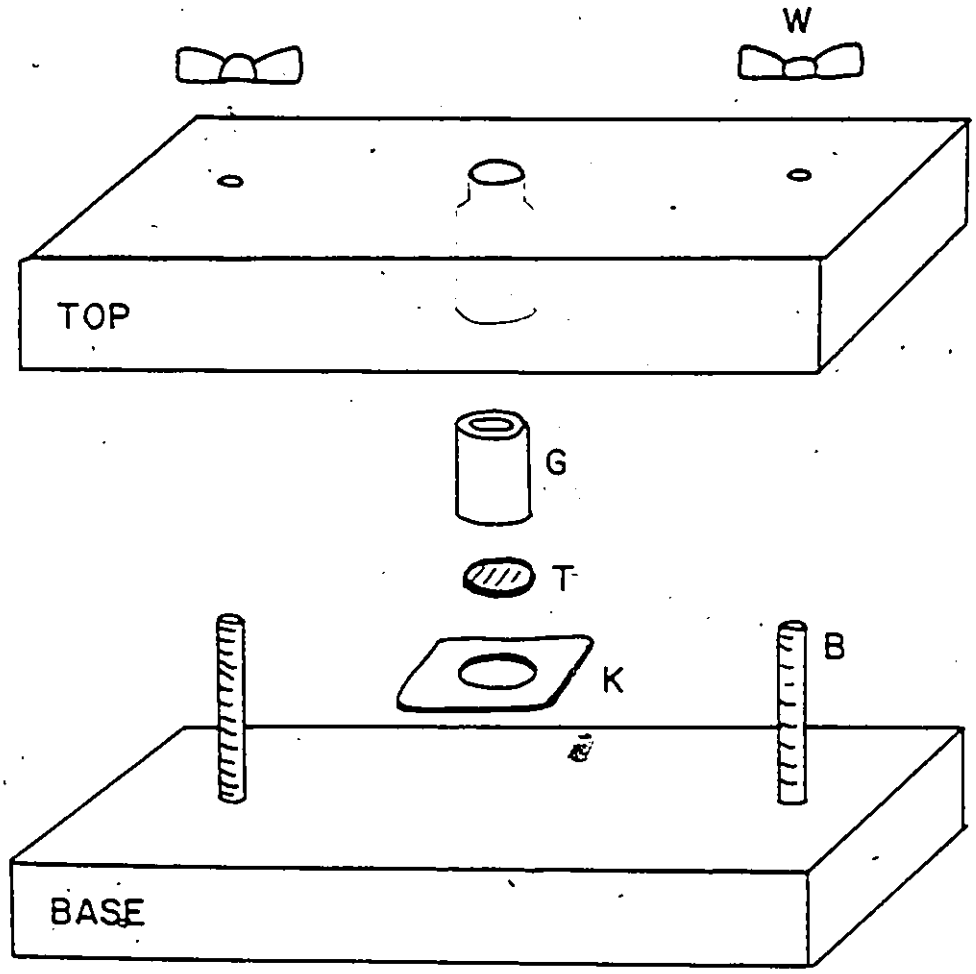
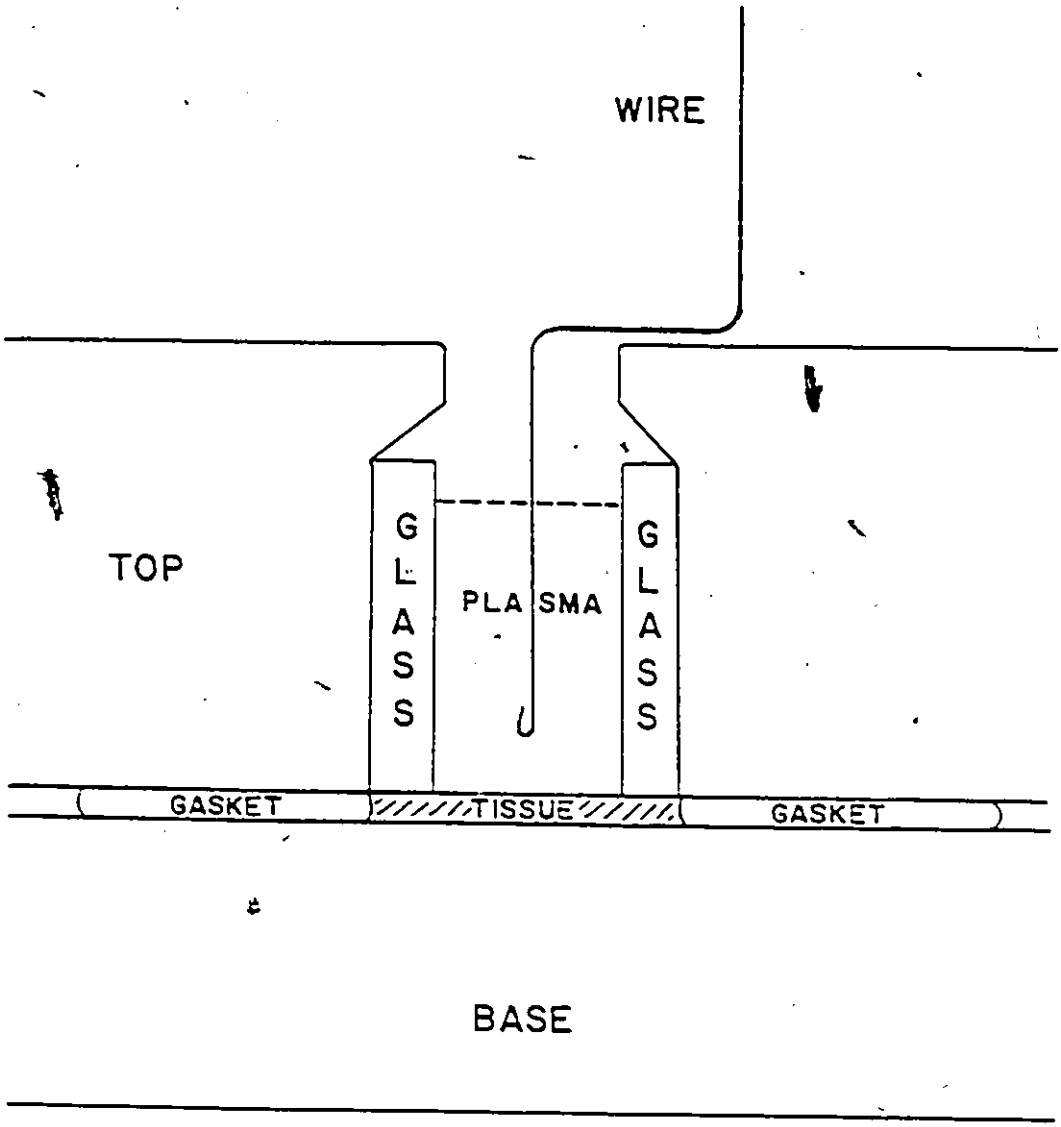


FIGURE 2b



new liner for the next coagulation assay. The central hole in the rubber gasket that surrounded the tissue, was cut with the same punch as the tissue so that the tissue fit precisely within the hole in the gasket. Both sides of the gasket were coated with a fine film of high vacuum grease so that a water-tight seal was formed when the base was secured to the top of the apparatus. The gasket was slightly thicker than the tissue so that, when the wing nuts were tightened, the bottom of the glass liner sat firmly on the tissue but did not crush the specimen, thereby reducing the possibility of the release of tissue factor that could have activated the extrinsic pathway of coagulation.

b) Method:

To prepare aortae for the determination of the procoagulant activity of their luminal surface, the vessels were prepared as described in the section on adherence of platelets to vessel walls in vitro. The coagulation chamber and siliconized glass liners were warmed to 37°C. Citrated platelet-poor plasma (one part 3.8 per cent sodium citrate to 9 parts blood) and $CaCl_2$ (0.4 mol/L) were prewarmed shortly before use. The thoracic aorta immersed in Eagle's medium was opened along the dorsal surface and circles of tissue were cut from the vessel by pressing down firmly with a stainless steel punch (diametre 0.9 cm). Six to 7 pieces of tissue were obtained from the thoracic aorta of a 2.5 to 3 kg

rabbit. To assemble the chamber, the top block was inverted on a warming plate in a 37°C water bath, a glass liner was inserted in the block, the gasket was placed on the top block so the central hole was in line with the glass liner, and a segment of tissue with the luminal side down was gently placed over the base of the glass chamber liner, the plexiglas base was placed over the plexiglass top, the apparatus was turned upright, and the wing nuts were secured. Plasma (0.19 mL) was added to the chamber and, ten seconds later, 0.01 mL of 0.4 mol/L $CaCl_2$ was added and the timer was started. The apparatus was rapidly rotated on the warming plate for 20 seconds to mix the reagents. A fine wire with a small hook in the end was passed through the plasma every 1 to 2 seconds. When clotting occured there was an increase in the viscosity of the plasma and gelatinous or fibrous material could be seen adhering to the wire hook. The wire was bent approximately one cm from the end so that the shoulder of the well prevented the hook from coming in contact with and damaging the tissue.

22. Light microscopy

For light microscopy, specimens of tissue fixed with formaldehyde, gluteraldehyde or paraformaldehyde were dehydrated through increasing concentrations of ethanol, and then embedded in Histowax, or cleared in xylene and embedded in Paraplast. Sections (4 um) were stained with haematoxylin

and eosin (H and E) for routine histology, Martius yellow-scarlet red-celestine blue (MSB) to demonstrate fibrin, and Verhoeff-van Gieson (VvG) to demonstrate elastic tissue.

23. Scanning Electron Microscopy

Segments of aorta aproximately 2.5 cm in length were cut open to expose the luminal surface and attached to a coverglass with cyano-acrylic glue (Eastman 910) (Richardson and Moore, 1980). The tissue was kept moist with 0.2 mol/L cacodylate buffer during the mounting procedure. The specimen was post fixed in osmium tetroxide, dehydrated through graded ethanol, critical point dried from CO_2, mounted on a SEM stub with double sided tape, and coated with gold. Specimens were examined in a Philips 501 SEM.

24. Transmission Electron Microscopy

Tissue for examination by electron microscopy was diced so that each sample had a surface area of approximately 1 mm^2. The specimens were post-fixed in 1 per cent buffered osmium tetroxide in 0.1 mol/L sodium cacodylate, embedded in Spurr's firm resin (Spurr, 1969), and sectioned on a Reichert Ultramicrotome. Ultrathin sections were collected on uncoated 200-mesh copper grids, stained with lead citrate (Venable and Coggeshall, 1965), and examined on a Philips 301 or an AMR 1000 (Advanced Metal Research) transmission electron microscope.

25. Statistical Analyses

All results are expressed as mean ± standard error. An analysis of variance, and a Student's 't' test or a Studentized range test was used for statistical comparisons. Statistical significance was defined at a p value of <0.05.

CHAPTER III

NEOINTIMAL INJURY

NEOINTIMAL INJURY

A. Introduction

Most of the studies on platelet response to vascular injury have examined the accumulation of platelets following injury to a previously undamaged vessel. In previous studies it was demonstrated that a layer of platelets with little evidence of fibrin formed on the subendothelium exposed by a single injury with a balloon catheter (Groves et al., 1977; Groves et al., 1979). However, Stemerman (1973) observed that platelet-fibrin thrombi formed on the surface of a vessel following injury to the neointima that had formed 4 weeks after removal of the endothelium. The presence of fibrin on the injured neointima indicated that coagulation had been activated on this surface. Since fibrin formed on the injured neointima, whereas there was little evidence of fibrin on the exposed subendothelium, it appeared that the properties of the surface exposed by injury to the neointima differed from the properties of the surface exposed by injury of a normal vessel.

Coagulation can be activated through two pathways and each of these pathways could possibly contribute to the development of platelet-fibrin thrombi on injured vessel walls. For example, the exposure of factor XII (Hageman factor) to collagen activates the intrinsic pathway of coagulation (Ratnoff and Rosenblum, 1957), whereas tissue factor (tissue

thromboplastin) elaborated at sites of injury activates the extrinsic pathway of coagulation (Howell, 1912; Nemerson and Bach, 1982). Thrombus formation on a damaged vessel can involve platelet adherence to connective tissue components, or the activation of coagulation by the injured surface. Since fibrin forms on reinjured or diseased vessels (Stemerman, 1973), and platelets are stimulated by thrombin and interact with fibrin during its polymerization (Niewiarowski et al., 1966), some of the platelet accumulation on the injured neointima might be dependent on the activation of coagulation. Therefore, it was important to determine a) the role that thrombin generation and fibrin formation play in the accumulation of platelets on an injured vessel, and b) the pathway of coagulation most likely to be responsible for the formation of platelet-fibrin thrombi on a reinjured vessel.

In his studies of the reaction to injury of the neointima, Stemerman (1973) did not examine whether the reactivity of an injured neointima to circulating blood constituents changed with time, whether there were changes in the turnover of platelets in the circulation as a result of their interaction with fibrin and activated coagulation factors on the injured vessel, or whether this affected the survival of platelets in the circulation. Therefore, the objectives of the experiments reported in this chapter were to determine:

1. the extent of damage produced by injury with a balloon catheter of a vessel that had previously been de-endothelialized.

2. the extent to which platelets accumulate on the surface of an injured neointima.

3. the extent to which platelets continue to interact with the vessels during specific periods following injury to the neointima.

4. the effect of injury to the neointima on platelet survival.

5. the role of activation of coagulation on platelet accumulation on the injured neointima.

6. the ability of injured neointima to activate coagulation compared to the procoagulant activity of the endothelium, subendothelium and undamaged neointima.

B. Results

1. Injury of the Neointima with a Balloon Catheter: Extent of Damage and Comparison with Single Injury

The passage of a balloon catheter through the lumen of a previously undamaged aorta removes the endothelial lining and exposes the subendothelial connective tissue. Figure 3 shows an aorta 4 days following such an injury. The areas of the vessel from which the endothelium is removed are stained with Evans blue dye that binds to albumin and is carried into the vessel wall in areas of increased permeability. This increased permeability to albumin-bound Evans blue persists until re-endothelialization occurs. Small unstained white regions surrounding intercostal arteries and other branch vessels were areas covered with endothelium that had not been removed by the balloon catheter or had proliferated and spread from intact endothelium lying within the branch vessels: the endothelium in these areas is protected from injury during the mechanical denudation produced by the passage of the balloon catheter.

Figures 4 and 5 show sections of an arterial wall following the passage of a balloon catheter through a previously undamaged aorta. Although the endothelium was removed from the vessel, as seen in the electron micrograph (Figure 5), the internal elastic lamina appears largely intact (Figures 4 and 5).

FIGURE 3

EVANS BLUE STAINED AORTA

FOUR DAYS FOLLOWING REMOVAL OF THE ENDOTHELIUM

To determine the extent to which the balloon catheter injured the aorta, some of the animals were given an injection of Evans blue dye (4.5 mg/kg) 30 minutes before they were killed. Dark areas of this aorta indicate the regions where the endothelium was removed and the albumin-bound dye penetrated the vessel. Pale regions represent areas of the aorta that were not damaged by the balloon catheter, and areas of endothelium that had proliferated onto the aorta from intact endothelium lining the intercostal arteries; albumin-bound dye does not penetrate intact endothelium significantly.

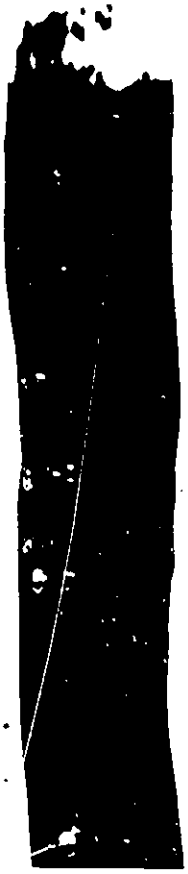


FIGURE 4

LIGHT MICROGRAPH OF A RABBIT AORTA

FOLLOWING A SINGLE INJURY WITH A BALLOON CATHETER

A rabbit aorta following the removal of the endothelium with a balloon catheter is shown in this light micrograph. The internal elastic lamina (arrow) appears intact. The tissue was stained with haematoxylin and eosin. x 250

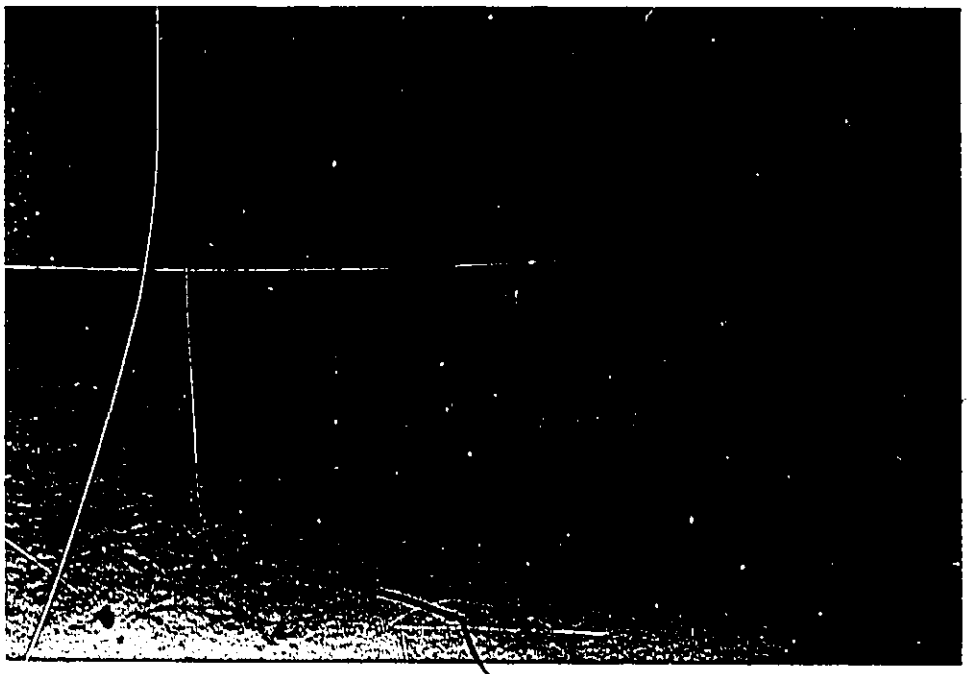


FIGURE 5

TRANSMISSION ELECTRON MICROGRAPH OF RABBIT AORTA FOLLOWING A SINGLE INJURY WITH A BALLOON CATHETER

Transmission electron micrograph of the surface of a rabbit aorta following de-endothelialization with a balloon catheter. The internal elastic lamina (E) exposed by the passage of a balloon catheter appears undamaged. Platelets (P) and a leukocyte (L) can be seen on the surface of the damaged vessel. X 6,500


P
L
E

Seven days after the removal of the endothelium, a smooth muscle cell-rich neointima has formed above the internal elastic lamina of the aortae (Figures 6, 7 and 8). Although most of the vessel is covered by a neointima approximately 4 cell layers thick, there is greater intimal thickening in regions adjacent to and opposite intercostal vessels (Figure 9), and in some areas there is little evidence of intimal thickening (Figure 7). The extent of neointimal thickening in these specimens was mapped morphologically by Dr. L. Jørgensen while he was on sabbatical in our laboratory.

Following balloon catheter injury to the aortic neointima that had formed 7 days following the removal of the endothelium, the neointimal smooth muscle cells can still be seen above the internal elastic lamina in some regions of the vessel (Figure 10), indicating that the balloon catheter did not remove all the smooth muscle cells of the neointima. Therefore, in these regions, the balloon catheter damaged the surface of the smooth muscle cells or exposed deeper layers of the neointima. In some areas it appears that the internal elastic lamina is exposed, indicating that, either 1) the neointima was removed by the balloon catheter, or 2) there was little intimal thickening present in these areas of the vessel before reinjury (Figure 9). These results show that the surface exposed to the flowing blood following injury of the neointima is different from the surface exposed by re-

FIGURE 6

SCANNING ELECTRON MICROGRAPHS OF RABBIT AORTA

SEVEN DAYS FOLLOWING THE REMOVAL OF THE ENDOTHELIUM

This micrograph shows the surface of the smooth muscle cell-neointima that formed by 7 days following the removal of the endothelium with a balloon catheter. Occasional leukocytes (L) are seen on this surface. x 400

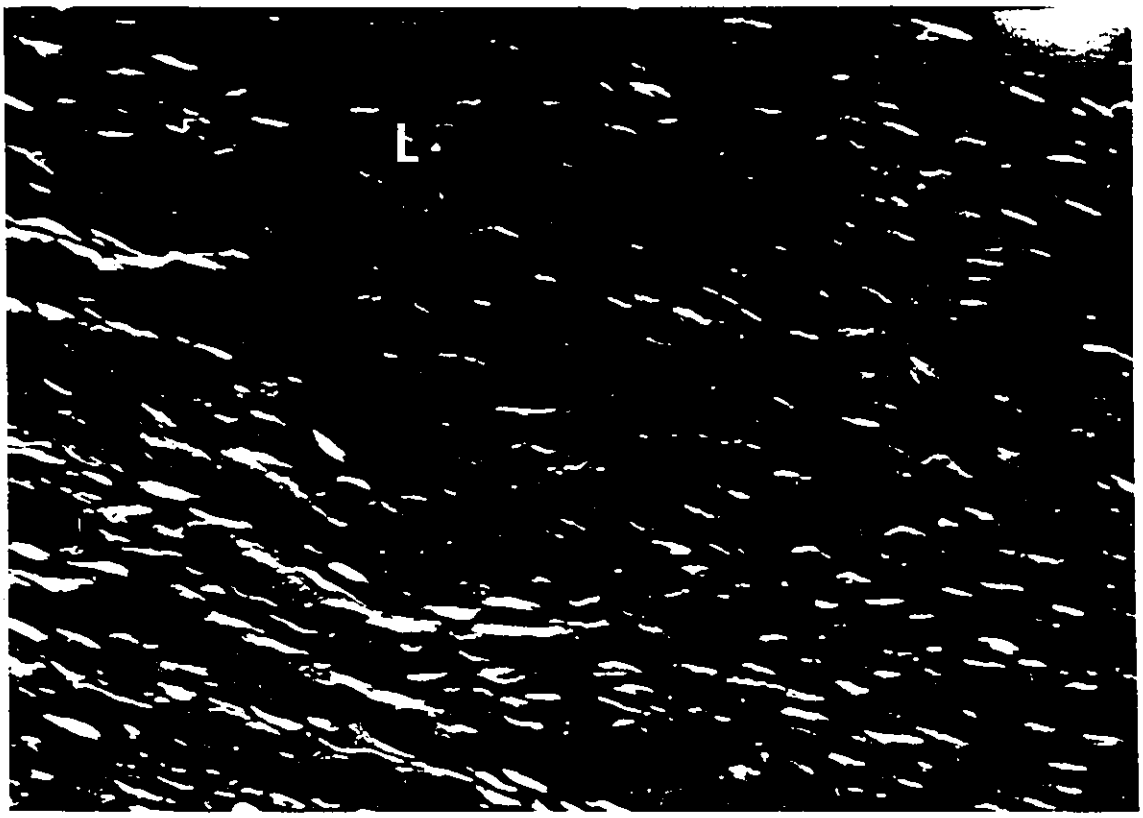

L

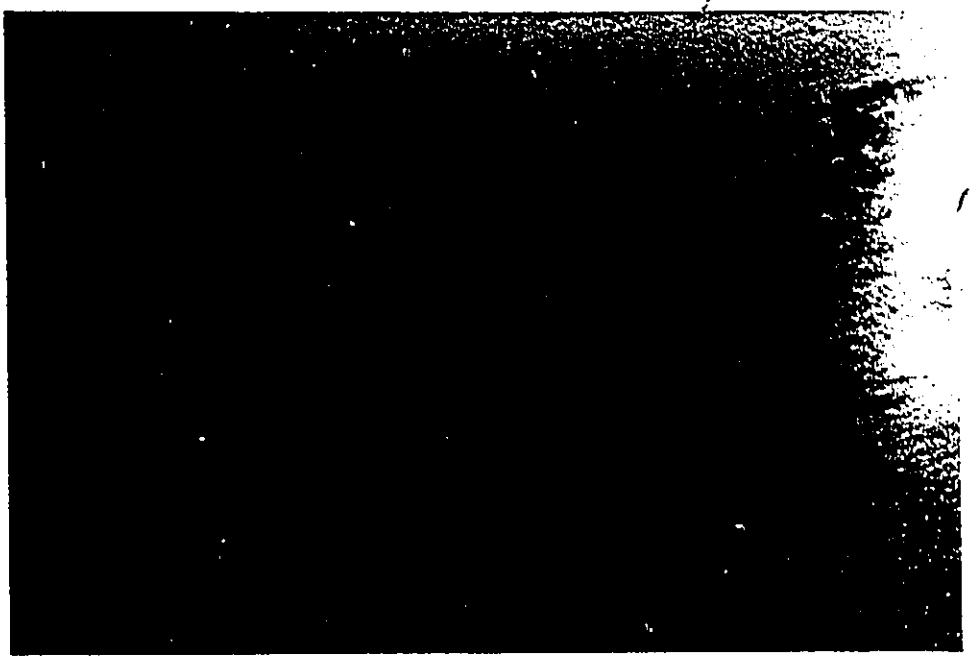
FIGURE 7

NEOINTIMA OF RABBIT AORTA

SEVEN DAYS FOLLOWING THE REMOVAL OF THE ENDOTHELIUM

These light micrographs show: a section of rabbit aorta one week following the removal of the endothelium with a balloon catheter. A. Thickened smooth muscle cell neointima lies above the internal elastic lamina (arrow). x 280 B. At the right is a thickened cushion-like neointima that lies lateral to the ostium of an intercostal artery. There is little intimal thickening in the region of the vessel at the left of the slide. The tissues were stained with hematoxylin and eosin. x 500

A



B

FIGURE 8

NEOINTIMA OF RABBIT AORTA

SEVEN DAYS FOLLOWING THE REMOVAL OF THE ENDOTHELIUM

Transmission electron micrograph of a section of rabbit aorta one week following the removal of the endothelium with a balloon catheter. A neointima composed of several layers of smooth muscle cells (M) lies above the internal elastic lamina (E). x 10,000


E

FIGURE 9

DRAWINGS OF THICKENED INTIMA OF RABBIT AORTA SEVEN DAYS FOLLOWING INJURY

These drawings illustrate the thickened intima observed by light microscopy of transverse sections of rabbit aorta. All these sections are at the level of branches of intercostal arteries in the lower thoracic aorta. There is considerable variation in the extent of intimal thickening; the greatest intimal thickening tends to occur adjacent to and opposite the orifices of branch vessels. These data were obtained in collaboration with Dr. Leif Jørgensen who mapped the extent of intimal thickening in the aorta of animals used in these studies.

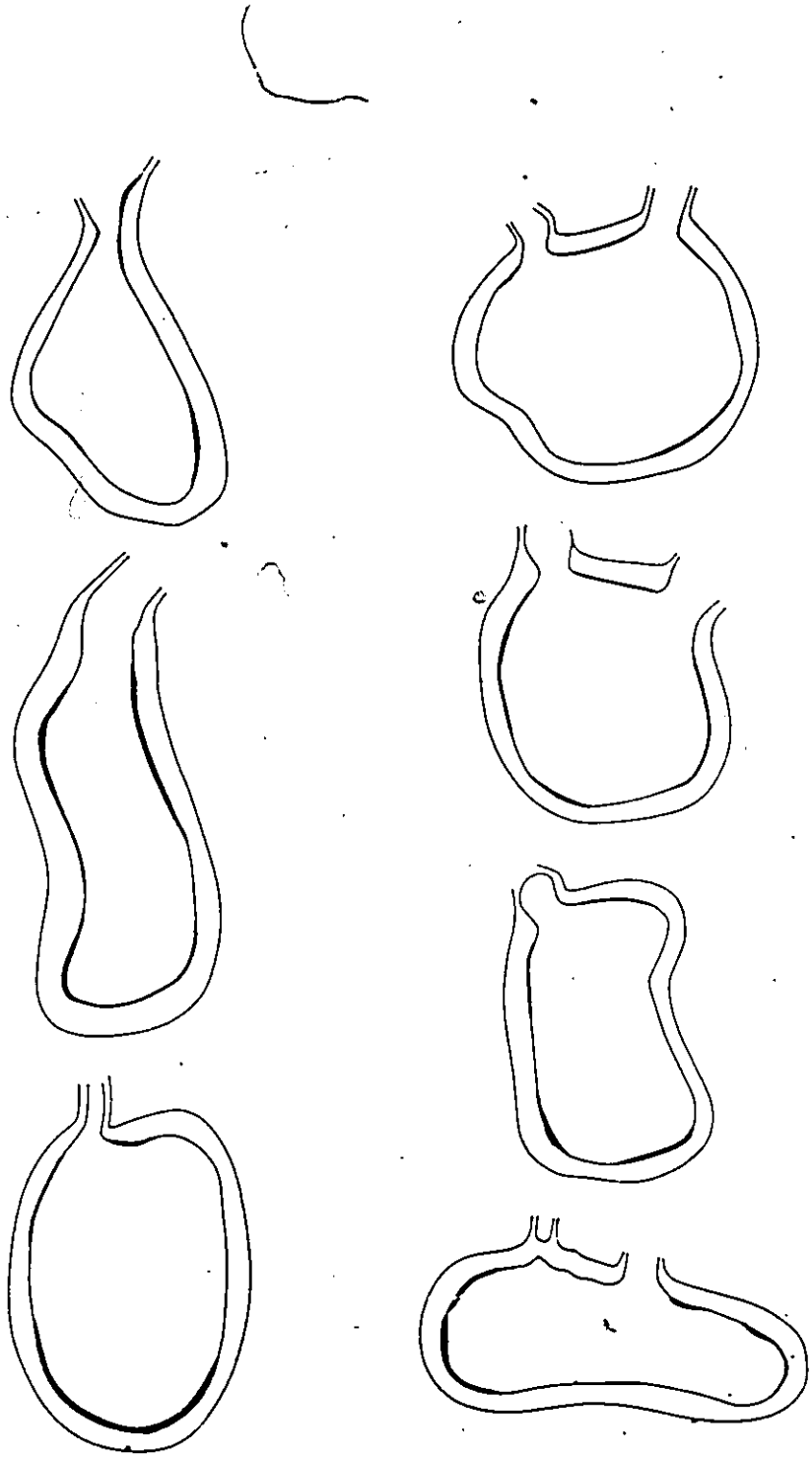


FIGURE 10

RABBIT AORTA FOLLOWING INJURY TO THE NEOINTIMA WITH A BALLOON CATHETER

This light micrograph of a rabbit aorta 30 minutes following injury with a balloon catheter to the neointima that formed by 7 days following de-endothelialization of a previously undamaged vessel, shows the thickened neointima lateral to the ostium of an intercostal artery. This section was stained with Verhoeff's elastic stain. The smooth muscle cell-rich neointima lies above the darkly stained internal elastic lamina. The media of the artery is also rich in darkly staining elastic tissue. x 200

moval of the endothelium from a previously undamaged vessel.

2. Platelet Accumulation on Injured Neointima In Vivo: Comparison with Accumulation on Subendothelium

Previously I showed that, following the removal of the endothelium from an apparently normal vessel, approximately 40,000 platelets/mm^2 accumulate on the exposed subendothelium by 10 minutes after injury (Groves et al., 1979). Although the number of platelets associated with the vessel appears to remain fairly constant during the first 24 hours following injury, 4 days after injury many of the platelets are lost from the vessel wall, and by 7 days few platelets remain associated with the aortae (Table I).

To determine the extent of platelet accumulation on a reinjured vessel, the accumulation of radiolabelled platelets following injury with a balloon catheter to the neointima that formed 4 weeks following removal of the endothelium was quantitated. The number of labelled platelets that accumulated by specific times following injury is shown in Table I. Approximately 50,000 platelets/mm^2 accumulated on the injured neointima 10 minutes following injury. Although there was no significant difference between the accumulation 10 minutes, 30 minutes, one hour or 3 hours following injury to the neointima, by one day following injury fewer platelets were associated with the vessels. Seven days following injury, little radioactivity remained associated with the

TABLE I

ACCUMULATION OF PLATELETS ON AORTAE

FOLLOWING EXPOSURE OF SUBENDOTHELIUM OR INJURY TO NEOINTIMA

TIME AFTER INJURY	PLATELET ACCUMULATION NO. PER SQ. MM.	
	SUBENDOTHELIUM	INJURED NEOINTIMA
10 MIN	40,300 ± 6,700	49,400 ± 5,000 a
30 MIN	40,300 ± 10,000	57,900 ± 14,200 b
1 HR	42,000 ± 1,500	65,100 ± 16,100 c
3 HR	40,700 ± 2,100	40,300 ± 6,700 d
1 DAY	43,200 ± 16,900	18,800 ± 4,900 e
4 DAYS	1,100 ± 100	13,600 ± 4,600 f
7 DAYS	0	7,600 ± 1,800 g

Values are mean ± standard error for 4 animals. ^{51}Cr-labelled platelets were injected 18 hours before removal of the endothelium from a normal vessel (results of previous studies), or injury to the neointima that had formed 4 weeks following exposure of the subendothelium. At specific times following injury the animals were perfused with Locke's-Ringer solution and fixed with paraformaldehyde. The aortae were removed from the animals, the associated radioactivity measured, and platelet accumulation calculated. An analysis of variance of the platelet accumulation on the injured neointima shows that there is no significant difference among the groups a, b, c and d (right column). Results of the Studentized range test show that, for the comparison between abcd vs e, $p<0.05$; abcd vs f, $p<0.005$; abcd vs g, $p<0.001$.

aortae.

These results indicate that, following neointimal injury, either: a) ^{51}Cr-labelled platelets were lost from the surface of the vessel, as occurred following exposure of the subendothelium (Groves et al., 1979), or b) labelled platelets with a high specific radioactivity were lost from the surface and replaced by fresh platelets from the circulation that had a lower specific radioactivity than the platelets that initially accumulated. Therefore, the aortae were examined morphologically to determine the extent of platelet accumulation on the vessels.

3. Morphology of Injured Neointima

Thirty minutes following injury of the smooth muscle cell-rich neointima with a balloon catheter, small platelet-fibrin thrombi are present on much of the surface of the injured vessels (Figures 11-15). These thrombi are aligned in the direction of blood flow, and this is particularly obvious at branch vessels where the thrombi are directed into the orifices in a slightly curved manner (Figure 13). Platelets are associated with fibrin on the surface of the injured neointima (Figures 16 and 17). Figure 18 shows a light micrograph of fibrin on the surface of a vessel stained with Martius scarlet blue. By comparison, figure 19 shows a layer of platelets with no evidence of fibrin on the surface of a vessel 30 minutes following exposure of the subendo-

FIGURE 11

THROMBI ON INJURED NEOINTIMA ADJACENT TO ORIFICE OF INTERCOSTAL ARTERY

Scanning electron micrograph of platelet thrombi on a rabbit aorta adjacent to the orifice of an intercostal artery 30 minutes following injury of the neointima with a balloon catheter. platelets (P). x 4,000

FIGURE 12

THROMBI ON INJURED NEOINITIMA OF RABBIT AORTA

Scanning electron micrographs of small thrombi (Th) at the orifice of an intercostal artery 30 minutes following injury of the neointima with a balloon catheter. A. The intact endothelium (E) lining the intercostal artery was protected from damage during the passage of a balloon catheter. x 1,000. B. A higher magnification of a platelet thrombus. platelets (P). x 4,000

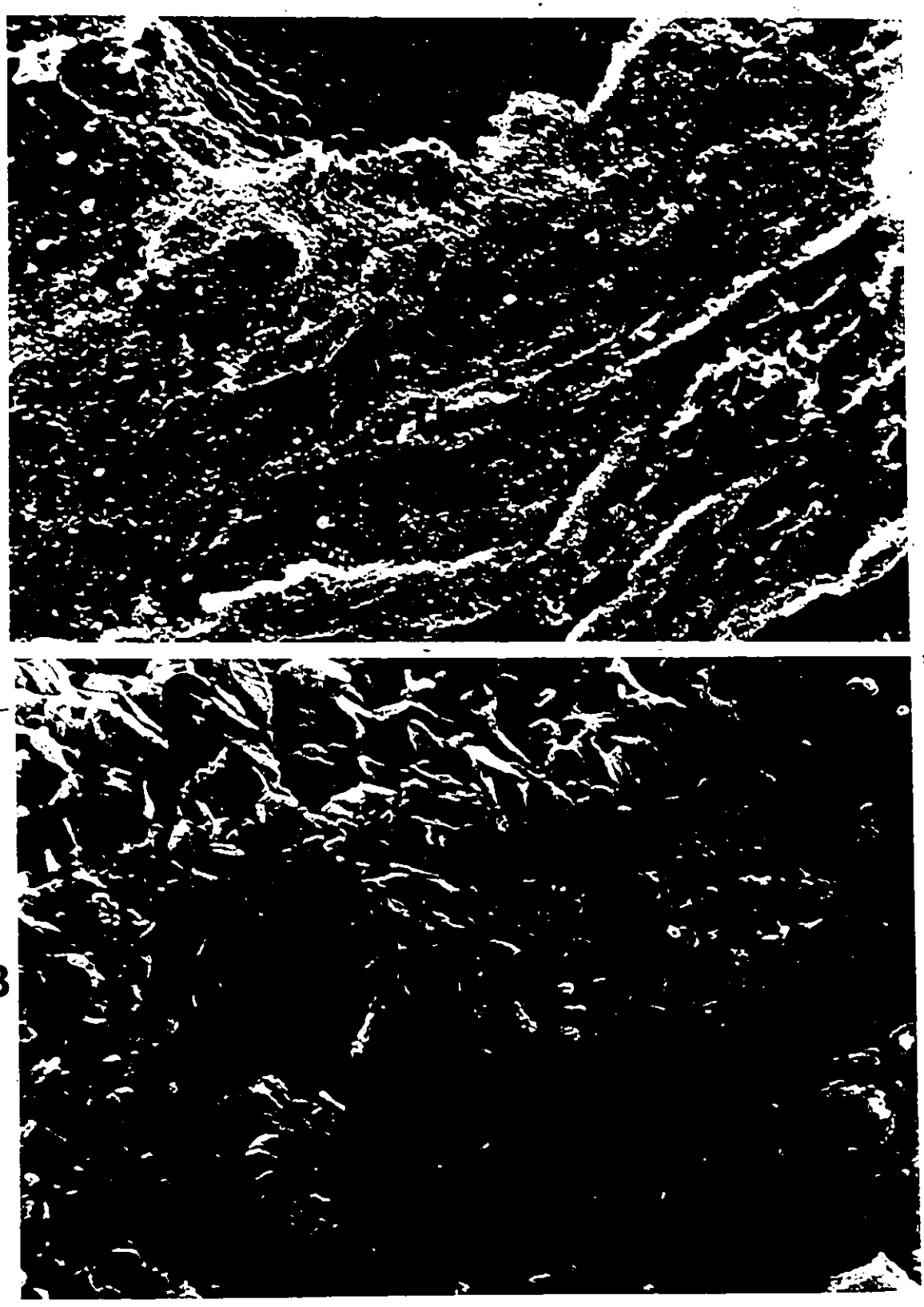

A
B

FIGURE 13

THROMBI ALIGNED IN THE DIRECTION OF FLOW AROUND ORIFICE OF INTERCOSTAL ARTERY

Scanning electron micrograph of thrombi on the surface of a rabbit aorta around the orifice of an intercostal artery 30 minutes following injury to the neointima with a balloon catheter. Thrombi are aligned in the direction of flow in the regions above and adjacent to the vessel orifice. The direction of blood flow is indicated by the arrow. x 600

FIGURE 14

LOWER LIP OF ORIFICE

OF INTERCOSTAL ARTERY

Scanning electron micrograph of the lower lip of the orifice of the intercostal artery seen in figure 13. In contrast to the appearance of the upper lip of this vessel, there are few thrombi in this region of the aorta 30 minutes following neointimal injury. However, numerous leukocytes (L) are present on the lower region of the lip. x 600.

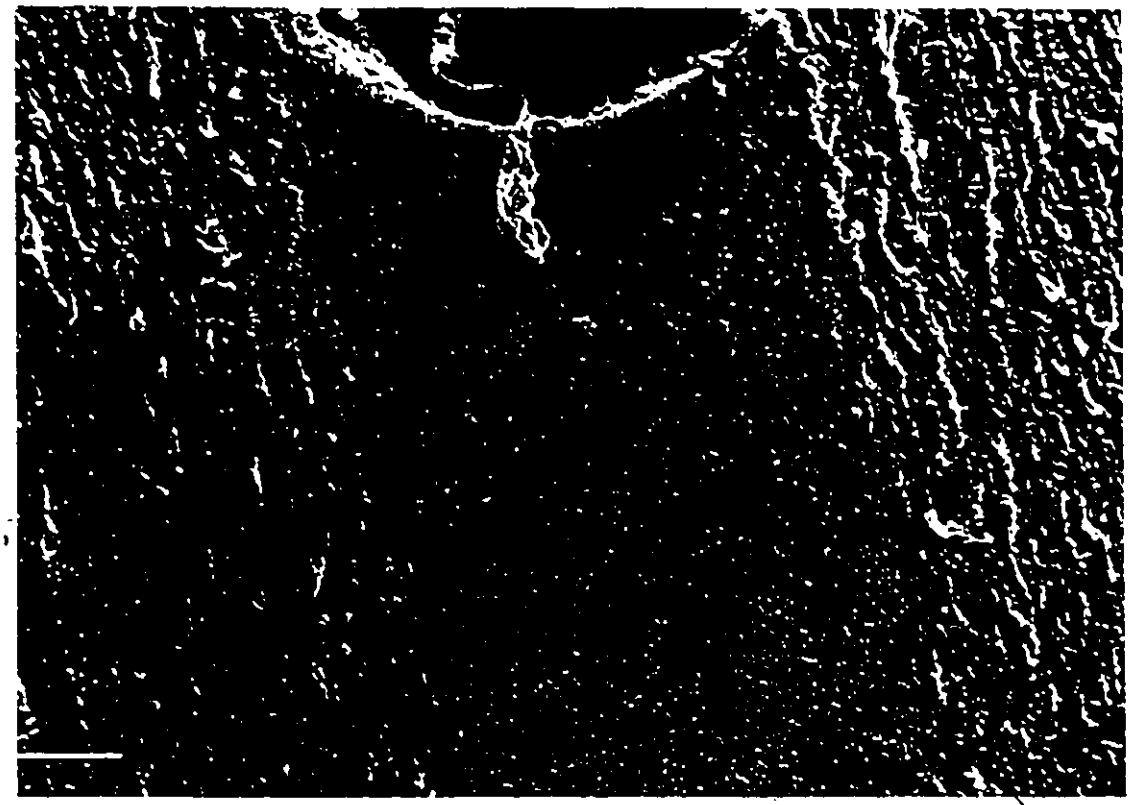


FIGURE 15

THROMBI ON SURFACE OF RABBIT AORTA

BETWEEN ORIFICES OF ADJACENT INTERCOSTAL ARTERIES

Scanning electron micrographs of a rabbit aorta 30 minutes following injury with a balloon catheter to the neointima that had formed 7 days after the removal of the endothelium. A. The thrombi (Th) are aligned in the direction of flow (indicated by the arrow). This region of the aorta lies between the orifices of adjacent intercostal arteries. x 800. B. Higher magnification of platelet-fibrin thrombi on the surface of the vessel. platelets (P), fibrin (F). x 1,600

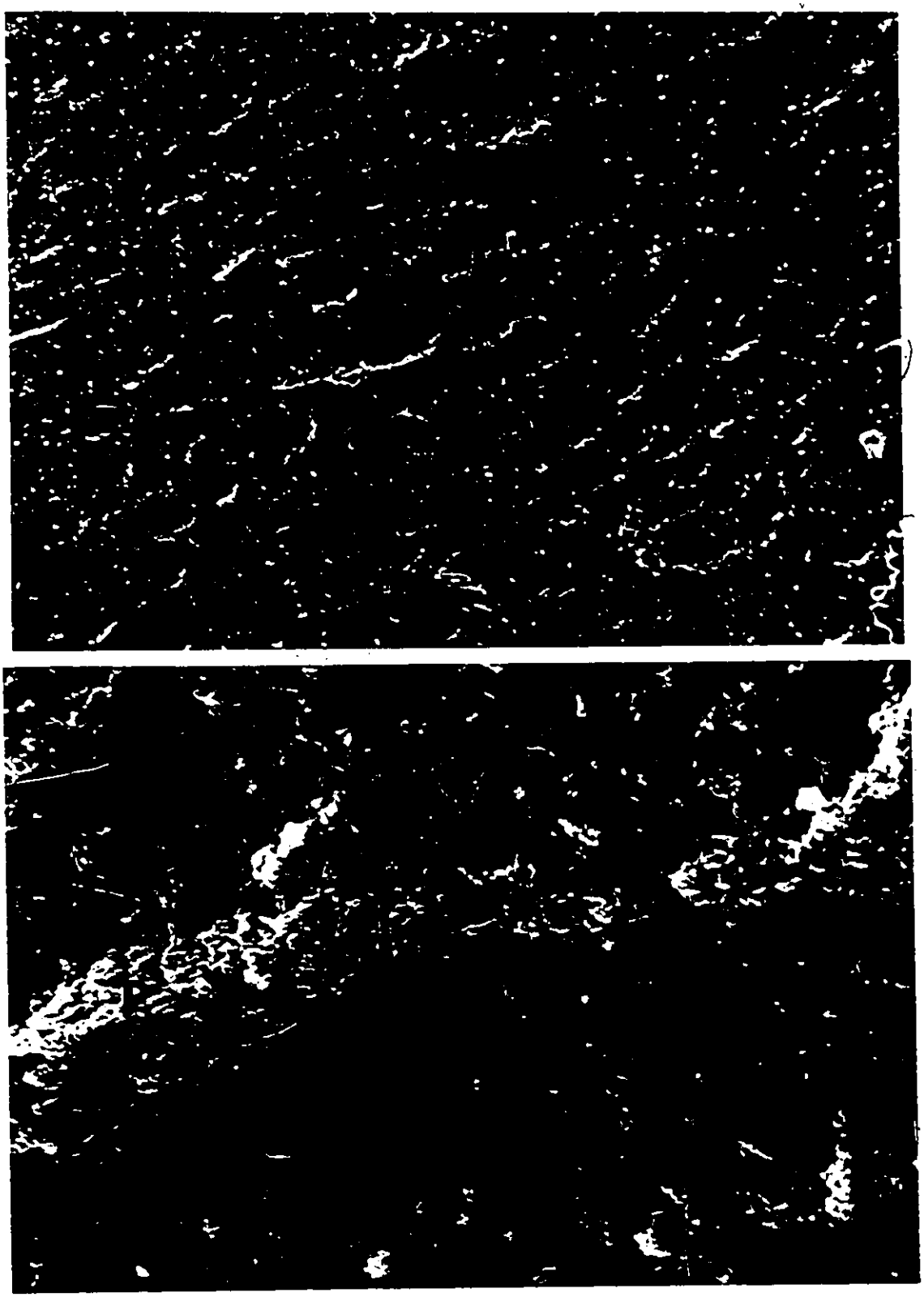

A
B

FIGURE 16

FIBRIN ON INJURED NEOINTIMA

OF RABBIT AORTA

Transmission electron micrograph showing fibrin (F) on the surface of a rabbit aorta 30 minutes following injury had with a balloon catheter to the neointima that formed 7 days following removal of the endothelium. x 14,000

FIGURE 17

PLATELET-FIBRIN THROMBI ON INJURED NEOINTIMA OF RABBIT AORTA

Transmission electron micrograph of platelets (P) and fibrin (F) associated with the surface of the vessel 30 minutes following injury to the neointima with a balloon catheter. X 9,500.

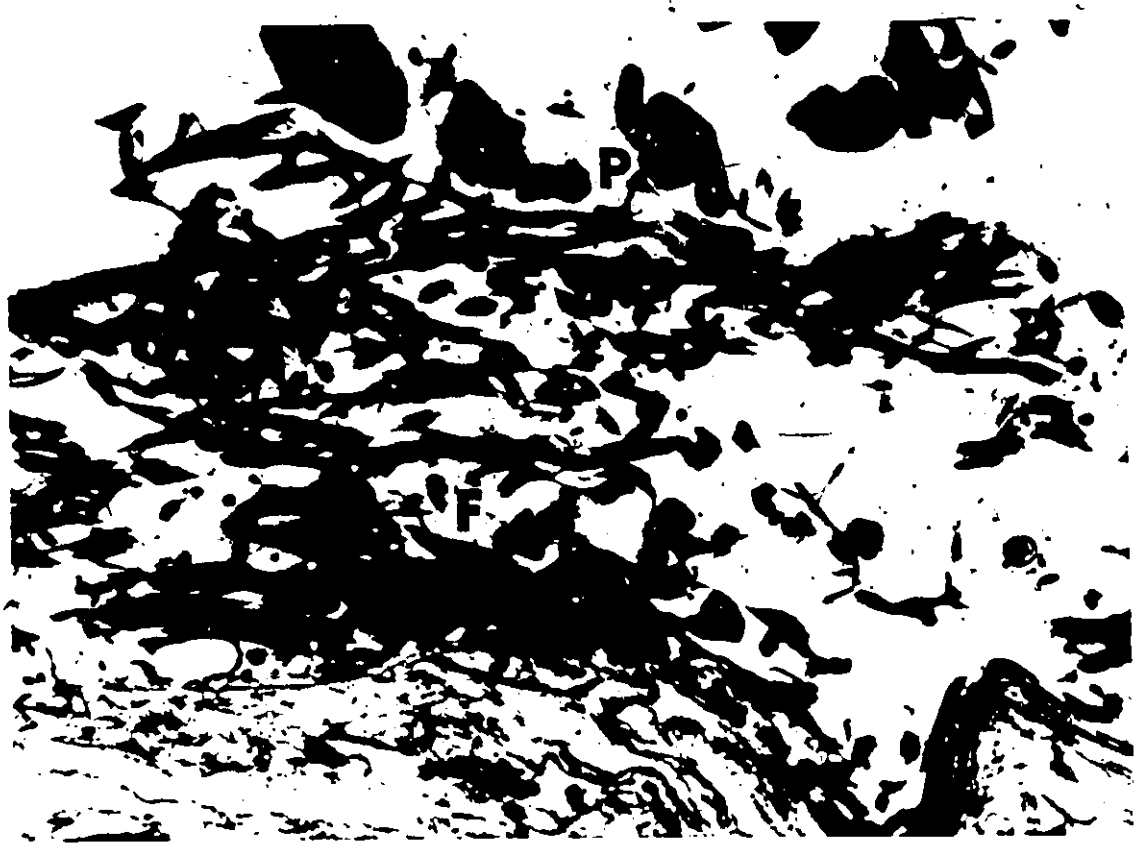

P
F

FIGURE 18

FIBRIN ON INJURED NEOINTIMA

Light micrograph of a rabbit aorta 30 minutes following injury to the neointima shows pink staining fibrin (F) on the surface of the vessel. The neointima that remained on the vessel following injury with a balloon catheter lies above the internal elastic lamina (arrow). The tissue is stained with Martius scarlet blue. x 500

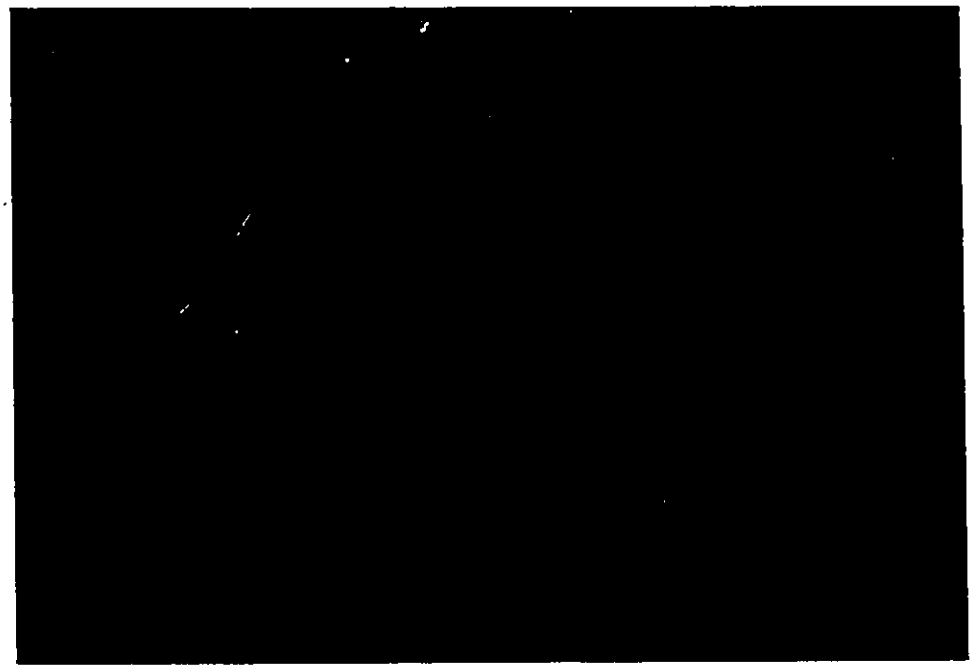


FIGURE 19

PLATELETS ON SUBENDOTHELIUM OF RABBIT AORTA

Transmission electron micrograph of a rabbit aorta 30 minutes following an injury with a balloon catheter to a previously undamaged vessel. The exposed subendothelium is covered by adherent platelets (arrows). The internal elastic lamina (E) appears intact. X 5,500

thelium with a balloon catheter. Occasional large platelet-rich thrombi are seen on the surface (Figure 20). As previously shown, whereas platelets appear to have accumulated on fibrin that formed on the vessel wall in some regions (Figure 17), in other areas, the platelets adhere directly to the surface of the injured vessel with little or no evidence of fibrin formation (Figure 21 and 22). In other regions of the reinjured vessel, very few platelets accumulate on the injured surface, and many of the platelets associated with the vessel are not spread extensively on the surface but retained their disc shape (Figure 23). Thus, platelet coverage on the injured neointima is not uniform.

Leukocytes are occasionally seen on the surface of the injured neointima (Figure 24A). More leukocytes appeared to accumulate in regions adjacent to and on the lower lip of branch vessels (Figure 15 and 24B).

Four days following injury to a 7-day neointima there appears to be little fibrin and few platelets on the surface of the vessel (Figure 25).

Overall, these results show that, although platelet-fibrin thrombi formed on much of the injured surface, in some areas there is no evidence of fibrin, and platelets adhered directly to the vessel wall, whereas in other areas there is little platelet accumulation. In comparison, following the removal of the endothelium a layer of platelets with little evidence of fibrin forms on the vessel. Thus, in these

FIGURE 20

THROMBI ON INJURED NEOINTIMA

ABOVE AN INTERCOSTAL ORIFICE

A. Scanning electron-micrographs of a platelet-rich thrombus on the surface of a rabbit aorta 30 minutes following injury to the neointima. This thrombus is located above the orifice of an intercostal artery. x 400. B. Higher magnification of "A". x 15,000

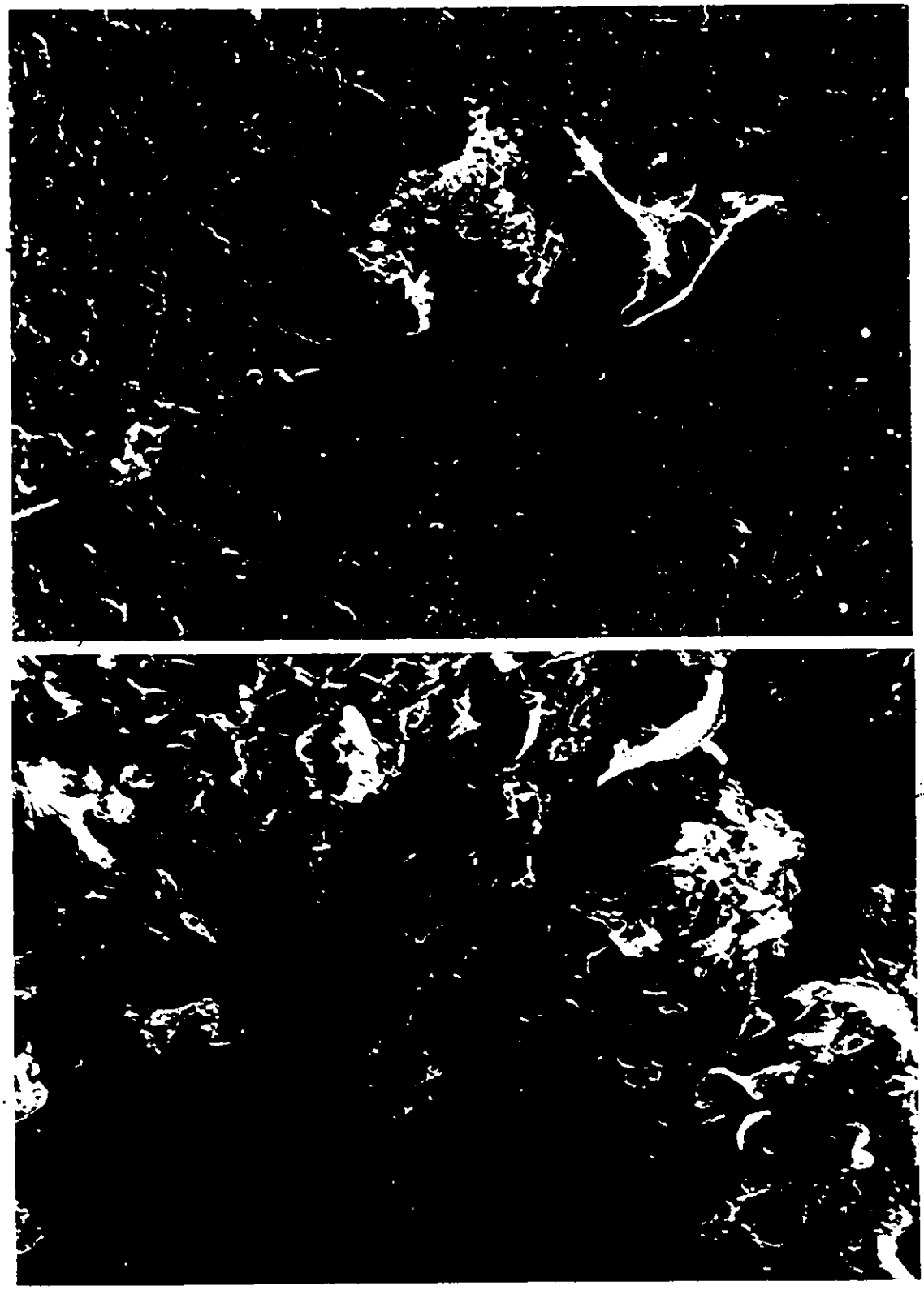

A
B

FIGURE 21

PLATELETS ON SURFACE OF RABBIT AORTA

FOLLOWING INJURY TO A PREVIOUSLY INJURED VESSEL

Scanning electron micrograph showing the surface of an aorta 30 minutes following injury to the neointima of a vessel that was de-endothelialized 7 days earlier. A. In this section significant fibrin formation is not apparent and many of the adherent platelets are not extensively spread; some of the platelets have retained their discoid appearance. x 1,600. B. Higher magnification. x 3,200

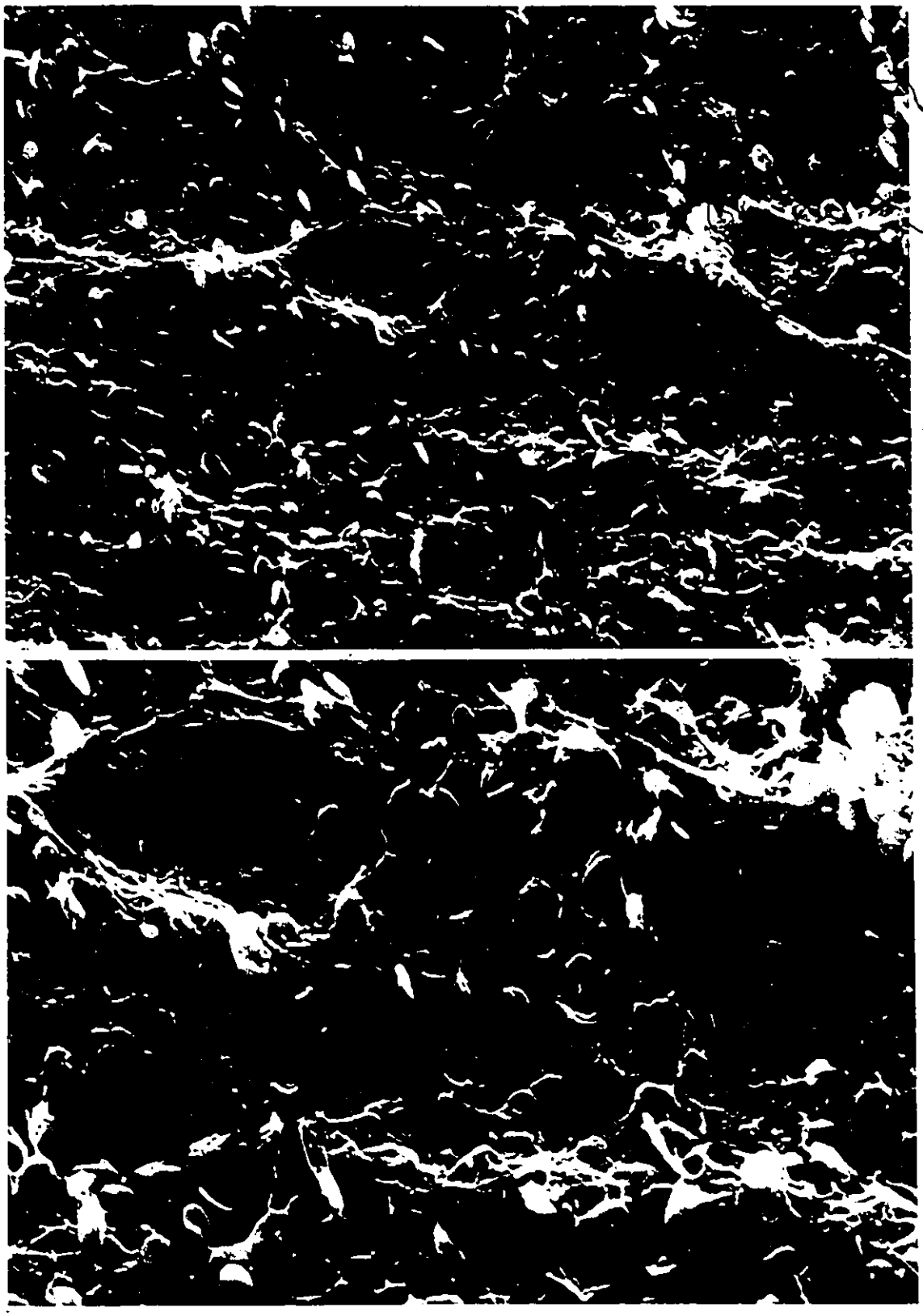

A
B

FIGURE 22

PLATELETS ON SURFACE OF DAMAGED AORTA

Transmission electron micrograph of platelets (arrows) on the amorphous material exposed by injury to the neointima with a balloon catheter. Constituents of the vessel wall include smooth muscle cells (M), elastic tissue (E) and collagen (C). x 5,400

FIGURE 23

SURFACE OF RABBIT AORTA

FOLLOWING INJURY TO A PREVIOUSLY INJURED VESSEL

Thirty minutes following injury with a balloon catheter to the neointima that had formed 7 days following the removal of the endothelium, few platelets (P) had accumulated on this area of the vessel. In this region, the surface appears morphologically similar to the subendothelium connective tissue (CT) exposed by a single injury with a balloon catheter. x 1,600

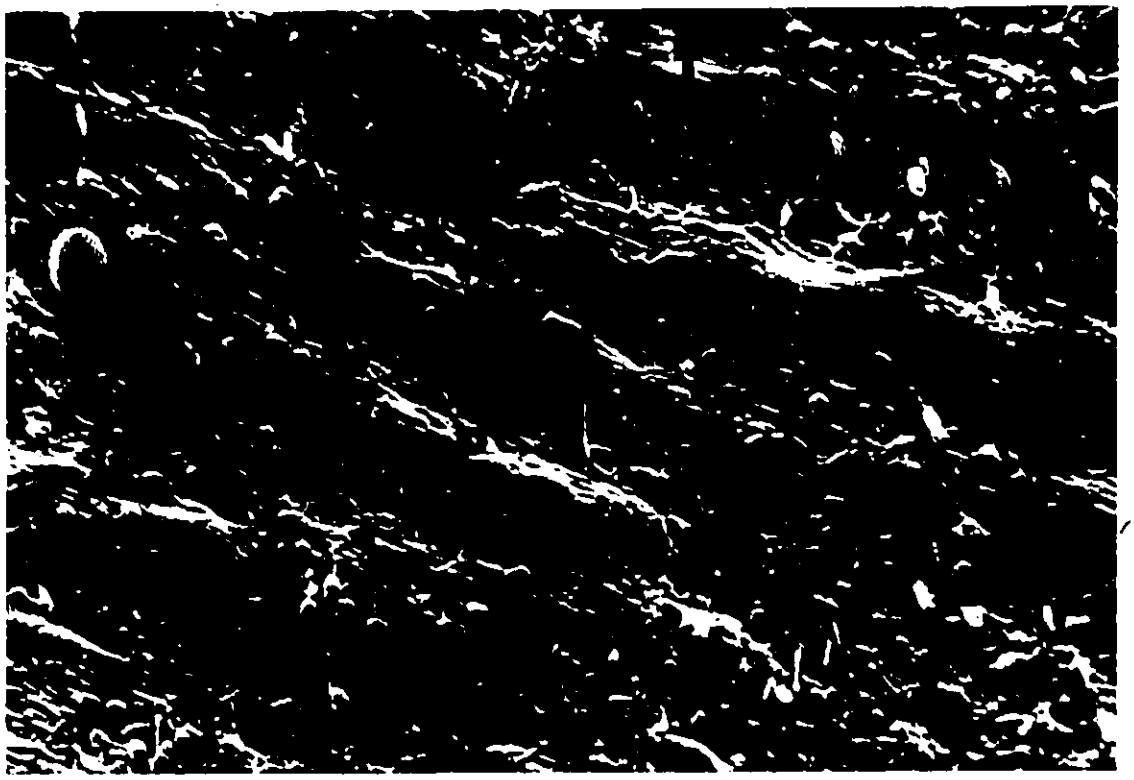


FIGURE 24

LEUKOCYTES AND PLATELETS

ON INJURED NEOINTIMA

Scanning electron micrographs of the surface of a vessel 30 minutes following injury to the neointima. A. Leukocytes can be seen in association with platelets and thrombi. x 850. B. Accumulation of leukocytes and numerous platelets on the injured surface near the lower margin of an intercostal vessel. x3,100

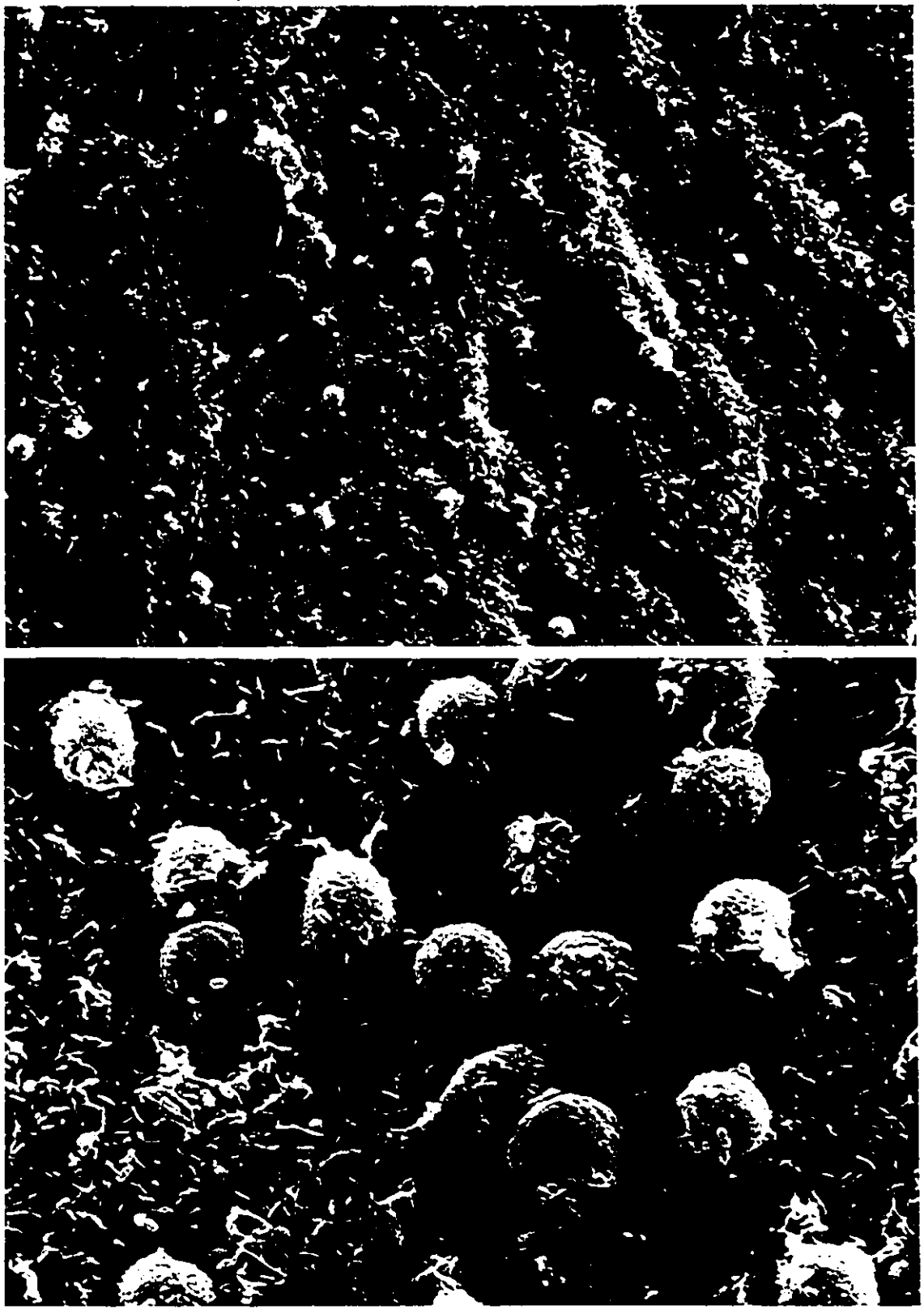
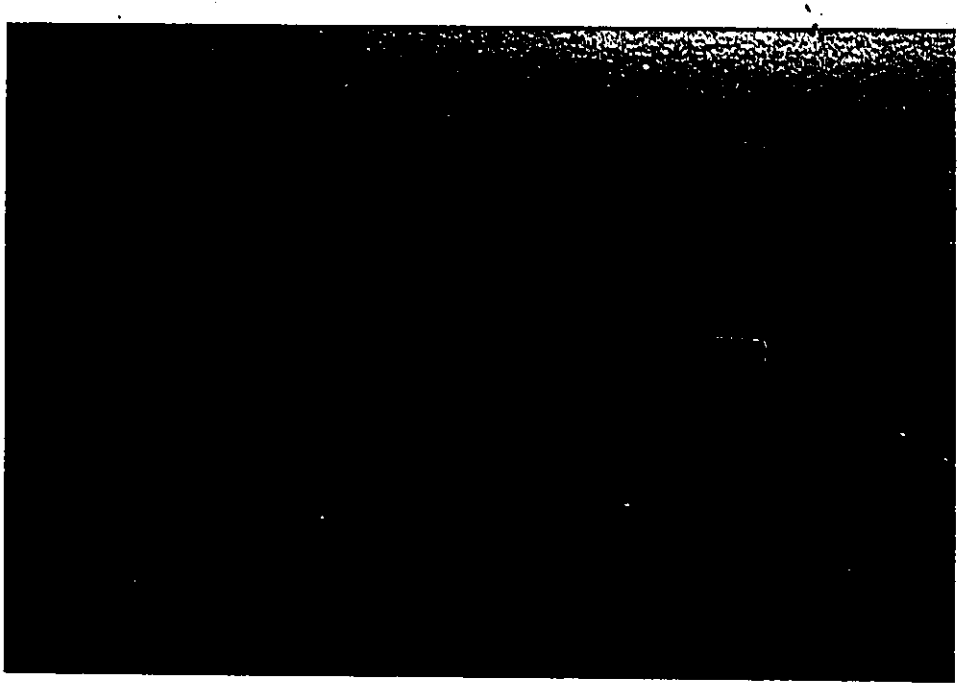

A
B

FIGURE 25

SMOOTH MUSCLE CELL-RICH NEOINTIMA

FOUR DAYS FOLLOWING INJURY TO THE NEOINTIMA

Light micrograph of a rabbit aorta 4 days following injury to a vessel that was de-endothelialized 7 days earlier. A smooth muscle cell-rich neointima is seen lying above the internal elastic lamina (arrow). Neither fibrin nor platelets are in evidence. The tissue was stained with haematoxylin and eosin. x 500



studies, the response of platelets following injury of a previously injured vessel is different from the response following injury of a previously undamaged vessel.

4. Platelet Interaction with the Neointima during Specific Periods following Injury

In previous studies, following the initial rapid accumulation of platelets on the exposed subendothelium, the vessel wall lost much of its reactivity and there was little further platelet accumulation on the injured surface (Groves et al. 1979). Since many of the platelets on the reinjured vessels were associated with fibrin, it appeared that the accumulation of these platelets on the injured neointima might be dependent on the activation of coagulation and the formation of fibrin. If platelet accumulation was dependent on fibrin formation, then it appeared likely that the surface would remain reactive to platelets while fibrin continued to form on the injured surface.

To determine whether the injured neointima also loses much of its reactivity following injury, or whether platelets continue to interact with and turnover on the injured surface, ^{51}Cr-labelled platelets were infused into rabbits at specific times following injury and allowed to circulate for 30 minutes before the animals were killed by perfusion-fixation.

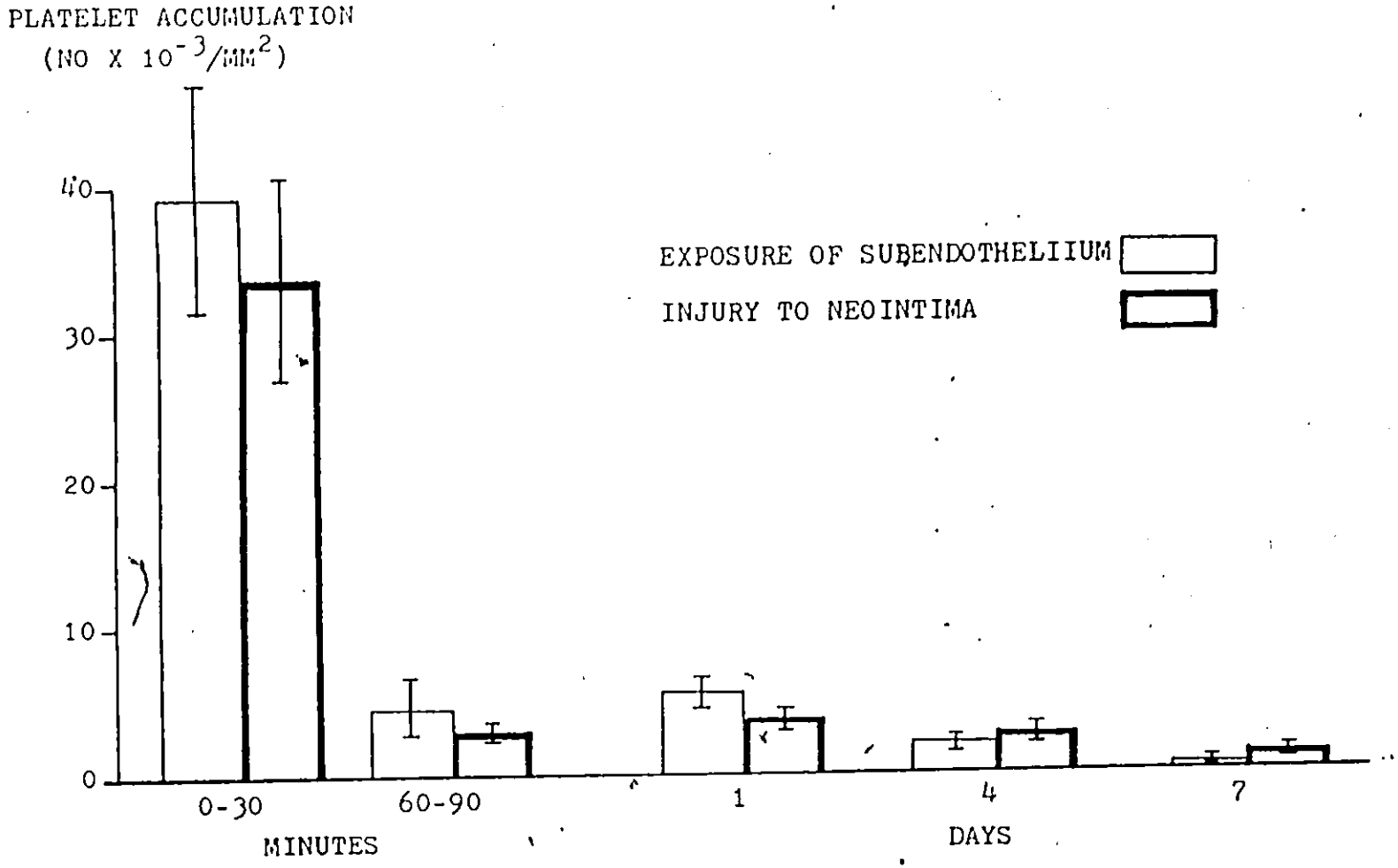
Figure 26 shows the results of this study. When

FIGURE 26

ACCUMULATION OF ^{51}Cr-LABELLED PLATELETS ON AORTAE DURING A 30 MINUTE PERIOD AT SPECIFIC TIMES FOLLOWING INJURY TO THE NEOINTIMA: COMPARISON WITH ACCUMULATION FOLLOWING EXPOSURE OF SUBENDOTHELIUM

This graph shows the accumulation of ^{51}Cr-labelled platelets on the aortae of rabbits during a 30 minute period a specific times following injury with a balloon catheter of the neointima that formed 4 weeks following de-endothelialization (dark bars). For comparison the light bars show the accumulation of platelets following exposure of the subendothelium (Groves et al., 1979). In one group of animals ^{51}Cr-labelled platelets were infused into the circulation before injury and the animals were killed by perfusion-fixation 30 minutes following injury (the first bars on the left). The other groups received an infusion of labelled platelets at 1 hr, or 1, 4 or 7 days following injury, and the animals were perfusion-fixed 30 minutes following the infusion of labelled platelets. There were 5 animals in each group. The error bars indicate the standard error of the mean for each group.

ACCUMULATION OF PLATELETS ON AORTAE DURING A 30 MINUTE PERIOD
AT SPECIFIC TIMES FOLLOWING INJURY TO THE NEOINTIMA:
COMPARISON WITH ACCUMULATION FOLLOWING EXPOSURE OF THE SUBENDOTHELIUM



radioactive platelets were in the circulation at the time of injury, the number of platelets that accumulated on the injured neointima by 30 minutes following injury was similar to the accumulation on the subendothelium 30 minutes following injury that was found in previous studies (Groves et al., 1979). However, when fresh ^{51}Cr-labelled platelets were injected into the circulation one hour following injury, few platelets accumulated on the injured neointima during the following 30 minutes. Similarily, when fresh ^{51}Cr-labelled platelets were infused one, 4, or 7 days following injury, few platelets accumulated on the vessel in a 30 minute period. Platelet accumulation on the injured neointima during a 30 minute period at specific times following injury was similar to the accumulation observed during the same period following exposure of the subendothelium. These results indicate that the surface of the injured neointima also loses much of its reactivity to platelets soon after injury.

Thus, although there is morphological evidence that coagulation is activated when the neointimal is injured, following the initial platelet accumulation on the injured neointma, the damaged surface rapidly loses much of its reactivity to circulating platelets.

5. Platelet Survival following Injury to the Neointima with a Balloon Catheter

As demonstrated previously, the number of platelets that interacted with a rabbit aorta following removal of the endothelium with a balloon catheter was not sufficient to affect platelet survival (Groves et al., 1979). Since, in the present study, the initial platelet accumulation on the injured neointima involved only a small percentage of the total circulating platelet population, and few platelets subsequently interacted with the damaged vessel, it appeared unlikely that neointimal injury would affect platelet survival.

To determine the effect of neointimal injury on platelet survival, ^{51}Cr-labelled platelets were infused into the circulation 2 hours before the neointima was injured with a balloon catheter. Blood samples for the measurement of platelet survival were obtained immediately before and at specific times following neointimal injury. Since the thrombogenicity of a vessel might be greater following injury to a thicker or older neointima, and greater numbers of platelets could interact with and accumulate on the vessel, the effect on platelet survival of injury to the neointima that had formed 7 and 14 days after removal of the endothelium was examined.

Table II shows the results of these studies. The recovery of infused ^{51}Cr-labelled platelets in the circulation

TABLE II

PLATELET SURVIVAL IN RABBITS

FOLLOWING INJURY TO THE NEOINTIMA WITH A BALLOON CATHETER

TREATMENT OF ANIMALS	PLATELET SURVIVAL IN HOURS	RECOVERY OF ^{51}Cr-PLATELETS AT 2 HOURS
STUDY A		
UNDAMAGED AORTAE	57.2 ± 8.4	77.8 ± 2.2
INJURY TO 7 DAY NEOINTIMA	67.9 ± 4.4	77.2 ± 0.9
STUDY B		
UNDAMAGED AORTAE	80.2 ± 4.3	81.5 ± 3.8
INJURY TO 14 DAY NEOINTIMA	80.4 ± 2.0	73.8 ± 8.0

Values are mean ± standard error. There were 6 animals in each group for study A and 4 in each group for study B. ^{51}Cr-labelled platelets were infused 2 hours before injury. The recovery of labelled platelets in blood samples bbtained immediately before injury and at specific times following injury was determined and the platelet survival was calculated with the computerized gamma function. Results of the Studentized range test showed that differences in platelet survival or platelet recovery between the rabbits with undamaged and those with injured aortae are not significant.

2 hours following infusion (immediately before injury) was greater than 75 per cent, and was not significantly different between the groups in either study. This indicated that 1) most of the infused platelets were not substantially altered by the labelling procedure and were capable of surviving in the circulation, and 2) the population of labelled platelets in the circulation at the time of injury was likely to be similar in both groups.

The mean platelet life span for the group of animals that received no injury was not significantly different from the mean platelet survival in animals that received an injury with a balloon catheter to the neointima that formed by 7 days following the removal of the endothelium (Table II, Study A). Similarily, platelet survival in animals that received no injury to the aorta, was not significantly different from survival in the group that received an injury to the neointima that formed by 14 days after the removal of the endothelium (Table II, Study B).

If approximately 40,000 platelets/mm^2 accumulated on the injured neointima (Figure 26), and there was little subsequent interaction with the 12 cm^2 of injured aorta of a 3 kg rabbit with a blood volume of approximately 150 ml, then it can be calculated that, the total number of platelets that associated with the injured vessel wall represented approximately

$\frac{40{,}000 \times 10^3 \times 12 \times 100}{150 \times 3 \times 10^8}$ = 0.1% of the total circulating platelet population. Since the platelets that associated with the injured vessel represent such a small percentage of the circulating platelets, and since few additional platelets accumulated on vessels under these conditions (Figure 26), it is not surprising that the measured platelet survival was not reduced.

6. Comparison of Platelet Adhesion to Injured Neointima In Vivo and In Vitro

The accumulation of platelets on the subendothelium and on the injured neointima in vivo appeared to be quantitatively similar. If, as indicated by the morphological studies, platelet accumulation on the injured neointima was dependent on fibrin formation, whereas platelet accumulation on the subendothelium was not associated with fibrin formation, then under test conditions where coagulation factors were absent, platelet accumulation on an injured neointima should be decreased in comparison to the accumulation on the subendothelium. Therefore the extent of platelet adhesion to injured neointima and to subendothelium in vivo, and also in vitro were compared; in the in vitro studies the vessels were exposed to washed platelets resuspended in a medium without coagulation factors.

In the in vivo studies, the number of platelets that

adhered to a vessel was similar 30 minutes following the exposure of the subendothelium or injury to the neointima (Table III). As previously demonstrated, few platelets accumulated on the neointima formed 7 days after de-endothelialization when it was exposed to labelled platelets for 30 minutes. However, in the in vitro studies, the number of platelets that adhered to the surface of the neointima of everted aortae mounted on a rotating probe, was substantially less than the platelet adherence to the subendothelium. These results are compatible with the hypothesis that much of the platelet accumulation on the injured neointima is dependent on the activation of coagulation with the generation of thrombin and formation of fibrin.

7. Effect of Heparin on Platelet Accumulation on Injured Neointima In Vivo

To determine the role of fibrin formation on platelet accumulation on injured neointima in vivo, the effect of inhibiting coagulation by administering heparin before injury was examined. Since heparin has been reported to affect platelet function, the effect of heparin on platelet accumulation on the subendothelium was also examined to determine whether the dose of heparin used in these studies had an affect on platelet accumulation under conditions where there was little evidence of fibrin formation. The effect of

TABLE III

PLATELET ACCUMULATION ON RABBIT AORTAE IN VIVO AND IN VITRO FOLLOWING EXPOSURE OF SUBENDOTHELIUM OR INJURY TO NEOINTIMA

SURFACE STUDIED	n	PLATELET ACCUMULATION NO. PER SQ. MM.
IN VIVO		
SUBENDOTHELIUM	10	43,400 ± 4,600 a
UNDAMAGED NEOINTIMA	4	2,000 ± 300 b
INJURED NEOINTIMA	12	42,400 ± 8,200 c
IN VITRO		
SUBENDOTHELIUM	18	36,800 ± 2,600 d
INJURED NEOINTIMA	10	15,200 ± 3,000 e

Values are mean ± standard error. 'n' equals number of animals. For the in vivo studies ^{51}Cr-platelets were infused before removal of the endothelium or injury to the neointima that had formed 7 days following de-endothelialization. Thirty minutes following injury the animals were killed by perfusion-fixation. To study platelet accumulation on the undamaged 7 day neointima, labelled platelets were infused and allowed to circulate for 30 min before the animals were killed by perfusion-fixation. For in vitro studies, everted aortae on the rotating probe were exposed to labelled platelets for 10 min. Results of the Studentized range test showed that, for the comparison between a vs b, $p<0.001$; b vs c, $p<0.001$; a vs c, not significant; d vs e, $p<0.01$.

several doses of heparin was examined. In these studies, a single dose of heparin (500 or 200 U/kg) prolonged the clotting time of blood in a glass tube to more than 30 minutes.

In the first study, the effect of treatment with 500 units of heparin per kg on the accumulation of platelets 60 minutes after removal of the endothelium or injury to the neointima was examined. Although the accumulation of platelets on the subendothelium of animals that recieved no heparin, and on the vessels of heparin-treated animals, was not significantly different, the accumulation of platelets on the injured neointima was significantly decreased by treatment with heparin (Table IV). Figure 27 shows platelets associated with the fibrin that formed on the injured neointima. In contrast, figure 28 shows adherent platelets but no fibrin on the surface of an aorta from an animal treated with heparin before injury to the neointima. Although platelet accumulation on the subendothelium in heparin-treated animals was not significantly different from accumulation in animals that received no heparin, heparin-treatment appeared to decrease platelet accumulation slightly, raising the possibility that heparin might have a slight effect on platelet adhesion to the vessel wall.

Since high concentrations of heparin inhibit platelet responsiveness to collagen and to ADP (Mohammad et al., 1981), the high dose of heparin (500 U/kg) used in this

TABLE IV

EFFECT OF HEPARIN ON PLATELET ACCUMULATION

ON SUBENDOTHELIUM OR INJURED NEOINTIMA IN VIVO

HEPARIN TREATMENT	SURFACE STUDIED	n	PLATELET ACCUMULATION NO. PER SQ. MM.	P
500 U/kg				
	SUBENDOTHELIUM			
	NO HEPARIN	8	68,400 ± 6,100	
	HEPARIN	8	53,900 ± 6,500	>0.05
	INJURED NEOINTIMA			
	NO HEPARIN	11	61,500 ± 11,800	
	HEPARIN	12	31,900 ± 7,600	<0.05
250 U/kg				
	SUBENDOTHELIUM			
	NO HEPARIN	4	39,500 ± 6,800	
	HEPARIN	5	25,900 ± 7,000	>0.05
	INJURED NEOINTIMA			
	NO HEPARIN	4	52,900 ± 9,900	
	HEPARIN	4	24,200 ± 3,600	<0.05
200 U/kg				
	SUBENDOTHELIUM			
	NO HEPARIN	6	43,200 ± 6,000	
	HEPARIN	5	29,000 ± 6,300	>0.05
	INJURED NEOINTIMA			
	NO HEPARIN	6	67,600 ± 13,000	
	HEPARIN	6	36,000 ± 8,200	>0.05*

Values are mean ± standard error. 'n' equals number of animals. ^{51}Cr-labelled platelets were infused 18 hr before injury. Heparin was given 10 min before injury with a balloon catheter. Animals were killed by perfusion-fixation 60 min following injury in the group that received 500 U/kg of heparin, and 30 min following injury in the groups that received 250 or 200 U/kg of heparin. Results of the Studentized range test are for the comparison between the group that received no heparin and the heparin-treated group in each study. *The result of the Student's 't' test is $p<0.025$.

FIGURE 27

PLATELET-FIBRIN THROMBUS ON THE SURFACE OF AN INJURED NEOINTIMA

This transmission electron micrograph shows platelets (P) and fibrin (F) in a thrombus on the surface of a vessel 60 minutes after the neointima was injured with a balloon catheter. Fibrin is associated with the vessel, and platelets are associated with this fibrin. x 8,000


P
F
P

FIGURE 28

PLATELETS ON THE SURFACE OF AN INJURED NEOINTIMA IN HEPARIN-TREATED ANIMALS

This transmission electron micrograph shows platelets (arrows) on the surface of a vessel 60 minutes after the neointima was injured with a balloon catheter in animals treated before reinjury with 500 units of heparin per kg of body weight. Smooth muscle cells (M). x 8,000

experiment might have both inhibited coagulation and interfered with platelet adhesion to the vessel surface. Therefore, the effects of lower doses of heparin on platelet accumulation were also determined. When a lower dose of heparin (250 U/kg) was used, although the platelet accumulation on the subendothelium of heparin-treated animals appeared slightly less than the accumulation in the group that received no heparin, this difference was not significant (Table IV). However, treatment with heparin produced a significant decrease in platelet accumulation on the injured neointima.

Platelet accumulation 30 minutes following exposure of the subendothelium in control animals and animals that received heparin (200 U/kg) was not significantly different. The difference in platelet accumulation on the injured neointima in the group treated with heparin and the group not treated with heparin was not significantly different by the Studentized range test. However, it appears possible that the lack of significance in the difference in accumulation between the groups was due to the high standard error for the values in the group that was not treated with heparin. The standard error for this group was much higher than for the other groups in these and other similar experiments.

Thus, treatment of animals with a dose heparin (200-500 U/kg) that prolonged clotting time in a glass tube to more that 30 minutes, produced no significant decrease in

platelet accumulation on the subendothelium, whereas this concentration of heparin significantly decreased platelet accumulation on the injured neointima. These results provide additional evidence that platelet accumulation on the injured neointima is dependent in part on activation of coagulation with the generation of thrombin and the formation of fibrin.

8. Activation of Coagulation by Injured Aortae

The observation that fibrin formed on the injured neointima, whereas little fibrin formed on the exposed subendothelium, indicated that the injured neointima was more capable than the exposed subendothelium of activating coagulation. To determine whether the ability of the injured neointima to activate coagulation was enhanced when compared to that of the subendothelium, a coagulation chamber was designed to be used for testing the procoagulant activity of the luminal surface of a vessel. The apparatus, described in detail in the section on materials and methods, consisted of a chamber with the walls formed by the luminal surface of a segment of siliconized glass tubing, and the floor formed by the luminal surface of the vessel. Plasma was added to the chamber, recalcified, and the coagulation time determined by measuring the time required for fibrin strands to form on a wire hook.

When there was no vessel in the chamber, the coagulation time of recalcified plasma was greater than 400

seconds (Table V). The coagulation time of plasma exposed to a vessel with an intact endothelium was 85.7 ± 11.6 seconds. The coagulation times of plasmas incubated with the subendothelium exposed by injury with a balloon catheter in situ after the animal had been exsanguinated, ex vivo after the vessel was removed from the animal, or in vivo 10 minutes before the animal was exsanguinated, were not significantly different. However, when compared to the coagulation time of plasma exposed to the endothelium, coagulation was significantly accelerated (ie. the coagulation time was decreased) by the surface of the aortae injured in situ, ex vivo or in vivo.

Since it was possible that the generation of thrombin on the surface of the vessels following exsanguination or during removal of the vessels, could enhance the procoagulant activity of the surface, in one study, heparin (3 U/mL blood) (a dose sufficient to inhibit thrombin generation in plasma) was given to animals prior to exsanguination. When the animals were treated with heparin, the coagulation times of plasma exposed to the endothelium was 97.3 ± 6.8 seconds (Table V). For heparin-treated animals, the coagulation times of plasma exposed to aortae injured in situ, ex vivo, or in vivo were significantly less than the clotting times of plasma exposed to the endothelium. There was no significant difference among the clotting times of plasma exposed vessels injured in vivo, in situ, or ex vivo. Although the

TABLE V

EFFECT OF INJURY AND HEPARIN TREATMENT ON AORTA-CHAMBER COAGULATION TIME OF PLATELET-POOR PLASMA

SURFACE STUDIED	n	COAGULATION TIME TREATMENT OF ANIMALS	
		NO HEPARIN	HEPARIN
NO VESSEL	16	406.0 ± 43.0	
ENDOTHELIUM	24	85.7 ± 11.6 a	97.3 ± 6.8 e
AORTA INJURED WITH A BALLOON CATHETER:			
IN SITU	18	55.7 ± 4.2 b	63.1 ± 5.0 f
EX VIVO	18	63.8 ± 4.4 c	73.6 ± 4.4 g
IN VIVO	12	61.2 ± 5.9 d	65.3 ± 6.2 h

Values are mean ± standard error. 'n' equals number of aortae. The clotting times of PPP exposed to the luminal surface of vessels was determined following injury with a balloon catheter in situ before the vessels were removed from the animals, injury ex vivo shortly before the coagulation assay, or injury in vivo 10 min before exsanguination. One group of animals received heparin prior to exsanguination. An analysis of variance shows that the differences among the groups injured with a balloon catheter and treated with heparin, and those not treated with heparin, are not significant. Results of the Studentized range test show that, for the comparison between a vs bcd, $p<0.05$; e vs fgh, $p<0.05$; a vs e, not significant.

coagulation times for vessels from the heparin-treated groups appeared to be slightly longer than the clotting times for the groups that received no heparin, the values were not significantly different. Thus, treatment of animals with heparin did not have an inhibitory effect on the activation of coagulation by the vessels.

Since platelets could contribute to the activation of coagulation, the coagulation times were determined for both platelet-rich plasma (PRP) and platelet-poor plasma (PPP) exposed to subendothelium or injured neointima. The coagulation time for PRP and for PPP exposed to endothelium, subendothelium, undamaged neointima, or to injured neointima are shown in Table VI. The coagulation times of PRP and PPP exposed to subendothelium were significantly shorter than the coagulation times of the plasmas exposed to endothelium, and the coagulation time of PPP exposed to injured neointima was shorter than the coagulation time of plasma exposed to the undamaged neointima. Although the coagulation time of PRP exposed to the injured neointima appeared to be shorter than the coagulation time for the undamaged neointima, these values were not significantly different. This lack of significant difference could be due to the high standard error in the values for the coagulation times for the undamaged neointima. The coagulation times of PRP were not significantly different from the coagulation times of PPP exposed to endothelium, to subendothelium, to undamaged neointima, or to

TABLE VI

AORTA-CHAMBER COAGULATION TIME OF

PLATELET-RICH AND PLATELET-POOR PLASMA

SURFACE STUDIED	COAGULATION TIME	
	PRP	PPP
NO VESSEL	253.6 ± 22.5	271.3 ± 9.5
ENDOTHELIUM	92.8 ± 10.7 a	88.0 ± 10.7 e
SUBENDOTHELIUM	56.1 ± 7.1 b	57.6 ± 4.7 f
7 DAY NEOINTIMA	111.2 ± 23.2 c	134.1 ± 7.3 g
INJURED NEOINTIMA	89.8 ± 8.7 d	88.3 ± 7.1 h

Values are mean ± standard error. The coagulation times following the recalcification of PRP and PPP exposed to the luminal surface of aortae were determined. The subendothelium was exposed or the neointima was injured with a balloon catheter in situ. For the studies with platelet-rich plasma (PRP) n=12, and with platelet-poor plasma (PPP) n=24. Results of the Studentized range test show that, for the comparison between a vs b, $p<0.05$; c vs d, not significant; e vs f, $p<0.05$; g vs h, $p<0.001$; b vs f, not significant; d vs h, not significant.

injured neointima.

Thus, although injury to a previously undamaged vessel or reinjury of a vessel wall enhanced the ability of the vessel surface to activate coagulation, in this system the procoagulant activity of the injured neointima was not greater than the procoagulant activity of the subendothelium.

C. Discussion

The results of these experiments show that repeating the injury to a previously damaged vessel leads to changes in the vessel wall that are more representative of the developing lesions of early atherosclerosis than those that develop in response to other forms of vascular injury. Following this form of vascular injury there is a) thickening of the vessel wall, b) a tendency for focal development of lesions around vessel orifices, c) formation of thrombi associated with the lesions, and d) activation of coagulation. In addition, since changes in the response of injured vessels are important in the progression and development of vascular disease, the properties of the surface exposed by repeated injury were examined and it was shown that vessels injured in this way lose their reactivity to circulating platelets with the passage of time.

1. Extent of Damage to the Neointima

In these studies, although a neointima of approximately 4 cell layers formed on most of the vessel by one week following the removal of the endothelium, intimal thickening was greater in regions adjacent to and opposite branch vessels. In some areas there were few neointimal cells. Although injury to the neointima exposed damaged smooth muscle cells on most of the vessel surface, connective tissue was exposed in a few areas where there might have been little

smooth muscle cell proliferation. In contrast, most of the surface exposed by a single injury with a balloon catheter is composed of subendothelial connective tissue (Groves et al., 1979).

2. Platelet Accumulation on the Injured Neointima

Although the surface of the neointima that formed by 7 days following the removal of the endothelium was essentially non-reactive to circulating platelets, when this surface was injured platelets rapidly accumulated on the vessel. Numerous platelet-fibrin thrombi formed on the injured neointima, particularly around the orifices of branch vessels. This response contrasts with the response to a single injury where a layer of platelets with little evidence of fibrin formed on the exposed subendothelium (Groves et al., 1979). In other areas of the reinjured vessel the layer of adherent platelets that formed on the vessel appeared similar to the layer of platelets that formed following a single injury with a balloon catheter, and there was little evidence of fibrin formation. These platelets appeared to be adherent to reinjured subendothelium or to connective tissue exposed by removal of neointimal smooth muscle cells. There were also occasional areas where few platelets adhered to the vessel. Although the nature of this thrombo-resistant surface is not known, it might be that in these regions of the vessel there was little injury of the vessel during the passage of the

balloon catheter and the underlying connective tissue was not exposed. Alternatively, the exposed surface of the injured vessel might have been composed of substances that are not reactive for platelets.

Although, in earlier studies Stemerman (1973) demonstrated that platelet-fibrin thrombi formed on vessels following injury to the neointima that formed by 4 weeks after the removal of the endothelium, the results of the present studies showed that platelet fibrin-thrombi formed on the surface of a vessel injured with a balloon catheter as soon as 7 days following the initial removal of the endothelium.

3. Reactivity of the Injured Neointima

Although platelets rapidly accumulated on the injured neointima, and much of this accumulation appeared to be associated with the activation of coagulation and the formation of fibrin, the surface of the injured vessel rapidly became non-reactive to the further accumulation of platelets. These findings are compatible with the observations of Piepgras and his colleagues (1976) who demonstrated that the surface of endarterectomized carotid arteries in cats became non-thrombogenic by approximately 4 hours following injury. Therefore, even when coagulation is activated and platelet accumulation appears to be associated with fibrin formation on a reinjured vessel, the surface of the vessel remains reactive for only a short period following injury. Thus,

most of the platelet-derived growth factor delivered to vascular smooth muscle cells would be made available by the platelets that rapidly adhered to the surface exposed by injury. This is supported by the findings of Goldberg and his colleagues (1980) who demonstrated that platelet factor 4, another alpha granule protein, could be demonstrated in the media by 10 minutes following injury, although there was little evidence of its presence 4 hours later.

4. Platelet Survival

Although endothelial injury is associated with a decrease in platelet survival (Kinlough-Rathbone, 1983), if the number of platelets that accumulate on an injured vessel represent only a small percentage of the circulating platelet population, and there is little platelet interaction with the vessel following the initial platelet accumulation, measurements of platelet survival are unaffected (Groves et al., 1979). In the present studies only about 0.1 per cent of the circulating platelet population became associated with the injured neointima, and few platelets subsequently accumulated on the vessel surface. This is likely attributable to the fact that the injured neointima rapidly became non-reactive to further platelet accumulation. Therefore, it was not surprising that platelet survival was not decreased following injury of the neointima.

However, when the aortae in rabbits or rats are ex-

posed to continuous injury with indwelling catheters platelet survival is shortened (Mueleman et al., 1977; Mueleman et al., 1980; Somers et al., 1980; Winocour et al., 1980; Winocour et al., 1982). Platelet survival is also shortened in monkeys exposed to continuous arterial injury by the infusion of homocysteine (Harker et al., 1976) or in humans with homocystinemia (Harker et al., 1977). Therefore, it appears that repeated or continuous vessel injury is required to shorted platelet survival. Thus, the results of previous studies and those presented in this thesis show that the measurement of platelet survival is of only limited value as an indicator of vessel injury and thrombosis.

5. Role of Activation of Coagulation in Platelet Accumulation on the Injured Neointima

In contrast with the platelet accumulation on the subendothelium exposed by a single injury with a balloon catheter, the mechanisms responsible for the accumulation of platelets on the injured neointima appear to involve the activation of coagulation with the generation of thrombin and the formation of fibrin. The reasons for this are that: 1) many of the platelets on the injured neointima were associated with fibrin, and 2) treatment with heparin that prevented the formation of fibrin reduced platelet accumulation by approximately 50 per cent. When heparin was present, probably only the non-coagulation-dependent adher-

ence of platelets to connective tissue occurred. In addition, since during the in vitro experiments the surface of the injured neointima was only exposed to platelets suspended in Eagle's media with added albumin, the components required for coagulation were not present. Thus, the decrease in platelet accumulation on the injured neointima in the present experiments was probably due to the fact that coagulation could not occur in these studies.

6. Activation of Coagulation by Injured Vessels

Although the ability of the endothelium to activate coagulation in vitro was substantially less than that of an injured vessel, the endothelial surface activated coagulation more rapidly than the empty chamber. Since small areas of endothelial damage were observed on other aortae prepared for study on the rotating probe device (Dejana et al., 1983), it was possible that coagulation was activated by exposure of the plasma to connective tissue in areas where the endothelium was damaged during the removal of the vessel from the animal and its preparation for assay. Since Maynard and coworkers (1976) reported that the transfer of cells to new culture media was sufficient to stimulate the cells to produce tissue factor, it was also possible that manipulating the vessel or exposing it to new culture media was sufficient to stimulate the endothelial cells to produce tissue factor that could accelerate coagulation.

In the present studies plasma coagulated more rapidly when exposed to the subendothelium than when exposed to the endothelium, indicating that injury enhanced the procoagulant properties of the vessel wall. Since the time required for plasma to clot following its exposure to vessels injured in situ or ex vivo was similar, it appears that the factor or factors produced by the vessel wall that were responsible for accelerating coagulation were not lost or inactivated between injury of the vessel in situ and assay of its procoagulant activity.

Since the injured vessel rapidly lost its reactivity to circulating platelets following the formation of a layer of platelets on the surface, it was considered possible that the layer of adherent platelets either might decrease the procoagulant activity of an injured vessel by concealing those factors on the injured vessel wall that were responsible for initiating coagulation, or might increase the activation of coagulation by making platelet factor 3 available to enhance coagulation. However, in the present studies, the coagulation time for vessels de-endothelialized in vivo and exposed to circulating platelets for 10 minutes was similar to the coagulation time for vessels injured in situ or ex vivo. Thus, it is not possible to conclude whether the platelets had no effect on the procoagulant properties of the vessel wall, or whether they inhibited coagulation by concealing some of the reactive sites on the injured vessel as

well as contributed to the activation of coagulation by making platelet factor 3 available.

Since the coagulation times of PRP or PPP were similar for each group, it appeared that platelets in the plasma did not play an important role in the activation of coagulation in this coagulation-chamber system.

In these studies the acceleration of coagulation by segments of vessel was not due to the effects of thrombin generated during exsanguination of the animals or removal of the vessels, since treatment of animals with heparin prior to exsanguination did not decrease the rate of activation of coagulation in plasma exposed to the vessels. In addition, these results indicate that either heparin was not adsorbed to components of the vessel wall or absorbed by the vessel, that any heparin associated with the vessel was not capable of inhibiting the coagulation of plasma exposed to the vessel surface, or that the quantity of heparin associated with the vessel was not sufficient to inhibit coagulation. Other investigators have reported in several studies reviewed by Salzman (1971), that although treatment with heparin made some surfaces non-thrombogenic, in other studies treatment with heparin did not decrease the thrombogenicity of surfaces. Therefore, there are several possible reasons why treatment of vessels with heparin did not inhibit their activation of coagulation in these studies.

Since fibrin formed on the neointima following injury

in vivo, whereas there was little evidence of fibrin formation on the subendothelium exposed by a single injury, it appeared likely that the procoagulant activity of the injured neointima was greater than that of the subendothelium. However, in the present studies, the procoagulant activity of the injured neointima and subendothelium were similar. The reason for these results remains unclear. It is possible that some of the procoagulant factors generated by the injured neointima were lost during preparation of the tissues for assay, or the conditions were not suitable for the vascular cells to generate or release these factors. Thus, although the coagulation-chamber was useful to demonstrate that the procoagulant activity of a vessel was increased following injury, the factors responsible for the formation of fibrin on the injured neointima in vivo could not be determined using this technique.

7. Conclusions

In conclusion these studies showed that:

1. Neointima thickening in response to removal of the endothelium is greatest in regions adjacent to and opposite branch vessels. These are the sites at which atherosclerotic lesions tend to occur.
2. Platelet-accumulation on the injured neointima is similar to the accumulation on the subendothelium.

3. Some, but not all of the platelet accumulation on the injured neointima is dependent on the activation of coagulation and the formation of fibrin.

4. The injured neointima loses its reactivity to platelets soon after the initial accumulation of platelets.

5. Injury to the neointima that results in activation of coagulation and the formation of platelet-fibrin thrombi does not decrease platelet survival.

6. Injury of the neointima increases the procoagulant properties of its surface.

CHAPTER IV

EFFECT OF PRODUCTS OF ARACHIDONIC ACID FORMED BY THE VESSEL WALL ON THE ACCUMULATION OF PLATELETS ON RABBIT AORTAE

EFFECT OF PRODUCTS OF ARACHIDONIC ACID FORMED BY THE VESSEL WALL ON THE ACCUMULATION OF PLATELETS ON RABBIT AORTAE

A. Introduction

When cells are stimulated by substances such as those generated at sites of inflammation or thrombus formation, phospholipases A_2 and C are activated to free arachidonic acid from membrane phospholipids (Bell et al., 1979; Broekman et al., 1980; Agranoff et al., 1983). Since several products of arachidonic acid metabolism can affect platelet function, these substances have the potential to affect platelet interaction with vessel walls in vivo. (The generation of products of the arachidonic acid pathway and their potential role in inhibiting platelet interaction with vessel walls is discussed in more detail in Chapter I.) In particular, arachidonic acid metabolites such as PGI_2 and products of the lipoxygenase pathway have been speculated to play a role in the response of vessels to circulating platelets.

1. PGI_2

It has been argued that PGI_2 generated by cells of the vessel wall is responsible for preventing platelet accumulation on normal vascular endothelium in vivo (Higgs et al., 1978). The observation that large amounts of PGI_2 are released from organs such as lung (Gryglewski et al., 1978), as well as from endothelial cells (Moncada and Vane, 1977),

lead Moncada and his colleagues (1978) to speculate that circulating PGI_2 functioned as an antithrombotic agent. However, there are several theoretical and practical points that mitigate against PGI_2 products being responsible for the non-thrombogenicity of normal blood vessels in vivo: 1) The concentrations of PGI_2 required to exert an antithrombotic effect are high (Cazenave et al., 1979a; Karniguian et al., 1978a; Karniguian et al., 1978b), and it is unlikely that normal endothelium would be sufficiently stimulated to produce these high concentrations; 2) the half-life of PGI_2 in the circulation is very short (Dollery et al., 1983), and the amount of PGI_2 in the circulation is so low that it is barely detectable (Haslam and McClenaghen, 1981; Blair et al., 1982; Dollery et al., 1983): 3) there is no evidence of an increased thrombotic tendency in individulas on long-term aspirin therapy; aspirin inhibits PGI_2 production and if the hypothesis were correct aspirin-treatment should enhance thrombosis.

A regulatory role for PGI_2 has not been excluded in injured or diseased vessels, however. It is possible that PGI_2 could limit thrombus formation at or near an injury site. Thrombin binds to endothelial cells (Awbrey, et al., 1979; Lollar and Owen, 1980) and stimulates PGI_2 production by endothelial cells (Weksler et al., 1978). PGI_2 produced under these conditions could limit the extension of thrombi at sites adjacent to injury. Furthermore, both the subendo-

thelium that is exposed following de-endothelialization and the injured neointima (Groves et al., 1979; Chapter III) rapidly lose their reactivity to circulating platelets. This raises the possibility that mechanical stimulation of the vessel wall by inflated balloon catheters that were used to injure the vessels could stimulate medial smooth muscle cells or smooth muscle cells of the neointima to produce PGI_2; the PGI_2 produced might inhibit further platelet accumulation on these injured surfaces.

2. Products of the Lipoxygenase Pathway

Arachidonic acid freed from membrane phospholipids of stimulated cells can be metabolized by the lipoxygenase pathway as well as by the cyclo-oxygenase pathway. Several products of this pathway have been reported to affect platelet function (See Chapter I). Buchanan and his colleagues (1983b) reported that inhibition of lipoxygenase result in the accumulation of radioisotopically-labelled platelets on cultured endothelial cells. However, since these platelets were labelled with ^3H-adenine and ^3H-labelled metabolites of adenine could be released from the platelets and taken up by the endothelial cells, it is possible that the radioactivity associated with the cultured endothelial cells was derived from metabolites of adenine and did not represent platelets adherent to the surface of the endothelial cells. Therefore, the role of products of the lipoxygenase pathway in the

non-reactivity of normal endothelium and the loss of reactivity of injured vessels remains uncertain.

3. Objectives of the Study

Thus, the specific objectives of these studies were to determine whether:

1. PGI_2 produced by undamaged vessel walls could be responsible for the non-thrombogenicity of normal endothelium.

2. PGI_2 produced by endothelial cells with thrombin bound to their surface limited the accumulation of platelets on the endothelium.

3. PGI_2 produced by vessel walls was responsible for the loss of reactivity of damaged vessel following the accumulation of a layer of platelets on the surface exposed when the vessel was de-endothelialized by a balloon catheter.

4. PGI_2 produced by vessel walls was responsible for the loss of reactivity of a vascular surface several days following injury.

5. PGI_2 produced by vessel walls was responsible for the loss of reactivity of vessels following the

accumulation of platelets on a surface where there was activation of coagulation and generation of thrombin, for example, on injured neointima.

6. products of the lipoxygenase pathway were responsible for the non-thrombogenic properties of the undamaged endothelial surface.

7. products of the lipoxygenase pathway were responsible for the loss of reactivity of a damaged vessel soon after injury.

B. Results

1. Effect of PGI_2 Produced by Vessel Walls on the Accumulation of Platelets on the Endothelium

To determine whether the production of PGI_2 by vessel walls is responsible for preventing platelet accumulation on the surface of normal endothelium, PGI_2 production by rabbit aortae was inhibited by treatment with aspirin, and the effect was examined on the accumulation of platelets on the aortic endothelium in vivo and in vitro.

As previously discussed in the Materials and Methods, some radioactivity became associated with the aortae on the rotating probe when they were exposed to supernatant from suspensions of ^{51}Cr-labelled platelets. Similarily, some radioactivity from suspensions of ^{51}Cr-labelled platelets injected into the animals became associated with normal damaged vessels. However, morphological examination of these vessels revealed no adherent platelets on the vessel surface. When platelet adherence was high (approximately 40,000 per mm^2), values for adherence determined radiometrically were similar to the values determined morphometrically. Thus, when the quantity of radioactivity associated with the vessels is very low, it may not represent the accumulation of labelled platelets, but rather the association of non-platelet bound radioactivity with the vessel wall. With platelets prepared and labelled as described in the Materials and Methods section, values for platelet accumulation of

approximately 1,500/mm^2 on vessels on the rotating probe, or 2,500/mm^2 on vessels in vivo can be attributed to radioactivity associated with the vessels that does not represent platelet accumulation on the vessel surface.

In vivo, the accumulation of ^{51}chromium on the endothelium 15 minutes following the infusion of aspirin (100 mg/kg) was similar to the accumulation of ^{51}Chromium on aortae in animals treated with Tyrode's solution (Table VII). Previous studies showed that accumulations of this amount of radioactivity did not represent platelet accumulation on the surface of the vessel, but was due to non-platelet-bound ^{51}chromium associated with the vessel. Therefore, these results indicate that there was no platelet accumulation on the endothelium in animals treated with aspirin or with Tyrode's solution.

Platelets might not accumulate on aortae in animals treated with aspirin because: 1) the surface has retained its non-reactivity to platelets through a mechanism that is independent of PGI_2 production, or 2) the platelets exposed to aspirin are incapable of accumulating on the endothelium. As shown in a later experiment (Table XI), when aspirin-treated platelets were infused into rabbits their accumulation on damaged vessels was similar to the accumulation of platelets that were not treated with aspirin. These results indicate that platelets in animals treated with aspirin were capable of accumulating on a reactive surface. Since abol-

TABLE VII

EFFECT OF ASPIRIN ON THE ACCUMULATION OF RADIOACTIVITY FROM A SUSPENSION OF ^{51}CR-PLATELETS ON AORTAE WITH NORMAL ENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM.	PGI_2 NG/MG DRY WEIGHT
TYRODE	3,000 ± 1,000	0.15 ± 0.05
ASPIRIN (100 MG/KG)	3,500 ± 1,000	0

Mean values ± SEM for 3 animals in each group. Two hours after the infusion of ^{51}Cr-platelets, the animals received an intravenous injection of aspirin (100 mg/kg). Fifteen min later, the animals were killed by perfusion-fixation. The aortae were removed, the radioactivity associated with them was measured, and the accumulation of platelets was calculated. (As pointed out in the materials and methods, this does not represent platelet accumulation, but rather non-specific accumulation of radioactivity.) In a separate group of animals the effect of this concentration of aspirin on PGI_2 production by the aorta was examined. Animals were given either Tyrode solution or aspirin intravenously 20 min before they were exsanguinated and the aortae were removed for the measurement of PGI_2 production by the bioassay (method 2) described in the Materials and Methods. In this method arachidonate was not added to the medium used for incubation of the vessels. Results of the Studentized range test show that the difference in platelet accumulation between the groups is not significant.

ishing PGI_2 production by vessel walls did not result in the accumulation of platelets on the endothelium, it appears that PGI_2 is not responsible for the non-thrombogenic properties of the endothelium.

When the effect of treatment of aortae with aspirin (2 mM) in vitro on the adherence of untreated platelets to the endothelium of vessels everted on a rotating probe was examined, the adhesion of platelets to the damaged vessels treated with aspirin and those not treated with aspirin was similar (Table VIII). The concentration of aspirin used in these studies was sufficient to abolish the production of PGI_2 by the cells of the vessel wall, even in the presence of added sodium arachidonate (Table VIII). Therefore, these results do not support the hypothesis of Moncada and his colleagues (1977).

2. Effect of PGI_2 Produced by Vessel Walls on the Adherence of Platelets to Thrombin-Treated Endothelium In Vitro

Since, PGI_2 production by endothelial cells exposed to thrombin might be important in limiting platelet accumulation on the surface of the vessel, particularly in regions adjacent to thrombi forming at sites of vascular injury, the effect on platelet accumulation of inhibiting PGI_2 production by 'undamaged' aortae treated with thrombin was examined in vitro. (Since some small areas of endothelial were injured during preparation and eversion of aortae on a probe

TABLE VIII

EFFECT OF TREATMENT OF UNDAMAGED AORTAE WITH ASPIRIN ON THE ADHERENCE OF PLATELETS IN VITRO

TREATMENT OF AORTAE	PLATELET ADHERENCE NO. PER MM. SQ.	PGI_2 PRODUCTION NG/MG DRY WT.
TYRODE	7,300 ± 1,000	3.5 ± 0.5
ASPIRIN	7,100 ± 1,400	0

Mean values ± SEM for 4 aortae in each group. The vessels were mounted on probes and incubated for 30 min in Eagle's medium containing 4 per cent albumin and either Tyrode solution or aspirin (2mM). The vessels mounted on a probe were rinsed in 2 changes of 10 ml portions of Eagle's medium before the adherence of platelets was tested. A segment of vessel was removed from the aortae after they were everted on the plastic tubing (but before they were rotated in a suspension of platelets), and the production of PGI_2 by the segments was measured by the bioassay method 2 described in Materials and Methods. Results of the Studentized range test show that the difference in accumulation between the groups is not significant.

[Figure 29B, page 248] the endothelium studied in these experiments was not completely undamaged.) Table IX shows that treatment of aortae with thrombin (20 U/ml) increased platelet adherence to the 'undamaged' endothelium of thrombin-treated vessels several fold when compared to the adherence to untreated vessels. However, the accumulation of platelets on the untreated aortae or those treated with thrombin was not enhanced by treatment of the vessels with aspirin (2 mM). Although the concentration of aspirin that was used in these experiments abolished the production of PGI_2 by the vessels that were not treated with thrombin, this concentration of aspirin was not sufficient to completely abolish the production of PGI_2 by the vessels that were stimulated by treatment with thrombin.

Since platelets can produce PGG_2 and PGH_2, that could be used by the cells of the aortae as precursors for the production of PGI_2, the effect of treating platelets with aspirin on their adherence to aortae that were treated with thrombin was also studied. In addition, the production of PGI_2 by these vessels was determined. Table X shows that the treatment of 'undamaged' vessels with aspirin did not alter the accumulation of aspirin-treated platelets on the vessels. Although thrombin treatment increased platelet accumulation on the 'undamaged' endothelial surface of both the aspirin-treated and control vessels, treatment of the vessels with aspirin did not enhance the accumulation of platelets when

TABLE IX

EFFECT OF TREATMENT OF AORTAE WITH ASPIRIN ON THE ACCUMULATION OF UNTREATED PLATELETS ON 'UNDAMAGED' RABBIT AORTAE EXPOSED TO THROMBIN

TREATMENT OF AORTAE	PLATELET ACCUMULATION NO. PER MM. SQ.	PGI_2 PRODUCTION NG/MG DRY WT.
TYRODE-TYRODE	7,500 ± 1,400 a	0.4 ± 0.1
ASPIRIN-TYRODE	6,600 ± 700 b	0
TYRODE-THROMBIN	31,400 ± 6,400 c	2.8 ± 0.8
ASPIRIN-THROMBIN	24,400 ± 3,300 d	0.9 ± 0.03

Mean values ± SEM for 4 aortae in each group. The vessels were mounted on a probe and incubated for 30 min in Eagle's medium containing Tyrode solution or aspirin (2 mM). The aortae were then rotated for 1 min at 200 rpm in either Tyrode solution or purified bovine thrombin (20 U/ml) in Tyrode solution, and rinsed twice in 10 ml portions of modified Tyrode solution before rotating in a suspension of ^{51}Cr-platelets to measure platelet accumulation. The Results of the Student's 't' test show that a vs b, not significant; a vs c, $p<0.02$; b vs d, $p<0.02$; c vs d, not significant. A segment of vessel was removed from the aortae after they were everted on the probe (but before they were rotated in a suspension of platelets), and the production of PGI_2 by the segment of vessel was measured by the bioassay (method 2) described in the Materials and Methods.

TABLE X

EFFECT OF TREATMENT OF AORTAE WITH ASPIRIN ON THE ACCUMULATION OF PLATELETS TREATED WITH ASPIRIN ON 'UNDAMAGED' RABBIT AORTAE EXPOSED TO THROMBIN

TREATMENT OF AORTAE	PLATELET ACCUMULATION NO. PER MM. SQ.	PGI_2 PRODUCTION NG/MG DRY WT.
TYRODE-TYRODE	6,000 ± 500 a	0.3 ± 0.1
ASPIRIN-TYRODE	8,000 ± 1,300 b	0
TYRODE-THROMBIN	32,300 ± 3,400 c	0.8 ± 0.1
ASPIRIN-THROMBIN	27,000 ± 3,000 d	0

Mean values ± SEM for 6 aortae in each group. Platelets were incubated for 15 min with 2 mM aspirin in the second washing solution. The vessels were incubated for 30 min in Eagle's medium containing Tyrode solution or aspirin (2 mM), rotated for 1 min at 200 rpm in either Tyrode solution or purified bovine thrombin (20 U/ml) in Tyrode solution, and rinsed twice in 10 ml portions of modified Tyrode solution before platelet accumulation was measured. The results of the Student's 't' test show that, for the comparison between a vs b, not significant; a vs c, $p<0.01$; b vs d, $p<0.01$; c vs d, not significant.

compared to the accumulation on vessels that were not treated with aspirin. Although the aspirin-treated vessels were capable of generating some PGI_2 when the platelets were not treated with aspirin (Table IX), when the platelets were treated with aspirin, the production of PGI_2 by the 'undamaged' vessels that were treated with aspirin was abolished, even when the vessels were exposed to thrombin. These results indicate that the vessel wall was capable of utilizing the PGG_2 and PGH_2 contributed by platelets for the generation of PGI_2.

Scanning electron micrographs of the surfaces of everted aortae used in these studies showed that platelets adhered only in regions where the endothelium was accidentally damaged (Figure 29). There was no evidence of platelet accumulation on the surface of the undamaged endothelium of vessels not treated with thrombin (29A), or on the undamaged endothelium of vessels exposed to thrombin (20 U/ml) before rotation of the vessels in suspensions of platelets (29C). However, platelet accumulation on damaged regions of the vessels (29B) was greatly enhanced when the vessels were treated with thrombin (29D).

Thus, since platelets did not accumulate on the endothelium when PGI_2 production was inhibited, it appears that the non-thrombogenicity of the endothelium is not due to the generation of PGI_2 by the vascular cells.

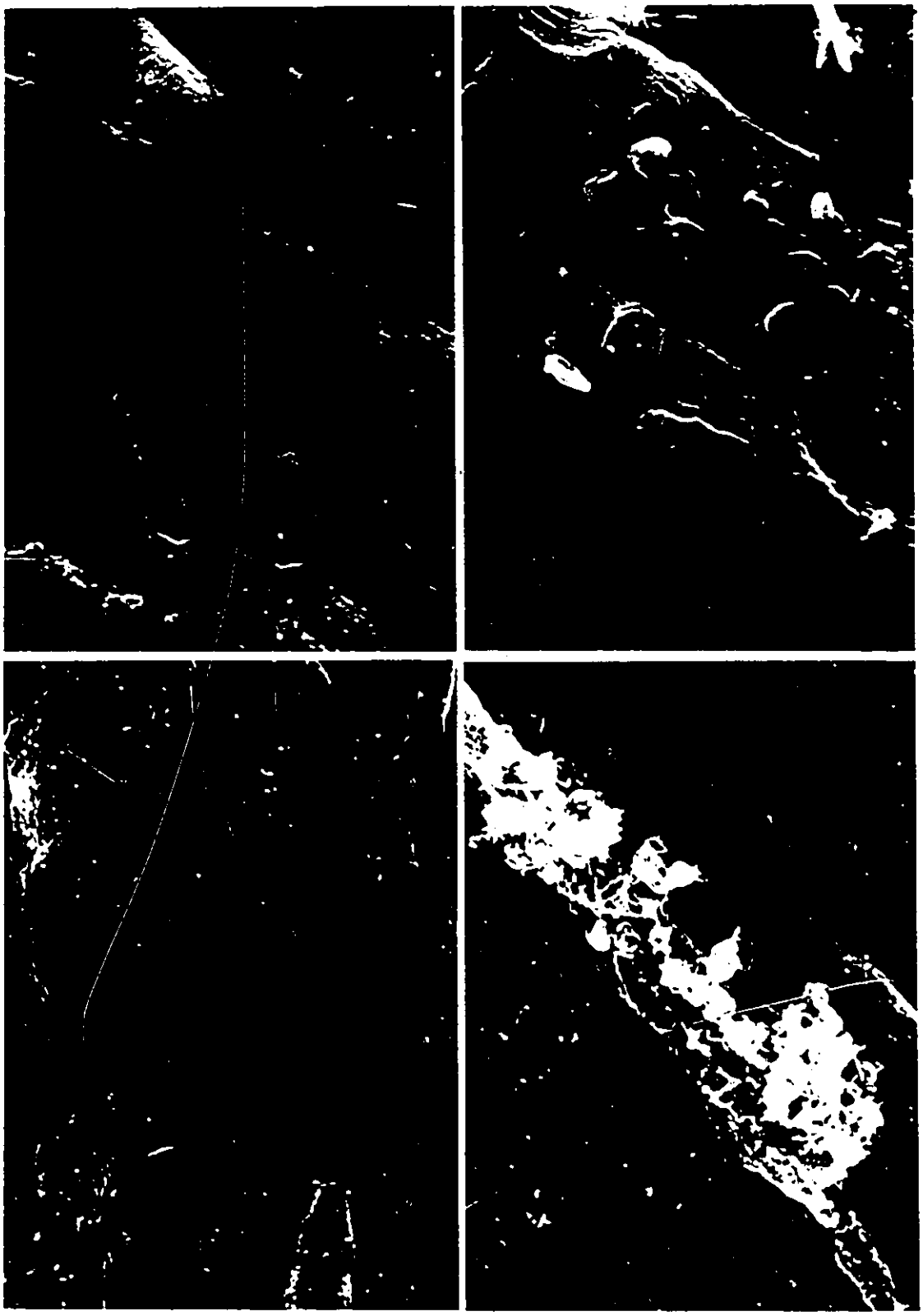
Although thrombin treatment did not promote platelet

FIGURE 29

SURFACE OF ENDOTHELIALIZED RABBIT AORTA

FOLLOWING EVERSION ON PROBE AND EXPOSURE TO THROMBIN

These scanning electron micrograph show the surface of previously undamaged rabbit aortae that have been everted on a probe and then rotated in suspensions of platelets. There is no evidence of platelet accumulation on the undamaged endothelium (A). Single platelets adhere to the subendothelium exposed by vessel injury (B). These areas of the vessel were likely damaged during the preparation and eversion procedure. There is no evidence of platelet accumulation on the endothelium on the undamaged endothelium when vessels mounted on a probe were exposed to thrombin (20 U/ml) before they were rotated in a suspension of platelets (C). However, platelet thrombi accumulated on the in regions of endothelial damage on vessels exposed to thrombin (D).


A

accumulation on undamaged endothelium, this treatment increased platelet accumulation in regions where the endothelium was damaged or lost. However, PGI_2 generation by the vessel wall was not capable of limiting the accumulation of platelets on these damaged areas of the thrombin-treated vessels under these experimental conditions.

3. Effect of PGI_2 Produced by Vessel Walls on the Accumulation of Platelets on the Subendothelium In Vivo and In Vitro

Although a de-endothelialized vessel is only capable of generating approximately 2 ng of PGI_2/mg of dry weight of tissue, this represents approximately 95 per cent of the total PGI_2 generated by the vessel wall (Moncada et al., 1977). Since cells of the vessel wall generate PGI_2 in response to stimuli, the removal of the endothelium with a balloon catheter might stimulate the vascular cells to generate sufficient PGI_2 to limit platelet accumulation soon after injury, and inhibit subsequent platelet accumulation on the vessel. Therefore, the effect of inhibiting vessel wall PGI_2 production with aspirin on the accumulation of platelets on the subendothelium in vivo and in vitro was examined.

In vivo

The accumulation of platelets in vivo on the aortae of rabbits 10 minutes following the removal of the endothelium

was similar for animals that were treated with aspirin (25 mg/kg) 10 minutes before injury, and for animals that received no aspirin (Table XI). Similarly, treatment with a higher dose of aspirin (100 mg/kg) did not affect platelet accumulation on the subendothelium of rabbit aortae. Although the lower concentration of aspirin (25 mg/kg) did not abolish the production of PGI_2 by the vessel wall, the higher concentration (100 mg/kg) was sufficient to inhibit PGI_2 production completely. Thus, the inhibition of the generation of PGI_2 by aortae by treating rabbits with aspirin produced no significant difference between the accumulation of platelets in the group that were treated with aspirin and the control group.

In vitro

Table XII shows the effect on platelet adhesion in vitro of treating platelets with aspirin to prevent the production of PGG_2 and PGH_2, that could be used by the vessels for the generation of PGI_2. As can be seen in table XII, 1) the adherence of aspirin-treated and control platelets to the subendothelium of untreated vessels was similar, and 2) treatment of the vessels with aspirin did not change the extent of adherence of control or aspirin-treated platelets. In addition, when vessels were treated with sodium arachidonate to provide substrate for the cyclooxygenase in the cells of the vessel wall and thereby enhance the possibility of

TABLE XI

EFFECT OF ASPIRIN ON PLATELET ADHERENCE

TO AORTIC ENDOTHELIUM AND SUBENDOTHELIUM IN VIVO

SURFACE STUDIED	TREATMENT OF ANIMALS	PLATELET ADHERENCE NO. PER MM. SQ.	PGI_2 PRODUCTION NG/DL
EXPERIMENT A			
ENDOTHELIUM		2,300 ± 900	0.15 ± 0.05
SUBENDOTHELIUM			
	NO ASPIRIN	47,900 ± 4,200 a	0.23
	ASPIRIN(25 MG/KG)	54,900 ± 6,500 b	0.04 ± 0.006
EXPERIMENT B*			
SUBENDOTHELIUM			
	NO ASPIRIN	34,800 ± 1,900 c	0.37 ± 0.1
	ASPIRIN(100 MG/KG)	31,800 ± 3,400 d	0
	INFUSED WITH ASPIRIN-TREATED PLATELETS	34,300 ± 4,300 e	

Mean values ± SEM for 12 or 3* animals in each group. Animals that were infused with ^{51}Cr-platelets 2 hr earlier, received an intravenous injection of aspirin 10 min before removal of the endothelium with a balloon catheter. Animals were killed by perfusion-fixation 10 min following injury and the radioactivity associated with the aortae was measured. One group was infused with aspirin-treated ^{51}Cr-platelets before injury. Results of the Student's 't' test show that there are no significant differences between adherence in groups a and b, or among groups c, d and e. In a separate group of animals Tyrode solution or aspirin was given 10 min before exsanguination and removal of vessels for measurement of PGI_2 production by the bioassay (method 2) described in Materials and Methods.

TABLE XII

EFFECT OF TREATMENT OF PLATELETS AND AORTAE WITH ASPIRIN ON THE ADHERENCE OF PLATELETS TO SUBENDOTHELIUM IN VITRO

TREATMENT OF: PLATELETS	AORTAE	PLATELET ADHERENCE NO. PER MM. SQ.	PGI_2 PRODUCTION NG/MG DRY WT.
TYRODE			
	TYRODE*	36,500 ± 4,700	0.4 ± 0.7
	ASPIRIN*	41,700 ± 3,100	0
ASPIRIN			
	TYRODE	42,900 ± 2,900	0.5 ± 0.05
	ASPIRIN	37,100 ± 1,300	0
	TYRODE + Na ARACHIDONATE	39,600 ± 1,600	1.5 ± 0.3
	ASPIRIN + Na ARACHIDONATE	38,200 ± 2,500	0

Mean values ± SEM for 6 or 3* aortae in each group. The suspensions of platelets were incubated with aspirin 1 mM or Tyrode solution in the second washing solution. Vessels were incubated in Eagle's medium containing either Tyrode solution or aspirin (2 mM) for 30 min, and 15 min after the beginning of incubation, either Tyrode solution or sodium arachidonate (0.05 mM) was added to the incubation solution. The aortae were rinsed twice in 10 ml portions of Eagle's medium before platelet adhesion was tested. An analysis of variance showed that there is no significant difference among the values for platelet adhesion in each of the groups. A segment of vessel was removed from the aortae after they were everted on the probe (but before they were rotated in a suspension of platelets), and the production of PGI_2 by the vessels was measured by the bioassay (method 2) described in the Materials and Methods.

PGI_2 production, the accumulation of platelets on the aortae was not diminished when compared to the accumulation on the vessels that were treated with aspirin to prevent the generation of PGI_2. In each of the groups the treatment of vessels with aspirin abolished the production of PGI_2.

Since the inhibition of the production of PGI_2 by the vessel walls does not alter the accumulation of platelets on the subendothelium of aortae in vitro or in vivo, it appears that the production of PGI_2 by the vessel is not responsible for the loss of reactivity of a vessel soon after the removal of the endothelium. In addition, the treatment of platelets with aspirin does not diminish their ability to accumulate on damaged vessels. These results indicate that the cyclooxygenase pathway is not required for platelet adhesion to a damaged vessel wall.

4. Effect of PGI_2 Produced by Vessel Walls on the Accumulation of Platelets on Aortae 4 or 7 Days following Removal of the Endothelium with a Balloon Catheter

Since the capacity of the vessel wall to generate PGI_2 begins to recover several days following removal of the endothelium (Eldor et al., 1981), it was considered possible that the PGI_2 produced by the vessel could be responsible for the non-reactivity of the injured surface during this period. Therefore, the effect on platelet accumulation of inhibiting PGI_2 production by vessels 4 or 7 days following injury was

examined.

When ^{51}Cr-labelled platelets were infused into animals before the removal of the endothelium, 29,100 platelets/mm^2 accumulated on the vessel during a 30 minute period following injury (Table XIII). However, when ^{51}Cr-labelled platelets, or labelled platelets and aspirin were infused into animals 2 days following injury, few platelets accumulated during the following 30 minute period on the vessels of aspirin-treated animal or animals that did not receive aspirin. The accumulation of platelets in the aspirin-treated groups was not significantly different from the accumulation in the control groups during a 30 minute period 4 or 7 days following injury.

Table XIV shows that 4 or 7 days following removal of the endothelium with a balloon catheter, the accumulation of platelets in vitro on vessels that were treated with aspirin and vessels that were not treated with aspirin was similar. The results of these experiments indicate that PGI_2 production by aortae was not responsible for preventing platelet accumulation on the vessels during a period of 7 days following the removal of the endothelium.

5. Effect of PGI_2 Produced by Vessel Walls on the Accumulation of Platelets on Injured Neointima In Vivo

Since thrombin is generated on the injured neointima, and thrombin stimulates vascular cells to generate PGI_2, it

TABLE XIII

EFFECT OF TREATMENT OF RABBITS WITH ASPIRIN
ON THE ACCUMULATION OF PLATELETS ON AORTAE IN VIVO
2, 4 OR 7 DAYS FOLLOWING DE-ENDOTHELIALIZATION

TIME OF INJURY WITH A BALLOON CATHETER	PLATELET ACCUMULATION NO. PER MM. SQ.	
	NO ASPIRIN	ASPIRIN (100 MG/KG)
FRESH INJURY	29,100 ± 1,900	
DAYS AFTER INJURY		
2	7,800 *	4,300 *
4	6,300 ± 3,000	3,500 ± 500
7	1,800 ± 500	3,200 ± 500

Mean values ± SEM for 3 animals (*n=2) in each group. ^{51}Cr-platelets were infused into rabbits immediately before removal of the endothelium, or ^{51}Cr-platelets and aspirin (100 mg/kg) were infused 2, 4 or 7 days following injury. Animals were killed by perfusion-fixation 30 min after the infusion of platelets. The radioactivity associated with the vessels was measured and accumulation of platelets on the vessels was calculated. Results of the Studentized range test show that the differences in accumulation between the group that received no aspirin and the group treated with aspirin at 4 days or at 7 days are not significant.

TABLE XIV

EFFECT OF TREATMENT OF AORTAE WITH ASPIRIN ON THE ADHESION OF PLATELETS TO THE VESSELS IN VITRO 4 OR 7 DAYS FOLLOWING DE-ENDOTHELIALIZATION

TIME OF INJURY WITH A BALLOON CATHETER	PLATELET ADHERENCE NO. PER MM. SQ.	
	TREATMENT OF AORTAE	
	NO DRUG	ASPIRIN
FRESH INJURY	47,100	
DAYS AFTER INJURY		
4	6,800	6,200
7	2,200	2,000

Mean values obtained for 2 aortae in each group. The vessels were incubated in Eagle's medium with 4 per cent albumin and Tyrode solution or aspirin (2 mM) for 15 min. The aortae were rinsed twice in 10 ml portions of Eagle's medium with 4 per cent albumin before platelet adhesion was tested. The radioactivity associated with the vessels was measured and the adherence of platelets to the vessels was calculated.

was considered possible that PGI_2 produced by the injured neointima might be capable of limiting platelet accumulation on the vessel. Therefore, the effect of treatment of aortae with aspirin on the accumulation of platelets in vivo on the neointima injured with a balloon catheter was determined. Table XV shows that the accumulation of platelets on aortae 10 minutes following injury to the neointima in animals that received no aspirin was not significantly different from the accumulation in the group that were treated with aspirin (100 mg/kg). The concentration of aspirin used in these studies did not abolish PGI_2 production by the vessels, although its generation was decreased by more than 90 percent; the accumulation of platelets on the surface of the injured neointima was not altered under these circumstances. Since the vascular cells are capable of generating new cyclooxygenase, it is possible that newly synthetized enzyme was responsible for the PGI_2 generated by the vessels treated with aspirin. Thus, although thrombin was generated and fibrin formed on the injured neointima, PGI_2 production by the vessel wall was not responsible for limiting platelet accumulation on this surface.

TABLE XV

EFFECT OF INHIBITING PGI_2 PRODUCTION BY TREATMENT OF RABBITS WITH ASPIRIN ON THE ACCUMULATION OF PLATELETS ON INJURED NEOINTIMA IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM.	PGI_2 PRODUCTION PER CENT PRODUCTION BY NORMAL VESSEL
NO INJURY		100
INJURY TO NEOINTIMA		
NO ASPIRIN	53,200 ± 11,200	96.4
ASPIRIN(100 MG/KG)	48,100 ± 7,900	9.3

Mean values ± SEM for 8 animals in each group. Seven days following removal of the endothelium and 2 hr following the infusion of ^{51}Cr-platelets, the animals received an intravenous injection of aspirin and 10 min later the neointima was injured with a balloon catheter. The animals were killed by perfusion-fixation 10 min following injury, the radioactivity associated with the aortae measured and the accumulation of platelets calculated. The Studentized range test showed no significant difference between the groups. PGI_2 production by the vessels was measured in another group of animals. These animals were given aspirin 10 min before they were exsanguinated and the vessels were removed for the measurement of their PGI_2 production by the bioassay (method 2) described in the Materials and Methods. Since synthetic PGI_2 was not available for a dose-response curve at the time of these studies, PGI_2 production was determined by measuring the of inhibition of release of platelet granule contents produced by the supernatant in which the vessels were incubated, and expressing the values as a percentage of the value for aortae from which the endothelium was not removed.

6. Effect of Products Generated by the Lipoxygenase Pathway on the Accumulation of Platelets on Rabbit Aortae

In Vitro

Since arachidonic acid released by stimulated cells can also be metabolized by the lipoxygenase pathway, products of this pathway might be capable of affecting platelet adhesion to vessel walls. Buchanan and co-workers (1983) reported that inhibition of the lipoxygenase pathway in cultured endothelial cells enhanced platelet accumulation on their surface. Therefore, the effect of inhibiting lipoxygenase on the accumulation of platelets on the endothelium and the subendothelium of aortae was determined in in vitro studies.

In these experiments, ETYA (15-hydroxy-5,8,11,13-eicosatetraenoic acid), NDGA (nordihydroguiaretic acid), or salicylate were used to inhibit the generation of products of the lipoxygenase pathway. When everted aortae with 'undamaged' endothelium were rotated in a suspension of ^{51}Cr-labelled platelets, only 2,300 platelets/mm^2 adhered to the vessel (Table XVI). Similarily, few platelets adhered to the endothelial surface of vessels treated with ETYA, NDGA or salicylate for 30 minutes before rotation in a suspension of ^{51}Cr-labelled platelets. As indicated previously in the Materials and Methods, this may not represent actual platelet adhesion, since much of this value likely represents non-

TABLE XVI

EFFECT OF INHIBITORS OF THE LIPOXYGENASE PATHWAY ON THE ADHERENCE OF PLATELETS TO ENDOTHELIUM IN VITRO

DRUG TREATMENT	PLATELET ADHERENCE NO. PER SQ. MM.
A. VESSEL TREATED WITH DRUG	
NONE	2,300 ± 300
ETYA 30 uM	3,700 ± 1,000
NDGA 10 uM	2,900 ± 500
SALICYLATE 100 uM	2,500*
SALICYLATE 500 uM	1,900*
B. PLATELETS + VESSELS TREATED WITH DRUG	
NONE	1,400 ± 600
ETYA 30 uM	1,300 ± 100
NDGA 10 uM	1,700 ± 100

Mean values ± SEM 4 aortae (*n=2) in each group. In experiments in which the vessels alone were treated, they were incubated with the drug in Eagle's medium containing 0.35 per cent albumin for 30 min before rotation. When the vessels and platelets were treated with drug, the vessels were treated as above, and in addition, the platelet suspensions were incubated with drug for 10 min before platelet adhesion was tested. An analysis of variance shows that the differences in platelet adherence among the groups in each study are not significant. When suspensions of these platelets were exposed to subendothelium they adhered to the surface in numbers similar those seen in other studies, indicating that they were capable of adhering to a reactive surface.

platelet bound radioactivity that became associated with the vessel. In addition, previous observations (Chapter III) indicate that some of this accumulation is due to the adherence of platelets in areas of endothelial damaged produced during the preparation and eversion of the vessels on the probe. When suspensions of the platelets used in these studies were exposed to everted de-endothelialized aortae on a rotating probe approximately 22,800 platets/mm^2 adhered to the surface of the vessels, indicating that these platelets were capable of adhering to a reactive surface.

It was possible that, when vessels were treated with the drugs prior to the rotation of the vessels on the probe, some of the drug was washed out of the tissue during the period of rotation, thereby diminishing the effect of the drug. Therefore, the effect of treating both the vessels and platelets with the drugs prior to and during rotation was also examined. There was little platelet accumulation on the endothelial surface when neither platelets or vessels were treated with drugs, or when both platelets and vessels were treated with NDGA or ETYA (Table XVI).

When the effect on the adherence of platelets to the subendothelium of treating both platelets and vessels with lipoxygenase inhibitors was examined, there was no significant difference between platelet adherence in the groups that were treated with drugs and adherence in the group that was not treated with drug (Table XVII). In addition, the treat-

TABLE XVII

EFFECT OF TREATMENT OF PLATELETS AND VESSELS WITH INHIBITORS OF THE LIPOXYGENASE PATHWAY ON THE ADHERENCE OF PLATELETS TO SUBENDOTHELIUM

DRUG TREATMENT OF: PLATELETS AND VESSELS	n	PLATELET ADHERENCE NO. PER SQ. MM.
NONE	9	41,800 ± 3,500
ETYA 30 uM	8	33,400 ± 4,200
NDGA 10 uM	4	38,100 ± 8,400
SALICYLATE 500 uM	4	39,400 ± 5,700

Mean values ± SEM. 'n' equals number of animals in each group. Everted vessels mounted on a probe were incubated with the drug in Eagle's medium containing 0.35 per cent albumin for 30 min and rinsed twice in 10 ml portions of Eagle's medium before rotation in a platelet suspension containing platelets treated with the drug for 10 min before platelet adhesion was tested. An analysis of variance shows that the differences in adherence among the groups are not significant.

ment of platelets with aspirin did not alter their adherence to vessels treated with ETYA, or with ETYA and aspirin (Table XVIII).

Since the inhibition of either the cyclooxygenase pathway or the lipoxygenase pathway could result in the diversion of arachidonic acid to the other pathway and enhance the generation of its products, the effect of preventing the release of arachidonic acid from membrane phospholipids, thereby making the substrate for either pathway unavailable, was examined. The results of these studies are shown in Table XIX, Experiment A. There was little platelet adherence to the endothelium when either platelets, vessels, both platelets and vessels were treated with mepacrine, or neither platelets nor vessels were treated with drug. These results are consistent with the view that the products of neither the cyclooxygenase nor lipoxygenase pathways are responsible for preventing platelets from accumulating on the endothelium under the conditions of these experiments.

When either platelets, vessels, or both platelets and vessels were treated with mepacrine to inhibit release of arachidonic acid by the phospholipaes, platelet adherence to the subendothelium was similar to the adherence when neither platelets or vessels were treated with drug (Table XIX, Experiment B).

The results of these experiments which examined the effects of products of arachidonic acid formed by the vessel

TABLE XVIII

EFFECT OF TREATMENT OF PLATELETS OR VESSELS WITH INHIBITORS OF THE LIPOXYGENASE PATHWAY ON THE ADHERENCE TO THE SUBENDOTHELIUM OF PLATELETS TREATED WITH ASPIRIN

DRUG TREATMENT OF: PLATELETS	VESSELS	n	PLATELET ADHERENCE NO. PER SQ. MM.
NONE	NONE	5	43,900 ± 6,700
NONE	ETYA	6	38,200 ± 7,300
ASPIRIN	NONE	4	36,900 ± 2,800
ASPIRIN	ETYA + ASPIRIN	4	32,900 ± 7,200

Mean values ± SEM. 'n' equals number of vessels in each group. Platelets were incubated with aspirin (1 mM) in the second washing solution for 15 min. Vessels were incubated with ETYA (30 uM) or a combination of aspirin (1 mM) and ETYA (30 uM) in Eagle's medium containing 0.35 per cent albumin for 30 min and then rinsed twice in 10 ml portions of Eagle's medium without any added drugs before platelet adhesion was tested. An analysis of variance shows that the differences in platelet adherence among the groups are not significant.

TABLE XIX

EFFECT OF MEPACRINE, AN INHIBITOR OF PHOSPHOLIPASE A_2, ON THE ADHESION OF PLATELETS TO ENDOTHELIUM OR SUBENDOTHELIUM

SURFACE STUDIED	MEPACRINE TREATMENT	PLATELET ADHERENCE NO. PER SQ. MM.
EXPERIMENT A		
ENDOTHELIUM	NONE	1,100 ± 200
	PLATELETS	1,700 ± 700
	VESSEL	1,400 ± 500
	PLATELETS + VESSEL	1,000 ± 100
EXPERIMENT B		
SUBENDOTHELIUM	NONE	26,500 ± 3,100
	PLATELETS	28,300 ± 7,800
	VESSEL	25,100 ± 4,600
	PLATELETS + VESSEL	28,800 ± 9,000

Mean values ± SEM for 4 aortae in each group. Platelets were incubated with mepacrine (100 uM) for 30 min in the second wash, and then washed a third time. Vessels were incubated with mepacrine (100 um) in Eagle's medium for 30 min, rinsed in fresh Eagle's medium for 1 min, and for 10 min in a second wash before platelet adherence was tested. An analysis of variance shows that the differences in platelet adherence among the groups are not significant. In experiment A, when suspensions of these platelets were exposed to subendothelium (data not shown) they accumulated in numbers similar to those seen in other studies, indicating that these platelets were capable of adhering to a reactive surface.

wall on the accumulation of platelets on rabbit aortae indicate that products of neither the cyclooxygenase nor lipoxygenase pathways are responsible for a) the non-reactivity of normal endothelium to platelets, b) limiting or otherwise modifying platelet interaction with damaged vessels, c) the loss of reactivity of a vessel soon after injury, or d) the non-reactivity of the surface of a vessel several days following injury.

C. Discussion

It has been speculated that products of the arachidonic acid pathway formed through both the cyclooxygenase and lipoxygenase pathways might play a role in preventing platelet accumulation on undamaged endothelium and limiting platelet accumulation on the surface of damaged vessels. The results of the studies described below indicate that, 1) PGI_2 produced by vessels does not appear to be responsible for the lack of thrombogenicty of undamaged vessels or the loss of reactivity of injured vessels, and 2) that products of the lipoxygenase pathway do not contribute significantly to the non-reactivity of normal endothelium, or to the loss of reactivity of an injured vessel.

1. PGI_2 Produced by Vessel Walls: Its Role in Inhibiting Platelet Accumulation on Vessel Walls

a) Role of PGI_2 in non-thrombogenicity of endothelium

The results of the present experiments show that the inhibition of PGI_2 production by aspirin had no effect on platelet accumulation on the endothelium of rabbit aortae either in vivo or in vitro. Although these results do not agree with the hypothesis that PGI_2 production by the vessel wall is responsible for the non-thrombogenicity of normal vascular endothelium (Moncada and Vane, 1979b), these findings are in agreement with those of Czervionke and co-workers

(1978) and Curwen and co-workers (1980), who reported that aspirin-treatment of endothelial cells in culture did not enhance platelet adherence to the cell surface. Although Dollery and Hensly (1978) initially suggested that circulating PGI_2 functions as an antithrombotic agent, it has subsequently been shown that little PGI_2 exists in the circulation (Dollery et al., 1983). Certainly the present studies show that inhibition of PGI_2 production does not lead to thrombus formation on vessel walls, which would be expected if the endothelial surface was thrombogenic and normal vascular endothelium were continuously stimulated to produce sufficient amounts of PGI_2 to inhibit platelet accumulation on their surface. Thus, it is not surprising that the inhibition of PGI_2 production by aspirin does not result in platelet accumulation on the endothelium. Rather, this indicates that PGI_2 generated by the vessel wall is not responsible for the non-thrombogenicity of its undamaged surface. It is more likely that other properties of endothelial cells are responsible for their lack of thrombogenicity.

i) Role of PGI_2 produced by endothelium exposed to thrombin

Thrombin generated at sites of thrombus formation on damaged or diseased vessels could affect platelet interaction with vessel walls in several ways. 1) Since thrombin is a potent stimulator of platelets, thrombin which might become bound to endothelial cells adjacent to sites of thrombus

formation could promote platelet accumulation and extension of thrombi onto the surface of endothelial cells. 2) Since exposure to thrombin stimulates PGI_2 production by endothelial cells, PGI_2 produced by endothelial cells with thrombin bound to their surface might be responsible for preventing platelet accumulation on these cells. 3) Since exposure of platelets to thrombin results in the activation of the arachidonic acid pathway with the generation of prostaglandin endoperoxides, PGG_2 and PGH_2 generated by the platelets might be made available to cells of the vessel wall as a substrate for the generation of PGI_2.

Although thrombin binds to endothelial cells (Dejana et al., 1985a), the results of these studies show that it does not lead to platelet accumulation on their surface. In addition, although the thrombin bound to the surface of the endothelial cells enhances their production of PGI_2 [which confirms the earlier findings of Weksler and her colleagues (1978)] the PGI_2 produced by these cells does not appear to be responsible for preventing platelet accumulation on their surface.

Although treatment with thrombin did not lead to platelet accumulation on intact endothelium in the present studies, platelet accumulation on damaged areas of a vessel was enhanced following exposure of the vessel to thrombin. These observations are compatible with the findings of Mohammed and Mason (1981), who showed that thrombin did not

promote platelet accumulation on the endothelium in their experiments in which perfused umbilical veins were exposed to thrombin. They attributed this lack of effect to the fact that thrombin could have been neutralized by the heparin and antithrombin III in the perfusate that was used in their studies. In the studies reported here, the suspending medium did not contain heparin or antithrombin III, therefore this cannot be the explanation for our findings. It appears likely that, in the present studies, platelets adhered to areas of the vessel wall damaged during removal of the aortae from the animals or during eversion of the vessels on the probe. The failure of platelets to adhere to the intact endothelium, even when thrombin was associated with its surface, might be due to the neutralization of the thrombin by the endothelial cells.

In the present studies heparin reduced platelet accumulation on damaged regions of thrombin-treated vessels (Dejana et al., 1983), indicating that increased platelet accumulation at sites of vessel injury was dependent on thrombin. Other investigators have reported that thrombin treatment increases platelet accumulation on 'undamaged' aortae (Essien et al., 1978) or on umbilical veins (Barnhart and Chen, 1978). However, since the 'undamaged' vessels were not examined morphologically, it was possible that platelets were adhering to areas of exposed subendothelium and not to undamaged endothelium. Since fibrin has been reported to

enhance platelet accumulation (Lough and Moore, 1975), the platelet aggregates on the thrombin-treated umbilical veins might have been associated with fibrin deposited on the endothelium and not directly adherent to the endothelium itself. Other investigators have reported that the inhibition by aspirin of PGI_2 production by thrombin-treated endothelial cells in culture increased the accumulation of platelet aggregates when thrombin was present in the suspending fliud (Czervionke et al., 1978). However, aspirin treatment did not enhance platelet accumulation when thrombin was not present in the suspending fluid. Therefore, it was possible that in the studies of Czervionke and his colleagues (1978), the interaction of thrombin with released fibrinogen was responsible for the formation of platelet aggregates and that these aggregates settled onto the surface of the endothelial cells grown in culture.

Dejana and her colleagues (1983) in this laboratory showed that ^{125}I-thrombin which became bound to the endothelium of rabbit aortae in vitro was displaced by heparin. This indicates that the lack of platelet accumulation on the endothelium cannot be attributed to the failure of thrombin to bind to the endothelial surface. However, it is possible that heparan sulfate on the surface of the endothelial cells (Wight, 1980) might neutralize the bound thrombin so it is not available to interact with platelets. This was not tested in the present studies. Dryjski and his colleagues

(1982) showded that, although thrombin bound to vascular endothelium retained its enzymatic activity, the bound thrombin was rapidly inactivated in the presence of anti-thrombin III. Thus, at sites of thrombus formation in vivo endothelial cells might decrease the effects of thrombin on platelets by binding thrombin so that it is not available to stimulate platelets.

The theory that platelets provide PGH_2 for use by the cells of the vessel wall in the synthesis of PGI_2 (Moncada et al., 1977; Weksler et al., 1977; Nordoy et al., 1978; Marcus et al., 1980) was supported by the findings of the present studies. The generation of PGI_2 by segments of aspirin-treated aortae in the presence of platelets and thrombin, was abolished by aspirin treatment of the platelets, indicating that the endoperoxides PGG_2 and PGH_2 generated by stimulated platelets were utilized by the vascular cells for the generation of PGI_2.

Thus, the results of these experiments show that PGI_2 production by the vessel wall is not responsible for 1) the non-thrombogenicity of the undamaged endothelium, 2) the lack of platelet interaction with the endothelium when thrombin is bound to its surface, or 3) limiting platelet accumulation in the regions of endothelial cell damage, even when thrombin associated with an injured vessel is responsible for some of the platelet accumulation in these areas.

b) Role of PGI_2 in limiting platelet accumulation on injured vessels

Although platelets rapidly accumulate on a vessel following exposure of the subendothelial connective tissue or injury of the neointima, few additional platelets accumulate on vessels following the formation of a layer of adherent platelets. The factors responsible for limiting platelet accumulation on a damaged vessel, have never been clarified. Since the stimulation of cells of a vessel from which the endothelium has been removed could theoretically lead to activation of phospholipases, the generation of products of the arachidonic acid pathway, such as PGI_2, that might be responsible for limiting platelet accumulation on the subendothelium or the injured neointima.

i) Subendothelium

We found that the capacity of the vessels to produce PGI_2 was considerably diminished when the aortic endothelium was removed, an observation that is in agreement with that of Moncada and co-workers (1977). They demonstrated that, 1) the removal of the endothelium results in a decrease in the capacity of the vessel wall to generate PGI_2, and 2) endothelial cells in culture are capable of producing more PGI_2 than are smooth muscle cells. However, these findings differ from those of other investigators. Baenziger and co-workers (1979) reported that there was no difference in

PGI_2 production by endothelial cells and smooth muscle cells in culture, and MacIntyre and co-workers (1978) were unable to demonstrate PGI_2 production by vascular smooth muscle cells.

Although the capacity of the vessel wall to produce PGI_2 was considerably reduced by the removal of the endothelium in the present study, it seemed reasonable to speculate that mechanical stimulation of the vessel produced by the inflated catheter during the removal of the endothelium could stimulate the cells of the vessel wall to increase PGI_2 production, so that the actual PGI_2 production by the de-endothelialized vessels might be sufficient to limit platelet accumulation on the subendothelium. However, the results of these studies show that treatment of the de-endothelialized aortae with aspirin in concentrations sufficient to inhibit PGI_2 production did not increase platelet accumulation on the subendothelium either in vitro or in vivo. Other investigators have reported that the inhibition of PGI_2 production by aspirin or other non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase activity resulted in enhanced platelet accumulation on damaged vessels under conditions where there was platelet aggregation and thrombus formation including: chronic damage of pulmonary arteries with the canine heartworm Dirofilaria immitis (Schwab et al., 1981), ligation of coronary arteries in baboons (Ruf et al., 1980), injury of rabbit aorta by 6 passages of a 5F Fogarty catheter inflated

to a pressure of approximately 735 mm Hg (Wu et al., 1981), and exposure of everted aortae to flowing blood in a perfusion chamber (Jaeger et al., 1979). In each of these cases, although the extent of injury was not quantitated, it appears likely that there was extensive thrombin generation and fibrin formation that lead to the accumulation of platelet thrombi on the injured surfaces. Under the conditions of the present experiments a layer of platelets formed on the exposed subendothelium in vivo with little if any evidence of fibrin formation, and the adherence of individual platelets and not platelet aggregates was determined in the in vitro studies, so that the PGI_2 effects studied were the effects upon platelet adhesion and not upon platelet aggregation. Although under the conditions of the experiments done by other investigators, PGI_2 production by the vessel wall might have been sufficient to inhibit platelet aggregation and thrombus formation, in the present experiments PGI_2 production by the vessel wall was not responsible for limiting platelet adherence to the subendothelium.

In studies that are to be presented in Chapter V, it was demonstrated that 100 nM PGI_2 was required to inhibit platelet adherence to subendothelium or collagen-coated glass, and other investigators have reported that 10-100 nM synthetic PGI_2 was required to inhibit platelet adherence to the aortic subendothelium (Cazenave et al., 1979a; Higgs et al., 1979; Weiss and Turitto, 1979). Since, in the present

experiments, vessel wall PGI_2 production (0.4 ng/kg dry weight) was much less than that required to decrease platelet adhesion or to inhibit the release of serotonin from adherent platelets, it was not surprising that the inhibition of the small amount of PGI_2 produced by the vessel wall did not enhance platelet accumulation on the subendothelium.

ii) Injured neointima

Although platelets and platelet-fibrin thrombi rapidly accumulate on the injured neointima, the number of platelets that accumulate on the reinjured vessel are similar to the number that accumulate on the freshly exposed subendothelium. It was considered possible that the amount of PGI_2 produced by a reinjured vessel would be greater than the amount generated following exposure of the subendothelium and might be sufficient to limit platelet accumulation on the injured neointima for the following reasons. 1) Other investigators have reported that the vessel wall regained some of its capacity to generate PGI_2 by one week following removal of the endothelium (although the ability of the injured vessels to produce PGI_2 did not return to normal values until approximately 10 weeks following denudation) (Eldor et al., 1981), 2) PGI_2 production by the injured neointima could be stimulated by thrombin generated at its surface, and 3) PGI_2 production could be stimulated by mechanical injury of the vessel. In addition, since the concentration of PGI_2 re-

quired to inhibit the response of platelets to the concentrations of thrombin made available at the surface of a re-injured vessel might be less than the concentration of PGI_2 required to inhibit platelet adhesion to components on damaged vessel walls, platelet accumulation on the injured neointima might be more susceptible to inhibition by PGI_2 than platelet accumulation on the subendothelium. However, in the present experiments inhibition of PGI_2 production by the vessel wall did not enhance platelet accumulation on the injured neointima that had formed 7 days following de-endothelialization. Thus, it appears that PGI_2 produced by the vessel wall is not responsible for limiting platelet accumulation on the surface of the injured neointima, even when coagulation is activated and thrombin is generated on the surface of the vessel.

c) Role of PGI_2 in Loss of Reactivity of Injured Vessels

Although we have demonstrated that the vessel wall is non-reactive to platelets at later times following injury (during a one week period of observation), even when the layer of adherent platelets are lost from the surface of the vessel, the factors responsible for the loss of reactivity of an injured vessel are not clearly understood. Since we and others have demonstrated that vessel walls regain some of their ability to generate PGI_2 by several days following removal of the endothelium (Eldor et al., 1981), and in our

studies the capacity of a vessel to generate PGI_2 was similar to that of an undamaged vessel by one week following injury, it was speculated that PGI_2 produced by a vessel might be responsible for its loss of reactivity at later times following injury. However, in the present studies the inhibition of PGI_2 production by treatment with aspirin during periods up to 7 days following injury did not increase platelet accumulation on rabbit aortae. This leads to the conclusion that vessel wall production of PGI_2 is not responsible for the non-reactivity of the surface exposed when adherent platelets are no longer present on the exposed subendothelial connective tissue, or for the lack of reactivity to circulating platelets of the neointimal smooth muscle cells that form much of the luminal surface by one week following injury.

2. Products of the Lipoxygenase Pathway: Their Role in the the Non-Thrombogenicity of Endothelium and Effect on Platelet Accumulation on Damaged Vessels

In 1983 Buchanan and his co-workers reported that a linoleic acid derived product of the lipoxygenase pathway, more recently identified as 13-hydroxyoctadecadienoic acid (Buchanan et al., 1985b), reduced platelet accumulation on endothelial cells in culture. They speculated that lipoxygenase products might be responsible for the non-reactivity of normal endothelium. In subsequent studies (Buchanan et

al., 1985a) demonstrated that although inhibition of lipoxygenase enhanced the production of PGI_2 and inhibition of cyclooxygenase enhanced the generation of products of the lipoxygenase pathway, in neither case was platelet accumulation affected. However, since they observed that when both lipoxygenase and cyclooxygenase were inhibited platelet accumulation on cultured endothelial cells increased, and platelet accumulation on a thrombogenic surface was decreased by treatment of the surface with 13-hydroxyoctadecadienoic acid, they concluded that this metabolite of lipoxygenase was responsible for the non-thrombogenicity of normal endothelium. However, in the present studies, the treatment of vessels or platelets with drugs that inhibit lipoxygenase alone or inhibit both lipoxygenase and cyclooxygenase, or prevent the release from membrane phospholipids of arachidonic acid to be used by either the lipoxygenase of cyclooxygenase pathways, did not result in platelet accumulation on the endothelium. These results indicate that products of the lipoxygenase pathway do not contribute significantly to the non-reactivity of normal aortic endothelium. In addition, Buchanan and his colleagues (1985b) also demonstrated that treatment of endothelial cells with thrombin decreased the production of 13-hydroxyoctadecadienoic acid. If the production of this substance by endothelial cells was responsible for the non-thrombogenicity of the endothelial surface, then treatment

with thrombin should lead to the accumulation of platelets on the endothelium. However, in the present studies, treatment with thrombin did not lead to platelet accumulation on endothelium. Thus, it appears unlikely that 13-hydroxyoctadecadienoic acid is responsible for the non-thrombogenicity of normal endothelium or of endothelium with thrombin bound to its surface.

These findings indicate that the non-thrombogenic properties of the endothelial surface are not due to: 1) products of the lipoxygenase pathway, 2) enhanced production of PGI_2 following the inhibition of lipoxygenase, 3) enhanced generation of products of the lipoxygenase pathway following the inhibition of cyclooxygenase, or 4) the combined effects of PGI_2 and products of the lipoxygenase pathways.

The results of these studies also show that inhibiting lipoxygenase, or preventing the release from membrane phospholipids of arachidonic acid to be used by either the lipoxygenase or cyclooxygenase pathways, does not enhance platelet accumulation on the subendothelium of rabbit aortae. Thus, the generation by the vessel wall of products of the lipoxygenase pathway is not responsible for limiting platelet accumulation on a freshly injured vessel.

3. Conclusions

In conclusion the results of the studies presented in this chapter show that:

1. PGI_2 generated by the vessel wall under the conditions of these studies is not responsible for:
   a) preventing platelet accumulation on vascular endothelium even when thrombin is bound to the endothelial cell surface.
   b) limiting platelet accumulation on injured vessels even when thrombin is generated on the vessel surface.
   c) the loss of reactivity of vessels following a single injury or reinjury of a previously damaged vessel.
2. Products of the lipoxygenase pathway are not responsible for preventing platelet accumulation on normal endothelium, or for limiting platelet accumulation on the surface of damaged vessels.

CHAPTER V

LOSS OF REACTIVITY OF INJURED VESSELS FOR PLATELETS: EFFECT OF BLOOD COMPONENTS

LOSS OF REACTIVITY OF INJURED VESSELS FOR PLATELETS: EFFECT OF BLOOD COMPONENTS

A. Introduction

Although it appears that the surface of the layer of platelets that rapidly covers the subendothelium exposed by vessel injury is responsible for the loss of reactivity of a vessel soon after injury, the factors responsible for the loss of reactivity of the vessel surface exposed to the circulating blood following the loss of the adherent platelets are not known. The results of studies presented in Chapter IV show that the products of arachidonate metabolism that are generated by the vascular cells are not responsible for the loss of reactivity of the surface of an injured vessel. However, other substances generated by the vessel wall, or the interaction of blood components including plasma or formed elements, might decrease the reactivity of an injured vessel (see Chapter I for a more detailed discussion).

Blood components might decrease reactivity of vessel walls in several ways including: 1) digestion of surface components such as collagen or elastin by proteolytic enzymes released from adherent platelets, leukocytes, or derived from other sources such as the activation of plasminogen; 2) alteration of the properties of the reactive sites by adsorbed plasma proteins; 3) loss of proteins such as von Willebrand factor or 4) masking of reactive sites by remnants of mem-

brane components left on the surface following the loss of adherent platelets, or following the contact of circulating red cells. Several of these possibilities were examined.

1. Platelets

Platelets that initially adhere to injury sites are lost over a period of several days. The adherent platelets might lyse and leave their membrane remnants on the vessel where they could mask reactive sites on the injured surface. Since platelet granules contain a number of proteolytic enzymes (Kaplan, 1981), and since released granule proteins penetrate the vessel wall (Goldberg and Stemerman, 1980), it is also possible that proteolytic enzymes released from adherent platelets might digest components of the injured vessel and alter the reactivity of the surface.

If platelet interaction with an injured vessel were responsible for its loss of reactivity, then the injured surface would likely remain reactive if platelet interaction with it was inhibited; if other factors were responsible for the loss of reactivity, then the injured vessel wall would likely become non-reactive even though platelet interaction with the surface was inhibited.

Three experimental approaches were used to determine whether the surface of the injured vessels became non-reactive even in the absence of platelets or other formed elements of the blood. These include: 1) decreasing the

number of platelets available to interact with the injured vessel wall by inducing thrombocytopenia (Evensen et al., 1968) in the animals before injuring their vessels; 2) decreasing platelet accumulation on the vessel surface with drugs that are known to inhibit platelet adhesion to surfaces, for example dipyridamole and PGI_2 (Packham et al., 1978); and 3) incubating in a culture media in vitro, vessels that were injured in situ.

In the past several experimental approaches have been used to induce thrombocytopenia in rabbits including: the administration of drugs, exposure to ionizing radiation, and injections of antiplatelet serum (Evensen et al., 1968; Moore et al., 1976). Each of these approaches has some problems for example, the exposure of animals to ionizing radiation might affect their vascular cells so that their response to injury is altered, and the use of antiplatelet serum could result in the formation of immune complexes that might affect platelet interaction with damaged vessels (Knicker and Cochrane, 1968). However, treatment with busulfan an alkanyl sulfonate that inhibits cell division by alkylating the purine base guanine, can be used at concentrations that inhibit platelet production while having little effect on the production of leukocytes (Evensen et al., 1968). Therefore, in this study busulfan was used to confirm the concentrations required to produce thrombocytopenia and to examine the effect of buslufan-induced thrombocytopenia on

the subsequent reactivity of injured vessels.

2. Plasma Factors

It is reported that the adsorption of plasma proteins alters the reactivity of a surface in vitro (Salzman, 1971; Packham et al., 1969). Since injured surfaces will be exposed to plasma proteins in vivo, adsorption of proteins could result in a rapid loss of reactivity. To test this hypothesis the reactivity of de-endothelialized aortae following incubation in vitro in plasma, serum, or Eagle's medium containing albumin was determined.

3. Red Blood Cells

The surface of circulating red cell membranes appears to be non-thrombogenic, since coagulation is not activated by exposure to these cells and platelets do not accumulate on their surface. Since red cells in the flowing blood are likely to come in contact with the surface of an injured vessel, and the accumulation of materials from red cell membranes might mask reactive sites on the damaged vessel wall, the possibility that circulating red cells might be responsible for the loss of reactivity of an injured vessel was examined.

4. Objectives of the Study

Thus, the specific objectives of these studies were to

determine whether the loss of reactivity of injured vessels results from:

1. the interaction of platelets or red blood cells with the injured vessel.

2. the adsorption of plasma proteins or the effects of other plasma factors at the injury site.

B. Results

1. Inhibition of Platelet Accumulation on Injured Aortae

To determine whether the loss of reactivity occurs independently of platelet interaction with an injury site, the accumulation of platelets on injured vessels was inhibited by using antiplatelet drugs, or by inducing thrombocytopenia before vessel injury to reduce the number of circulating platelets available to interact with the vessels. Changes in the reactivity of an injured vessel that occurred during incubation of the vessel in vitro in the absence of platelets, were also examined.

a) Thrombocytopenia: Effect on the Subsequent Reactivity of Injured Vessels

i) Effect of Busulfan on Platelet Count

The results of studies to determine the dose of busulfan and the time required for this treatment to reduce the platelet count below 20,000/mm^3 are shown in Table XX (busulfan was administered through an esophageal catheter). On the eleventh day after the administration of busulfan at a dose of 25 mg/kg on day 0 and day 2, the circulating platelet count decreased to less than 20,000/mm^3.

TABLE XX

EFFECT OF TREATMENT WITH BUSULFAN

ON WHOLE BLOOD PLATELET COUNT IN RABBITS

DAY	n	PLATELET COUNT NO. PER CUBIC MM.
0	16	552,800 ± 45,000
2	16	625,900 ± 32,600
4	15	608,200 ± 26,600
6	8	396,700 ± 27,300
7	7	241,900 ± 24,800
9	15	53,200 ± 6,400
10	15	32,200 ± 3,100
11	11	16,600 ± 2,500

Values are mean ± standard error. 'n' equals the number of animals in each group. Busulfan (25 mg/kg) was administered on day 0 and day 2.

ii) Effect of Thrombocytopenia on Subsequent Reactivity of the Injured Vessel to Circulating Platelets

In these studies, when the platelet count had fallen below 20,000/mm^3 on the eleventh day following treatment with busulfan, the aortic endothelium was removed with a balloon catheter. One group of animals was infused with ^{51}Cr-labelled platelets 8 hours following de-endothelialization of the aorta with a balloon catheter and perfusion-fixed 30 minutes following the infusion of platelets. A control group of animals that received no busulfan treatment, and a group of animals that were treated with busulfan were infused with ^{51}Cr-labelled platelets before the aortic endothelium was removed and killed by perfusion-fixation 30 minutes following injury to the aortae. The results of these studies are shown in Table XXI. Platelet accumulation on aortae of busulfan-treated animals exposed to circulating ^{51}Cr-labelled platelets for a 30 minute period 8 hours following injury with a balloon catheter was significantly less than the accumulation on the aortae of control animals 30 minutes following injury. These results raised the possibility that in the thrombocytopenic animals much of the reactivity of the injured vessels was lost by 8 hours following injury. However, the platelet accumulation in the 30 minute period following injury in the busulfan-treated group infused with labelled platelets immediately before removal of the endothelium, was similar to the accumulation during a 30 minute period 8 hours following

TABLE XXI

EFFECT OF BUSULFAN-INDUCED THROMBOCYTOPENIA ON THE ACCUMULATION OF PLATELETS ON THE SURFACE OF AORTAE DE-ENDOTHELIALIZED WITH A BALLOON CATHETER

DRUG TREATMENT	INJECTION OF ^{51}CR-PLATELETS	PERFUSION -FIXATION	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NO BUSULFAN)	BEFORE INJURY	30 MIN AFTER INJURY	24,200 ± 1,400
BUSULFAN	BEFORE INJURY	30 MIN AFTER INJURY	13,300 ± 3,000
BUSULFAN	8 HR AFTER INJURY	30 MIN AFTER INJECTION OF PLATELETS	12,000 ± 2,300

Values are mean ± standard errors for 6 animals in each group. Control animals were infused with ^{51}Cr-platelets before injury and perfusion-fixed 30 min folowing injury. Busulfan-treated animals were infused with ^{51}Cr-platelets 8 hr following injury with a balloon catheter killed by and perfusion-fixation 30 min following the infusion of platelets. The per cent of infused ^{51}Cr-platelets recovered in the circulation immediately before perfusion-fixation was 90.1 ± 3.6 per cent in the group that received no busulfan, and 105.5 ± 3.4 per cent in busulfan-treated animals. Results of the Studentized range test showed that, for the comparison between the control group and either group treated with busulfan, $p<0.05$. The difference between the groups treated with busulfan is not significant.

injury in the busulfan-treated animals. These results indicate that the decrease in rate of platelet accumulation on the aortae of busulfan-treated animals 8 hours following injury compared to the rate of accumulation on freshly injured surface, could not be solely attributable to loss of reactivity of the injured vessel with time, but rather that the drug had some effect on the properties of the vessel wall itself, or the decreased accumulation resulted from the lower numbers of circulating platelets in the busulfan-treated animals.

To examine this possibility, the effect of treatment of animals with busulfan on the adherence of platelets to the subendothelium of their aortae in vitro was examined. Animals were treated with busulfan (25 mg/kg on day 0 and day 2), and on day 11 the animals were exsanguinated, the endothelium of the aortae removed with a balloon catheter, and the vessels removed and everted on a probe for the determination of platelet adherence. In these studies, 18,700 $\pm$ 3,200 platelets/mm^3 adhered to the aortae from busulfan-treated animals, compared to the 30,400 $\pm$ 5,500 platelets/mm^3 that adhered to the aortae from control animals. These results indicate that the reactivity of the vessels was decreased by treatment of the animals with busulfan.

The results of the in vivo studies with busulfan were complicated by the observation that, although the weight of both groups was the same before treatment with drug, by the end of the period of the study the weight of the busulfan-treated animals decreased by approximately 15 percent com-

pared to the control group (busulfan-treated 2.63 ± 0.11 kg; control 3.09 ± 0.09 kg). The hematocrit was also decreased by approximately 25 percent (busulfan-treated 26.2 ± 1.6; control 35.5 ± 1.0) and the erythrocyte sedimentation rate was increased.

Since changes in hematocrit, or properties of the vessel wall could influence platelet accumulation on the surface of an injured vessel, the effects of thrombocytopenia on the reactivity of injured vessels could not be studied in animals that were treated with busulfan.

b) Anti-Platelet Drugs: Effect on Platelet Accumulation on Injured Vessels

Although a number of drugs are known to affect platelet function including platelet accumulation on collagen-coated glass or de-endothelialized vessels in vitro (Mustard and Packham, 1978), it was not known whether these drugs would inhibit platelet accumulation on the damaged surface of vessel walls in vivo. Therefore, the effect on platelet accumulation of treatment with several anti-platelet drugs was examined to determine which drug inhibited platelet accumulation to the greatest extent in vivo.

1) Dipyridamole

Results of studies on the effect of dipyridamole on platelet adhesion to surfaces in vitro are shown in

Table XXII. Dipyridamole (100uM) reduced platelet accumulation on collagen-coated glass and subendothelium when compared to the accumulation when no drug was used. The release of ^{14}C-serotonin from the granules of platelets adherent to either surface was also reduced.

Table XXIII shows that the incubation of vessels with dipyridamole produced no inhibition of platelet accumulation on the vessel, indicating that the reduction in platelet accumulation was not due to changes in the properties of the vessel wall produced by dipyridamole.

To determine the effect of dipyridamole on platelet adhesion to the subendothelium of rabbit aortae in vivo, dipyridamole was administered intravenously 10 minutes before the removal of the endothelium, and the aortae were perfusion-fixed 10 minutes following de-endothelialization. Platelet accumulation in animals treated with dipyridamole (12.5 mg/kg) was significantly reduced when compared to accumulation in the control group (Table XXIV). When a lower dose of dipyridamole (2.5 mg/kg) was used, the extent of reduction in platelet accumulation was less than that produced by the higher dose of drug. Treatment with aspirin, at concentrations sufficient to completely inhibit cyclooxygenase and abolish the generation of PGI_2, did not diminish the inhibitory effect of dipyridamole on platelet accumulation. Thus, treatment with dipyridamole inhibited platelet accumulation in vivo, and the inhibitory effect of dipyridamole on

TABLE XXII

EFFECT OF DIPYRIDAMOLE ON THE ADHERENCE OF PLATELETS TO SUBENDOTHELIUM OR COLLAGEN-COATED GLASS IN VITRO

SURFACE EXPOSED TO PLATELETS	DIPYRIDAMOLE (uM)	PLATELET ADHERENCE NO. PER MM. SQ.	PER CENT RELEASE OF ^{14}C-SEROTONIN
COLLAGEN-COATED GLASS	0	89,900 ± 3,100	43.9 ± 2.0
	1	79,900 ± 3,700	43.9 ± 3.0
	10	79.900 ± 3,800	35.2 ± 3.0*
	100	61,300 ± 5,300***	17.7 ± 2.8***
SUBENDOTHELIUM	0	30,700 ± 2,500	31.8 ± 4.6
	1	27,200 ± 2,500	28.2 ± 3.6
	10	23,200 ± 2,500	24,7 ± 3.1
	100	16,400 ± 2,700**	15.3 ± 3.8*

Values are mean ± standard error (collagen-coated glass n=8; subendothelium n=6). Dipyridamole was incubated with platelets suspended in Eagle's medium containing 4 per cent albumin and apyrase for 10 min before the addition of red cells (hematocrit 40 per cent). Platelet count for experiments with collagen-coated glass, 300,000/mm^3; with subendothelium, 500,00/mm^3. Imipramine (1μM) was added to inhibit the reuptake of serotonin released by the platelets. Results of the Student's 't' test are for the comparison with the control not treated with drug (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

TABLE XXIII

EFFECT OF INCUBATING AORTAE WITH DIPYRIDAMOLE ON THE ADHERENCE OF PLATELETS TO SUBENDOTHELIUM IN VITRO

TREATMENT OF VESSELS	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NO DIPYRIDAMOLE)	20,500 ± 5,900
DIPYRIDAMOLE	29,100 ± 3,300

Values are mean ± standard errors for 8 vessels. Vessels were incubated in Eagle's medium containing 4 per cent albumin for 30 min at 37 °C. To treat the aortae with drug, dipyridamole (0.22 mg/ml) was added to the medium before incubation of the vessels. The vessels were rinsed in 2 changes of Tyrode's solution before they were rotated in a suspension of ^{51}Cr-labelled platelets suspended in Eagle's medium containing 4 per cent albumin, apyrase and red cells (hematocrit 40 per cent). The dose of dipyridamole used was equivalent to the theoretical maximum plasma concentration that could be attained following the intravenous injection of 12.5 mg/kg of the drug. Results of the Studentized range test show that the difference in platelet adhesion between the groups is not significant.

TABLE XXIV

EFFECT OF DIPYRIDAMOLE ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	n	PLATELET ACCUMULATION NO. PER SQ. MM.
PLACEBO	15	41,100 ± 3,800
DIPYRIDAMOLE (12.5 mg/kg)	15	18,100 ± 3,000***
PLACEBO	9	37,400 ± 3,700
DIPYRIDAMOLE (2.5 mg/kg)	9	22,200 ± 3,600*
PLACEBO	6	56,200 ± 8,600
DIPYRIDAMOLE (2.5 mg/kg) + ASPIRIN (100mg/kg)	6	25,200 ± 7,800**

Values are mean ± standard error. 'n' equals number of animals in each group. Animals received an infusion of ^{51}Cr-labelled platelets 18 hr before the removal of the endothelium. Dipyridamole, placebo (vehicle for dipyridamole), or aspirin was given intravenously 10 min before injury. The aortae were perfusion-fixed 10 min following injury. Results of the Studentized range test are for the comparison between the placebo and the drug-treated groups (* $p<0.025$, ** $p<0.05$, and *** $p<0.005$). The level of dipyridamole in plasma samples taken from 5 animals 15 min after they had received an intravenous injection of dipyridamole (12.5 mg/kg) was 13.5 ± 1.1 µg/ml.

platelet adhesion was not dependent on the generation of PGI_2 by the vessel wall.

Since much of the platelet accumulation on the injured neointima is dependent on the activation of coagulation with the generation of thrombin and the formation of fibrin, while platelets appear to adhere directly to subendothelium exposed by a single injury with a balloon catheter, the effect of dipyridamole on platelet accumulation following injury of rabbit aorta that had been de-endothelialized 7 days previously was examined. When dipyridamole is administered intravenously 10 minutes before balloon injury to the neointima, and the aortae were perfusion-fixed 10 minutes following injury, platelet accumulation was reduced when compared to the accumulation in the control group that received no drug (Table XXV). Platelet accumulation in the group treated with the vehicle in which the drug was dissolved was not significantly different from the accumulation in the control group.

Thus, treatment with dipyridamole inhibits platelet accumulation on collagen-coated glass and subendothelium in vitro, and on subendothelium and injured neointima in vivo.

ii) $\underline{PGI_2}$

Since the half-life of PGI_2 in the circulation is extremely short, a continuous infusion of the drug was used to determine its effect on platelet accumulation on injured vessels in vivo. A continuous infusion of PGI_2 was begun one

TABLE XXV

EFFECT OF DIPYRIDAMOLE

ON THE ACCUMULATION OF PLATELETS ON INJURED NEOINTIMA IN VIVO

TREATMENT OF ANIMALS	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NO DIPYRIDAMOLE)	48,100 ± 6,300
PLACEBO	47,800 + 6,900
DIPYRIDAMOLE (12.5 mg/kg)	33,200 ± 5,100

Values are mean ± standard error for 7 animals. The animals received an intravenous injection of dipyridamole or placebo (vehicle for dipyridamole) 10 min before the neointima that had formed 7 days following the removal of the endothelium was injured with a balloon catheter. ^{51}Cr-labelled platelets were infused into the circulation of the animals 18 hr before neointimal injury. Aortae were perfusion-fixed 10 min following injury. The result of the Studentized range test for the comparison between the dipyridamole-treated group and either the vehicle-treated group or the group that received no dipyridamole is $p<0.05$.

minute before the exposure of the subendothelium with a balloon catheter, and maintained until the aortae were perfusion-fixed 10 minutes following injury. The effects on platelet accumulation of infusing several doses of PGI_2 are shown in Table XXVI. The infusion of PGI_2 at a rate of 850 or 425 ng/kg/min significantly reduced platelet accumulation when compared to the accumulation in the controls that received no drug. However, lower doses of PGI_2 were less effective as inhibitors of platelet accumulation.

The extent of inhibition of platelet accumulation on the aortic subendothelium in vivo produced by the infusion of 850 ng/kg/min of PGI_2 was examined in a larger study (Table XXVII). In these experiments the infusion of PGI_2 (850 ng/kg/min) significantly reduced platelet accumulation compared to the controls that received no drug. Although PGI_2 also produced a decrease in blood pressure (Figure 30), this extent of hypotension was well tolerated by the animals.

iii) Dipyridamole and PGI_2

In Vitro

In earlier studies it was demonstrated that 2 mM aspirin completely abolished PGI_2 production by de-endothelialized aortae (Table XI). However, this concentration of aspirin had no effect on platelet accumulation on the surface of damaged vessels (Table XXVIII). On the other hand, treat-

TABLE XXVI

EFFECT OF INFUSING DIFFERENT CONCENTRATIONS OF PGI_2 ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	n	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NO PGI_2)	3	45,100 ± 5,900 a
PGI_2 (ng/kg/min)		
850	2	6,200 ± 500 b
425	3	13,200 ± 2,000 c
212	2	30,200
106	2	23,300

Values are mean ± standard error. 'n' equals number of animals in each group. The infusion of PGI_2 was begun 1 min before removal of the endothelium from the aortae of rabbits that were infused with ^{51}Cr-labelled platelets 18 hr earlier. The aortae were perfusion-fixed 10 min following balloon injury. Results of the Studentized range test show that, for the comparison between a vs b, $p<0.001$;
b vs c, $p<0.05$.

TABLE XXVII

EFFECT OF INFUSING PGI_2 ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	n	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NO PGI_2)	11	48,900 ± 5,800
PGI_2 (850 ng/kg/min)	10	6,400 ± 1,100

Values are mean ± standard error. 'n' equals number of animals in each group. The infusion of PGI_2 into the aorta was begun 1 min before the removal of the endothelium from the aortae of rabbits that had received an infusion of ^{51}Cr-labelled platelets 18 hr earlier. The aortae were perfusion-fixed 10 min following de-endothelialization with a balloon injury. The result of the Studentized range test for the comparison between the groups is $p<0.001$.

FIGURE 30

EFFECT OF INFUSION OF PGI_2
ON SYSTOLIC BLOOD PRESSURE IN RABBITS

This figure shows the effect of infusing PGI_2 (850 ng/kg/min) on the systolic blood pressure in rabbits. A solution of PGI_2 was infused through a cannula introduced through the left common carotid artery and passed into the ascending aorta to approximately 0.5 cm above the aortic valve. A pressure-sensitive transducer for monitoring blood pressure was introduced into the right common carotid artery and connected to a Mingograf 34. The recording of blood pressure was begun before the infusion of PGI_2 was started and was continued after the infusion was stopped. In this study a continuous infusion of PGI_2 was begun at time '0' and maintained for 11 minutes. In other studies, the changes in blood pressure produced by a continuous infusion of PGI_2 for periods up to 8 hours were similar to those observed in this study.

EFFECT OF PGI_2 INFUSION ON SYSTOLIC BLOOD PRESSURE

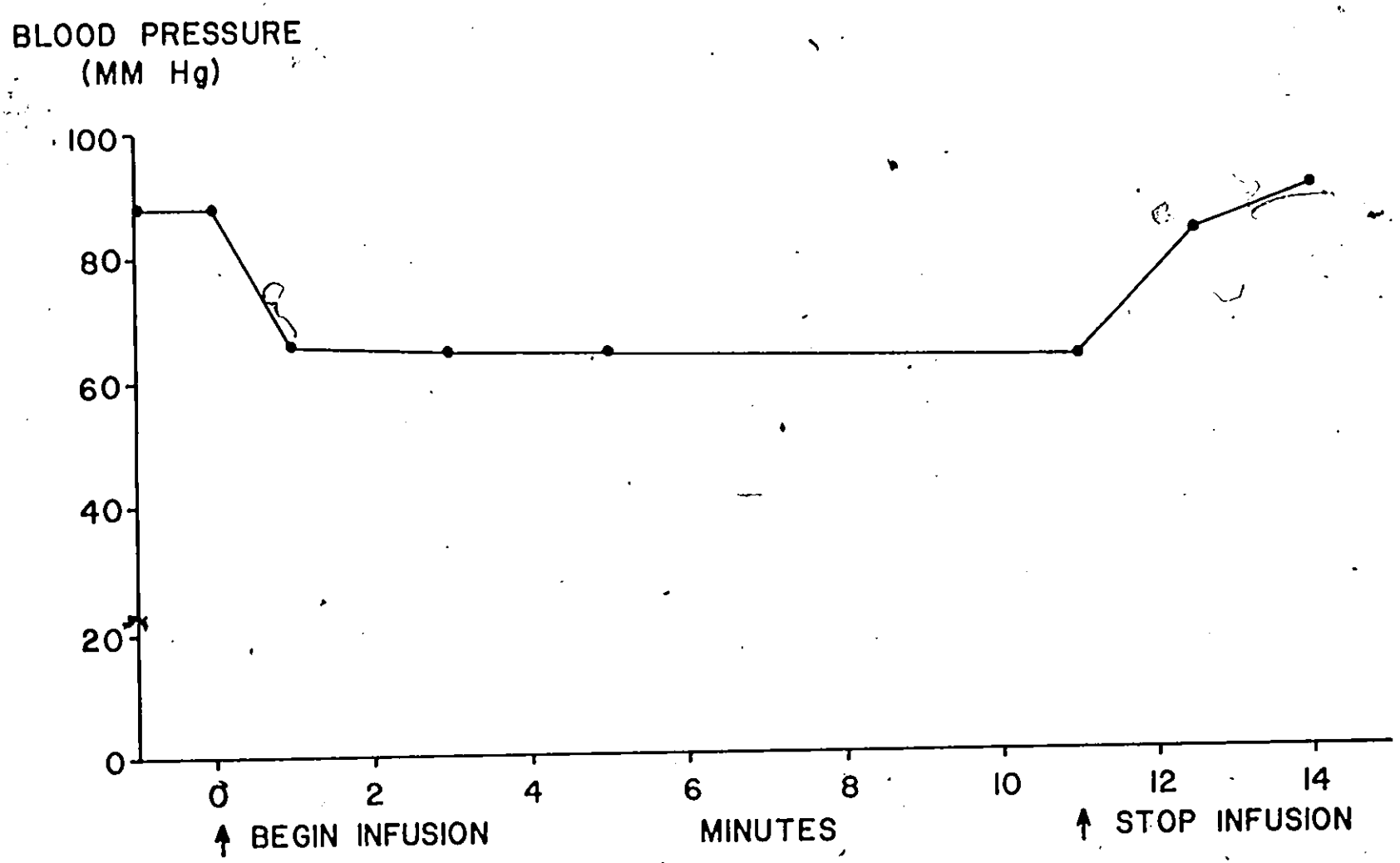


TABLE XXVIII

EFFECT OF DIPYRIDAMOLE ON THE ADHERENCE OF PLATELETS TO SUBENDOTHELIUM OF AORTAE PRETREATED IN VITRO WITH ASPIRIN

TREATMENT OF AORTA	TREATMENT OF PLATELET	PLATELET ADHERENCE NO. PER MM. SQ.
ASPIRIN (mM)	DIPYRIDAMOLE (uM)	
0	0	68,400 ± 4,200
10	0	69,400 ± 2,000
0	100	47,500 ± 3,200*
10	100	51,800 ± 2,300*

Values are mean ± standard error for 8 aortae. Dipyridamole was incubated with ^{51}Cr-labelled platelets suspended in Eagle's medium containing 4 per cent albumin and apyrase for 10 min before the addition of red blood cells (hematocrit 40 per cent). Aortae were incubated in Tyrode solution containing aspirin and 0.35 per cent albumin for 15 min at room temperature before they were rinsed twice and mounted on the probe. The platelet count in the suspension was 500,000/mm^3. Results of the studentized range test for the comparison of platelet adherence when neither aortae or platelets were incubated with drug was $p<0.05$.

ment of platelets with dipyridamole reduced their adherence to both aspirin-treated vessels and those not treated with aspirin (the difference in platelet adherence between groups was not significant). Therefore, treatment of vessels with aspirin, in a dose sufficient to inhibit PGI_2 production, did not diminish the inhibitory effect of dipyridamole on platelet adhesion in vitro. Since PGI_2 stimulates adenylate cyclase it increases concentrations of cAMP, and this is maintained by dipyridamole which inhibits cAMP phosphodiesterase so that it cannot breakdown cAMP. If aspirin blocks production of PGI_2 so that it is not available to stimulate an increase in cAMP, but dipyridamole still inhibits platelet adhesion (even when PGI_2 production is blocked by aspirin), then the inhibitory effect of dipyridamole on platelets could not be due to its inhibition of phosphodiesterase.

To determine whether the inhibition of platelet adhesion by dipyridamole could be due to its inhibition of phosphodiesterase activity, we also examined the effect of treatment of platelets with both PGI_2 and dipyridamole. The effects of treating platelets with dipyridamole and PGI_2, on their adhesion to collagen-coated glass are seen in Table XXIX. Although treatment with dipyridamole (10 μM) or PGI_2 (0.1 μM) reduced platelet adhesion, the extent of inhibition produced by the combination of dipyridamole and PGI_2 was no greater than the extent of inhibition produced by PGI_2 when used alone. These results provide further evidence that the

TABLE XXIX

EFFECT OF DIPYRIDAMOLE AND PGI_2

ON THE ADHERENCE OF PLATELETS TO COLLAGEN-COATED GLASS

INHIBITOR		n	PLATELET ADHERENCE NO. PER MM. SQ.
DIPYRIDAMOLE (μM)	PGI_2 (μM)		
0	0	24	65,500 ± 1,600
10	0	6	56,300 ± 3,000*
0	0.10	4	47,500 ± 700**
10	0.10	4	52,700 ± 700**

Values are mean ± standard error. Four segments of glass were used in each experiment. 'n' equals the number of experiments. Dipyridamole and PGI_2 were incubated with ^{51}Cr-labelled platelets suspended in Eagle's medium containing 0.35 per cent albumin and apyrase for 10 minutes before the addition of red blood cells (hematocrit 40 per cent). The platelet count in the suspension was 300,000/mm^3. Results of the Student's 't' test are for the comparison with platelet adherence when neither drug was used (* $p<0.01$; ** $p<0.001$).

inhibitory effect of dipyridamole on platelet adhesion is not dependent on its phosphodiesterase activity.

In Vivo

The effect of dipyridamole and PGI_2 on platelet accumulation on the subendothelium was studied in vivo, to determine which treatment produced the greatest inhibition. The results of these studies show that the extent of reduction in platelet accumulation was greater when both drugs were used, than when either drug was used alone (Table XXX).

Since thrombin plays a large role in platelet accumulation on injured neointima in vivo, and both PGI_2 and dipyridamole have an inhibitory effect on the response of platelets to thrombin, a higher concentration of these drugs was used in the studies on platelet accumulation on the injured neointima. Under these conditions, the extent of reduction in platelet accumulation on the injured neointima in vivo produced by treatment with the combination of dipyridamole and PGI_2 was no greater than when PGI_2 was used alone (Table XXXI). Thus, maximal inhibition of platelet accumulation on the injured neointima could be achieved by the infusion of a high concentration of PGI_2 (850 ng/kg/min) alone.

iv) PGI_2 and Heparin

The extent of reduction in platelet accumulation on injured subendothelium produced by the combination of PGI_2

TABLE XXX

EFFECT OF DIPYRIDAMOLE AND PGI_2 ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NEITHER DRUG)	58,200
DIPYRIDAMOLE (2.5 mg/kg)	31,500
PGI_2 (106 ng/kg/min)	15,800
DIPYRIDAMOLE + PGI_2	7,500

Values are the mean for 2 animals. The animals were infused with ^{51}Cr-labelled platelets 18 hr before the removal of the endothelium. Dipyridamole was administered intravenously 10 min before injury. The infusion of PGI_2 was begun 1 min before balloon injury. The aortae were perfusion-fixed 10 min following the removal of the endothelium.

TABLE XXXI

EFFECT OF DIPYRIDAMOLE AND PGI_2 ON THE ACCUMULATION OF PLATELETS ON INJURED NEOINTIMA IN VIVO

TREATMENT OF ANIMALS	PLATELET ADHERENCE NO. PER SQ. MM.
CONTROL (NEITHER DRUG)	48,400 ± 4,700 a
DIPYRIDAMOLE (12.5 mg/kg)	12,400 ± 3,800 b
PGI_2 (850 ng/kg/min)	5,300 ± 1,700 c
DIPYRIDAMOLE + PGI_2	3,600 ± 300 d

Values are mean ± standard error for 4 animals. The animals were infused with ^{51}Cr-labelled platelets 18 hr before injury to the neointima that formed by 7 days following the removal of the endothelium. Dipyridamole was administered intravenously 10 min before injury. The infusion of PGI_2 was begun 1 min before the neointima was injured with a balloon catheter and continued until the aortae were perfusion-fixed 10 min following neointimal injury. Results of the Studentized range test showed that, for the comparison between a vs b, $p<0.025$; a vs c, $p<0.01$; b vs d, $p<0.025$; c vs d, not significant.

(850 ng/kg/min)(which has primarily an anti-platelet effect) and a single injection of heparin (500 U/kg)(which is primarily an inhibitor of coagulation that will therefore inhibit the production of thrombin and its effects on platelets) was no greater than the reduction in platelet accumulation produced by PGI_2 alone (left hand column of Table XXXII). When the combination of PGI_2 and heparin was used to treat animals who received an injury to the neointima that had formed 7 days after de-endothelialization, platelet accumulation was also not significantly less than when PGI_2 was used alone (left hand column of Table XXXIII). This observation indicated that, although thrombin generation is demonstrated to play a major role in the response of circulating platelets to injured neointima, treatment with PGI_2 alone is sufficient to inhibit the accumulation of platelets on the neointima. Although heparin at the concentration used in these studies also partially inhibited platelet accumulation on the exposed subendothelium, PGI_2 was a more effective inhibitor and the extent of inhibition was not enhanced by combined therapy.

c) Reactivity of Injured Vessels: Effect of Inhibiting Platelet Accumulation on Damaged Vessels

Since dipyridamole and PGI_2 inhibited platelet accumulation on injured aortae in vivo, these drugs were used to inhibit platelet accumulation on injured aortae, so that changes in the reactivity of the vessels that might occur in

TABLE XXXII

EFFECT OF PGI_2 AND HEPARIN ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM. TIME AFTER INJURY 10 MIN	20 MIN
CONTROL (NO DRUG)	55,000 ± 9,600 a	38,300 ± 3,300 e
PGI_2 (850 ng/kg/min)	7,900 ± 1,800 b	37,000 ± 5,500 f
HEPARIN (500 U/kg)	28,900 ± 5,500 c	24,800 ± 3,400 g
PGI_2 + HEPARIN	8,700 ± 5,200 d	24,500 ± 9,100 h

Values are mean ± standard error for 3 animals. The animals were infused with ^{51}Cr-labelled platelets 18 hr before removal of the endothelium. Heparin was administered intravenously 10 min before injury and the infusion of PGI_2 was begun one min before injury. The aortae were perfusion-fixed 10 min following the removal of the endothelium or 10 minutes following the end of the PGI_2 infusion. Results of the Studentized range test show that, for the comparison between a vs b, $p<0.025$; a vs c, not significant; b vs d, not significant; c vs d, $p<0.025$; a vs e, not significant; b vs f, $p<0.025$; c vs g not significant; d vs h, not significant. An analysis of variance shows that there is no significant differences between groups e, f, g and h.

TABLE XXXIII

EFFECT OF PGI_2 AND HEPARIN

ON THE ACCUMULATION OF PLATELETS ON INJURED NEOINTIMA IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM. TIME AFTER INJURY 10 MIN	20 MIN
CONTROL (NO DRUG)	69,400	74,200
PGI_2 (850 ng/kg/min)	17,600 ± 1,900 a	63,700 ± 25,200 d
HEPARIN (500 U/kg)	38,900 ± 13,200 b	19,900 ± 6,100 e
PGI_2 + HEPARIN	11,600 ± 2,800 c	37,900 ± 7,800 f

Values are mean ± standard error for 3 animals (control n=2). The animals were infused with ^{51}Cr-platelets 18 hr before injury to the neointima that formed by 7 days following the removal of the endothelium. Heparin was administered 10 min before the injury to the neointima, and the infusion of PGI_2 was begun one min before injury. The animals were killed by perfusion-fixation 10 min following neointimal injury or 10 min following the end of the PGI_2 infusion. Results of the Studentized range test show that, for the comparison between a vs b, not significant; a vs c, $p<0.05$; a vs d, $p<0.025$; b vs e, not significant; c vs f, $p<0.025$.

the absence of platelet interaction, could be examined.

i) Dipyridamole

Although a single intravenous injection of dipyridamole (12.5 mg/kg) given 10 minutes before removal of the endothelium inhibited platelet accumulation on the exposed subendothelium during the 10 minutes following injury (Table XXIV), the duration of the inhibitory effect of a single injection of drug was not known. Table XXXIV shows the effect of this dose of dipyridamole (12.5 mg/kg), given 10 minutes before exposure of the subendothelium, on the accumulation of platelets at specific times following injury. Platelet accumulation in the dipyridamole-treated group 10 minutes following injury was reduced by approximately 70 per cent when compared to the accumulation in placebo-treated animals. Platelet accumulation was similarly reduced 30 minutes and one hour following injury. However, by two hours following injury, platelet accumulation was increased when compared to the accumulation during the first hour following injury; 4 hours after injury platelet accumulation was not significantly different from the accumulation in the control group 10 minutes after injury. Thus, although a single injection of dipyridamole inhibited platelet accumulation during the first hour following injury, the extent of inhibition was decreased 2 hours and the inhibitory effect was lost by 4 hours following injury. These results also indi-

TABLE XXXIV

EFFECT OF A SINGLE INJECTION OF DIPYRIDAMOLE ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	TIME AFTER INJURY	n	PLATELET ACCUMULATION NO. PER SQ. MM.
PLACEBO	10 MIN	9	52,500 ± 8,400 a
DIPYRIDAMOLE	10 MIN	9	16,900 ± 1,800 b
	30 MIN	5	13,400 ± 1,500 c
	1 HR	6	17,900 ± 2,700 d
	2 HR	6	32,100 ± 5,200 e
	4 HR	6	43,900 ± 8,100 f

Values are mean ± standard error. 'n' equals number of animals in each group. Dipyridamole (12.5 mg/kg) was injected intravenously 10 min before the removal of the endothelium. The aortae were perfusion-fixed at the given times following injury. Results of the Studentized range test show that, for the comparison between a vs b, $p<0.01$; b vs d, not significant; b vs e, $p<0.025$; a vs f, not significant. An analysis of variance showed that there is no significant differences among the groups b,c and d, or among the groups a, e and f.

cate that the surface of an injured vessel remains reactive to circulating platelets after the plasma concentration of dipyridamole decreases below inhibitory levels between one and 2 hours following de-endothelialization.

The effect of inhibiting platelet accumulation over a longer period of time was then examined. Since a single injection of dipyridamole (12.5 mg/kg) is only sufficient to inhibit platelet accumulation on the injured vessel for up to one hour following injury, a dose of 12.5 mg/kg of dipyridamole was administered before injury and 5 mg/kg given hourly for 3 hours to maintain a plasma concentration of the drug that would inhibit platelet accumulation during a 4 hour period following injury. The results of these studies are shown in Table XXXV. Platelet accumulation in the dipyridamole-treated group 4 hours following injury was significantly reduced compared to accumulation in the placebo-treated group. However, 8 hours later (12 hours following injury and 9 hours following the last injection of drug or placebo) platelet accumulation in the drug-treated group was significantly greater than accumulation in the drug-treated group at 4 hours, and not significantly different from accumulation in the placebo-treated group 4 hours following injury. Thus, although maintaining the plasma concentration of dipyridamole with repeated administrations of the drug during a 4 hour period following injury inhibits platelet accumulation on the injured vessel, when the plasma

TABLE XXXV

EFFECT OF DIPYRIDAMOLE ADMINISTERED OVER A 4 HOUR PERIOD ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM. TIME AFTER INJURY 4 HR	12 HR
PLACEBO	38,100 ± 3,200 a	35,100 ± 10,100 c
DIPYRIDAMOLE	15,500 ± 5,400 b	26,200 ± 3,000 d

Values are mean ± standard error for 6 animals. Dipyridamole (12.5 mg/kg) was administered intravenously 10 min before the removal of the endothelium, and 5 mg/kg was administered hourly 1, 2 and 3 hr following injury. Animals were killed by perfusion-fixation 4 hr following de-endothelialization of the aortae with a balloon catheter (1 hr following the last injection of dipyridamole), or 12 hr following injury (9 hr following the last injection of dipyridamole). Results of the Studentized range test showed that, for the comparison between a vs b, $p<0.025$; c vs d, not significant; a vs c, not significant; b vs d, not significant.

concentration of dipyridamole decreased below inhibitory levels platelets accumulated on the surface of the injured vessel. These results indicate that: 1) platelets that were exposed to inhibitory concentrations of the drug are capable of accumulating on a reactive surface when the plasma concentration of the drug falls below inhibitory levels, and 2) the surface of the injured vessels remains reactive to platelets following a 4 hour period during which platelet interation with the vessel is inhibited.

The effect of inhibiting platelet accumulation by administering dipyridamole over a period of 8 hours is shown in Table XXXVI. Dipyridamole, administered before injury (12.5 mg/kg) and hourly (5 mg/kg) for 7 hours following the removal of the endothelium, reduced the platelet accumulation one hour following the last injection of drug when compared to accumulation following placebo-treatment. Eight hours later (9 hours following the last injection of drug or 16 hours following injury) platelet accumulation in the drug-treated group remained significantly less than accumulation in the placebo-treated group. Although the plasma concentration of dipyridamole 8 hours following injury (one hour following the last injection of drug) was 0.352 µg/ml, 16 hours following injury (9 hours following the last injection of drug) little dipyridamole remained in the plasma (0.002 $\pm$ 0.001 µg/ml). Dipyridamole was present at a detectable level in only one of the samples examined, and the

TABLE XXXVI

EFFECT OF DIPYRIDAMOLE ADMINISTERED OVER AN 8 HOUR PERIOD ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM.	
	TIME AFTER INJURY 8 HR	16 HR
PLACEBO	32,700 ± 6,600 a	23,800 ± 3,400 c
DIPYRIDAMOLE	12,700 ± 2,500 b	13,400 ± 1,500 d

	PLASMA DIPYRIDAMOLE UG. PER ML.	
	TIME AFTER INJURY 8 HR	16 HR
DIPYRIDAMOLE	0.352 ± 0.18	0.002 ± 0.001

Values are mean ± standard error for 9 animals. Dipyridamole (12.5 mg/kg) was administered intravenously 10 min before the removal of the endothelium, and 5 mg/kg was given hourly for 7 hr. Aortae were perfusion-fixed 8 hr following injury (one hr following the last injection of dipyridamole), or 16 hr following injury (9 hr following the last injection of dipyridamole). Results of the Studentized range test showed a vs b, $p<0.01$; c vs d, $p<0.01$; a vs c, not significant; b vs d, not significant.

concentration of dipyridamole in that specimen was considerably less than that required to affect platelet function. Thus, it appears that, when platelet accumulation on an injured vessel is inhibited, much of the reactivity of the surface is lost by 8 hours following injury.

ii) $\underline{PGI_2}$

Although dipyridamole inhibits platelet accumulation on an injured vessel, the extent of accumulation is decreased by only about 60 per cent. In addition, platelet accumulation on injured vessels can only be examined after the concentration of dipyridamole in the plasma has fallen below inhibitory levels; this requires approximately 2 hours after injection of the drug. The use of PGI_2 to inhibit platelet accumulation has two advantages when compared to the use of dipyridamole: 1) since PGI_2 is a more potent inhibitor of platelets the extent of inhibition of accumulation is greater, and 2) since the half-life of the drug in the circulation is extremely short (Rosenkranz et al., 1980) its effects are rapidly lost and vessel wall reactivity can be studied soon after the end of the infusion of PGI_2.

In these studies, the continuous infusion of PGI_2 (850 ng/kg/min), reduced platelet accumulation on the subendothelium 10 minutes following injury by approximately 85 per cent by (Table XXXVII), similar to the extent of reduction seen in previous experiments (Table XXV). As demonstrated in

TABLE XXXVII

EFFECT OF INFUSING PGI_2 FOR 10 MINUTES ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM IN VIVO

TREATMENT OF ANIMALS	PLATELET ACCUMULATION NO. PER SQ. MM.	
	TIME AFTER INJURY	
	10 MIN	20 MIN
CONTROL (NEITHER DRUG)	55,300 ± 5,500	
PGI_2 (850 ng/kg/min)	7,300 ± 1,300	38,400 ± 3,200

Values are mean ± standard error for 7 animals. The animals were infused with ^{51}Cr-platelets 18 hr before the removal of the endothelium. The infusion of PGI_2 was begun one min before injury and continued for 10 min following injury. The animals were killed by perfusion-fixation at the end of the PGI_2 infusion, or 10 min following the end of the infusion of drug. The result of the Studentized range test for the comparison between the groups infused with PGI_2 and perfusion-fixed at 10 and at 20 min following injury is $p<0.001$.

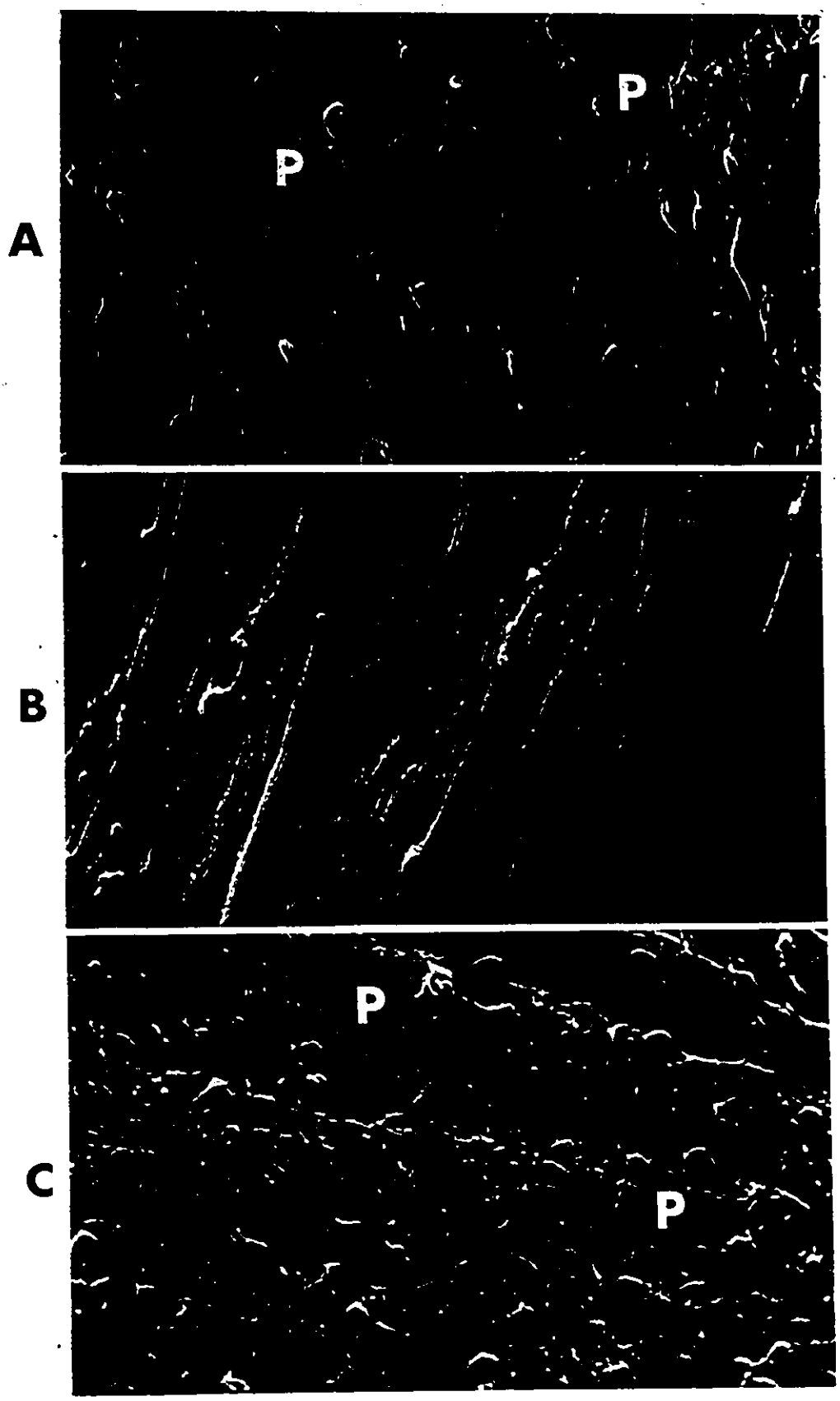
the Materials and Methods, when the amount of radioactivity associated with a vessel is very low, it may be due to non-platelet-bound radioactivity that remains associated with the vessel. A layer of platelets covering the surface of a rabbit aorta 10 minutes after the removal of the endothelium is shown in the scanning electron micrograph figure 31A. When an infusion of PGI_2 was begun before the removal of the endothelium and maintained until perfusion-fixation 10 minutes after injury, few platelets are seen on the subendothelium (Figure 31B). However, when the infusion of PGI_2 was stopped 10 minutes following injury, and the aortae were perfusion-fixed 10 minutes later (20 minutes following injury), there was considerable additional platelet accumulation on the injured vessel (Figure 31C). The rapid accumulation on the injured vessels following the end of the PGI_2 infusion indicates that the inhibitory effect of the drug is rapidly lost, and the surface of the vessel remains highly reactive to circulating platelets. These results also show that the half-life of PGI_2 in the circulation of rabbits is extremely short.

The infusion of PGI_2 for longer periods of time substantially reduced the number of platelets that had accumulated on the subendothelium at the end of the infusion. A continuous infusion of PGI_2 (850 ng/kg/min) for 8 hours decreased platelet accumulation when compared to the accumulation in the control group that received no drug, or in the

FIGURE 31

EFFECT OF INFUSION OF PGI_2 ON ACCUMULATION OF PLATELETS 10 MINUTES FOLLOWING REMOVAL OF THE ENDOTHELIUM

These micrographs show the surface of rabbit aortae 10 minutes following the removal of the endothelium. A) A layer of platelets covers the surface of the vessel in control animals (not treated with PGI_2). B) No adherent platelets are seen on the subendothelium shown in this micrograph: an infusion of PGI_2 (850 ng/kg/min) was begun before injury and maintained until the animal was killed by perfusion-fixation 10 minutes following injury. C) A layer of platelets covers the vessel 10 minutes after the end of a 10-minute infusion of PGI_2. P- platelets, SE- subendothelium. x 2,700.


A
P
P
B
C
P
P

placebo-treated group, and there was no additional platelet accumulation during the 30 minutes following the end of the PGI_2 infusion (Table XXXVIII). Scanning electron micrographs of these vessels are shown in figure 32. A layer of platelets covers the surface of a vessel 8 hours following the removal of the endothelium in control animals (figure 32A). However, when an infusion of PGI_2 was begun before injury and maintained until the animals were perfusion-fixed 8 hours following injury, few platelets are seen on the vessel (Figure 32B), and 30 minutes following the end of an 8 hour infusion of PGI_2, there is no morphological evidence of additional accumulation of platelets on the surface (Figure 32C). Thus, when platelet accumulation was inhibited by the continuous infusion of PGI_2, the surface became essentially non-reactive to circulating platelets by 8 hours following injury, indicating that an injured vessel can become non-reactive to circulating platelets independently of platelet accumulation on the surface.

Since the surface exposed by neointimal injury is different from the surface exposed by removal of the endothelium, and much of the platelet accumulation on the injured neointima is dependent on the activation of coagulation with the generation of thrombin and the formation of fibrin, it was considered possible that the surface of the injured neointima might retain its ability to activate coagulation. Activated components of coagulation might continue to promote

TABLE XXXVIII

EFFECT OF INFUSING PGI_2 FOR 8 HOURS ON THE ACCUMULATION OF PLATELETS ON SUBENDOTHELIUM OR INJURED NEOINTIMA IN VIVO

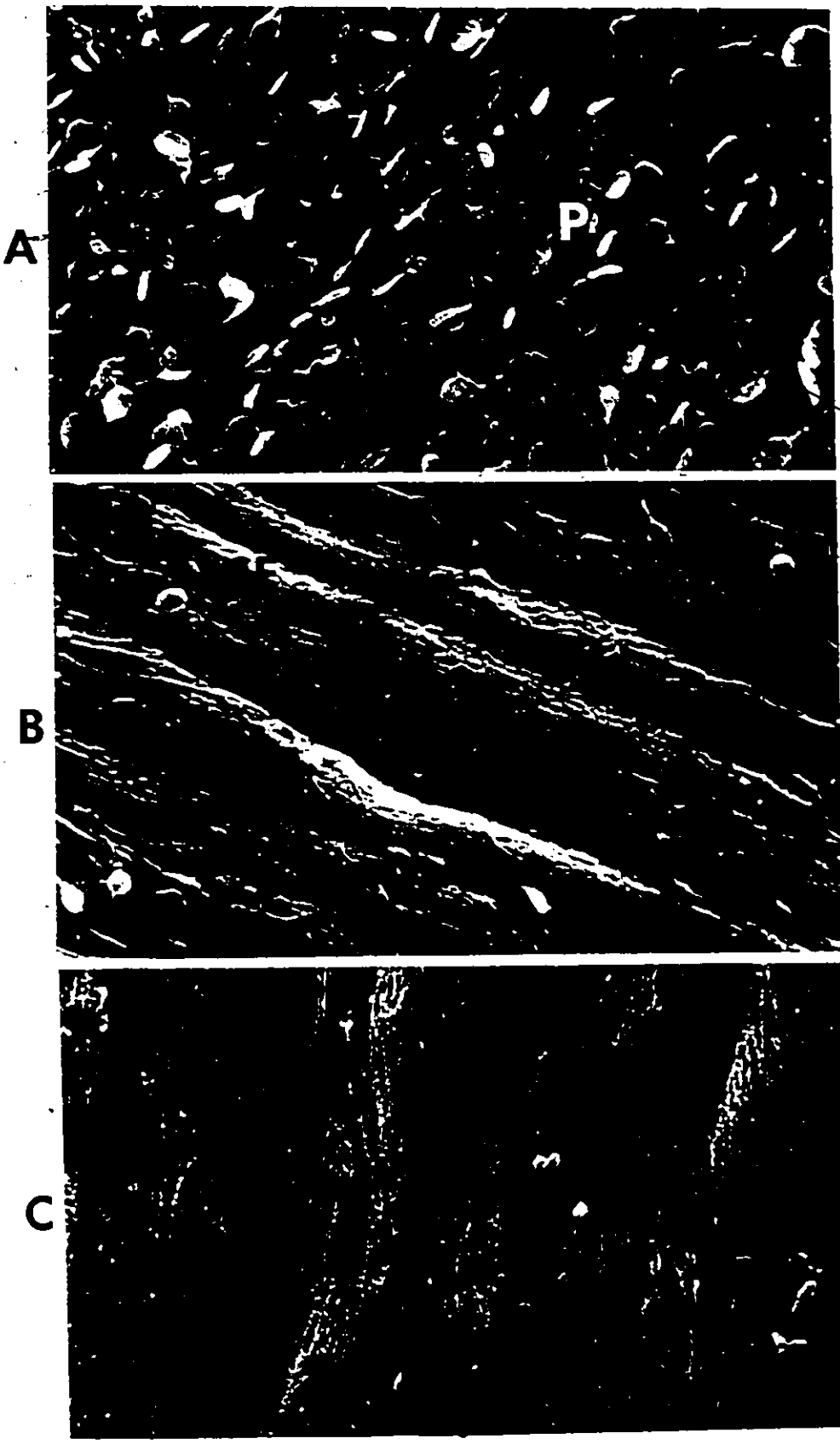
SURFACE EXPOSED TO PLATELETS	DRUG TREATMENT	PLATELET ACCUMULATION NO. PER SQ. MM. TIME AFTER INJURY 8 HR	8.5 HR
SUBENDOTHELIUM			
	CONTROL (NO PGI_2)	36,600 ± 4,100 a	
	PLACEBO	30,700 ± 4,200 b	
	PGI_2	8,600 ± 4,400 c	5,800 ± 1,800 d
INJURED NEOINTIMA			
	CONTROL (NO PGI_2)	18,800	
	PGI_2	4,000	4,400

Values are mean ± standard error for 4 animals with exposed subendothelium and 2 animals with injured neointima. Animals were infused with ^{51}Cr-platelets 18 hr before the removal of the endothelium, or injury to the neointima that formed by 7 days following removal of the endothelium. The infusion of PGI_2 (850 ng/kg/min) was begun one min before injury and was continued for 8 hr following injury. Animals were killed by perfusion-fixation at end of the PGI_2 infusion, or 30 min following end of the infusion. Results of the Studentized range test showed that, for the comparison between a vs b, not significant; a vs c, $p<0.05$; c vs d, not significant.

FIGURE 32

EFFECT OF INFUSION OF PGI_2 ON ACCUMULATION OF PLATELETS 8 HOURS FOLLOWING REMOVAL OF THE ENDOTHELIUM

These micrographs show the surface of rabbit aortae 8 hours following the removal of the endothelium. A) A layer of platelets covers the surface of the vessel in control animals (not treated with PGI_2). B) Few adherent platelets are seen on the subendothelium shown in this micrograph: an infusion of PGI_2 (850 ng/kg/min) was begun before injury and maintained until the animal was killed by perfusion-fixation 8 hours following injury. C) There are few platelets on the vessel 30 minutes after the end of an 8-hour infusion of PGI_2. P- platelets, SE- subendothelium. x 2,700.


A
P
B
C

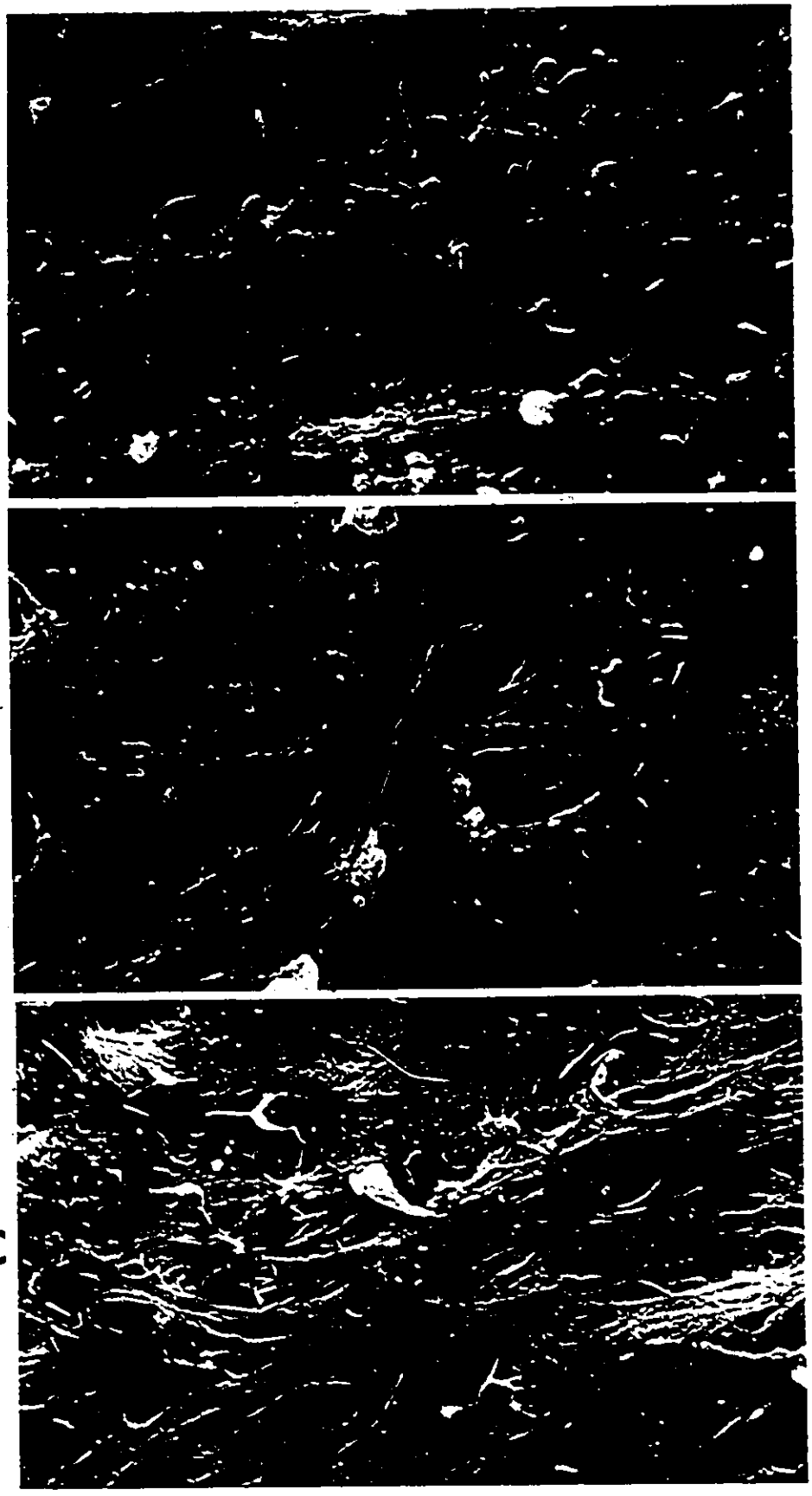
platelet accumulation on the surface of the vessel, even when those components of the injured vessel that were responsible for platelet adhesion to the surface were altered. In addition, since a substantial amount of the platelet accumulation on the injured neointima appears to be independent of the activation of coagulation, it is also possible that components of the injured neointima to which the platelets adhere might not lose their reactivity as rapidly as the components of a freshly injured vessel.

The results of studies on the effect of infusing PGI_2 for 8 hours on the accumulation of platelets on the injured neointima at the end of the period of infusion, and during the 30 minute period following the end of the infusion are also shown in Table XXXVIII. Platelet accumulation at the end of the infusion (8 hours following injury) is 4,000 per square millimeter, compared to 18,800 per square millimeter in the control that received no drug, and there is no appreciably accumulation of platelets during the 30 minutes following the end of the PGI_2 infusion. Micrographs of these vessels are shown in figure 33. Although there is a layer of platelets on the vessel 8 hours following injury of the neointima in control animals (Figure 33A), there are few platelets on the smooth muscle cell-rich surface following a continuous infusion of PGI_2 for 8 hours (Figure 33B), and 30 minutes after the end of an infusion of PGI_2 there are few platelets on the surface (Figure 33C). These results indi-

FIGURE 33

EFFECT OF INFUSION OF PGI_2 ON ACCUMULATION OF PLATELETS 8 HOUR FOLLOWING INJURY OF THE NEOINTIMA

These micrographs show the surface of rabbit aortae 8 hours following injury of the neointima that had formed 7 days following removal of the endothelium. A) There are numerous platelets on the surface of the vessel in control animals (not treated with PGI_2). B) Few platelets are seen on the smooth muscle cell-rich surface of the injured neointima, when an infusion of PGI_2 (850 ng/kg/min) was begun before injury and maintained until the animal was killed by perfusion-fixation 8 hours following injury to the neointima. C) Few platelets are seen on the vessel 30 minutes after the end of an 8-hour infusion of PGI_2. CT- connective tissue, P- platelets, M- smooth muscle cells. x 2,700.


A
B
C

cate that the surface of the injured neointima also loses its reactivity to circulating platelets by 8 hours following injury. Thus, even when platelet interaction with vessel walls is inhibited, the surfaces exposed by a single injury or by reinjury of a previously injured vessel, lose their reactivity to circulating platelets by approximately 8 hours following injury.

iii) PGI_2 and Heparin

The results of experiments on the effect of administering heparin and a continuous infusion of PGI_2 for ten minutes following injury on the accumulation of platelets 100 minutes following exposure of the subendothelium or injury to the neointima are shown in the right hand columns of tables XXXIII and XXXIV. Although platelet accumulation is inhibited immediately following the end of the PGI_2 infusion (10 minutes after injury), by 10 minutes after the end of the PGI_2 infusion (20 minutes following injury), platelet accumulation on both the subendothelium and the injured neointima is similar to the accumulation in the controls. Although treatment with heparin appears to produce a slight decrease in platelet accumulation on subendothelium, the extent of reduction is substantially less than the reduction in accumulation on the injured neointima.

These results indicate that even when rabbits are treated with heparin: 1) the inhibitory effect of PGI_2 is

rapidly lost following the end of the infusion, and 2) the injured surfaces of both the subendothelium and the injured neointima remain reactive during this period. The observation that platelet accumulation on the injured neointima increased following the end of the infusion of PGI_2 in animals treated with heparin, indicated that components of the vessel wall, that were responsible for the platelet accumulation which was independent of the activation of coagulation, remained reactive during this period.

2. Effect on Reactivity of Injured Aortae of Incubating Injured Vessels In Vitro

If the loss of reactivity of an injured vessel is not dependent on platelet interaction with the surface, but due to factors such as the adsorption of plasma proteins, the effects of other plasma factors such as enzymes, or the effects of inhibitory substances generated by the vessel wall, then injured vessels incubated under suitable conditions in vitro should also lose much of their reactivity. Therefore, the effect of incubating damaged aortae in vitro on the reactivity of the vessels was examined.

De-endothelialized rabbit aortae were everted on probes and rotated in oxygenated media containing penicillin and streptomycin at 37 ^{o}C and pH 7.4 for 8 hours. Following incubation, the vessels were rotated in a suspension of ^{51}Cr-

TABLE XXXIX

EFFECT OF INCUBATING DE-ENDOTHELIALIZED AORTAE IN EAGLE'S MEDIUM FOR 8 HOURS ON THE ADHESION OF PLATELETS TO THE SUBENDOTHELIUM

TREATMENT OF DE-ENDOTHELIALIZED AORTAE	PLATELET ADHERENCE NO. PER SQ. MM.
FRESH INJURY	19,300
EAGLE'S MEDIUM (8 HR INCUBATION)	20,200

Values are the mean for 2 experiments. De-endothelialized aortae were everted on the probe and rotated at 140 rpm for 8 hr in oxygenated Eagle's medium containing 4 per cent albumin with 50 units of penicillin and streptomycin per milliliter. Fresh medium was introduced into the incubation tubes at a rate of 1/20 of total volume per min. The vessels were rinsed in 2 changes of Eagle's medium before they were rotated in a suspension of ^{51}Cr-platelets for measurement of platelet adherence.

labelled platelets suspended in Eagle's medium with 4 per cent albumin and red blood cells. Table XXXIX shows that platelet accumulation on the aortae following 8 hours of incubation in Eagle's medium was not significantly different from the accumulation on the freshly injured control vessels. These results of these studies suggest that substances generated by the vessel wall are not responsible for the loss of reactivity of an injured vessel.

Since it was possible that factors present in the plasma might be responsible for the loss of vessel wall reactivity, the effect of incubating collagen-coated glass or de-endothelialized vessels in citrated plasma was examined. Table XXXX shows that platelet adhesion to collagen-coated glass or to subendothelium was essentially unchanged following incubation in citrated plasma, when compared to adhesion to freshly-coated glass.

Since calcium is required for a number of enzyme reactions and cellular processes, the citrate in plasma could make calcium unavailable for cellular processes or surface changed that might be responsible for the loss of vessel wall reactivity. Therefore, the effects of incubating vessels in serum are also examined. To inactivate any complement that could be activated during coagulation and might interact with and effect the properties of the surface of the vessel, the serum was heat inactivated at 56 ^{o}C for one hour. Table XXXXI shows that platelet adherence to the vessels is

TABLE XXXX

EFFECT OF INCUBATING DE-ENDOTHELIALIZED AORTAE OR COLLAGEN-COATED GLASS IN PLASMA FOR 8 HOURS ON THE ADHESION OF PLATELETS TO THE SURFACE

TREATMENT OF SURFACES	PLATELET ADHERENCE NO. PER SQ. MM.
COLLAGEN-COATED GLASS:	
FRESHLY COATED	47,900
INCUBATION IN PLASMA	49,800
DE-ENDOTHELIALIZED AORTAE:	
FRESH INJURY	20,800 ± 6,800
INCUBATION IN PLASMA	25,200 ± 4,000

Values are mean ± standard error (collagen-coated glass n=2; de-endothelialized aortae n=4). De-endothelialized aortae or segments of collagen-coated glass were incubated for 8 hr in citrated plasma containing penicillin and streptomycin (50 U/ml) for 8 hr at 37 °C. Fresh plasma was introduced into the incubation tubes at a rate of 1/20 of total volume per min. At the end of the 8 hr incubation period the adhesion of ^{51}Cr-platelets to the vessel was tested. The result of the Studentized range test showed that the difference between platelet adherence to freshly injured aortae and to vessels incubated in plasma is not significant.

TABLE XXXXI

EFFECT OF INCUBATING DE-ENDOTHELIALIZED AORTAE IN SERUM FOR 8 HOURS ON THE ADHESION OF PLATELETS TO THE SUBENDOTHELIUM

TREATMENT OF DE-ENDOTHELIALIZED AORTAE	PLATELET ADHERENCE NO. PER SQ. MM.
FRESH INJURY	32,100 ± 5,500
SERUM (8 HR INCUBATION)	33,200 ± 2,700

Values are mean ± standard error for 4 experiments. De-endothelialized aortae were everted on the probe and rotated at 200 rpm in oxygenated serum containing heparin (2 U/ml), and penicillin and streptomycin (50 U/ml) for 8 hr at 37 °C. Fresh serum was introduced into the incubation tubes at a rate of 1/20 of total volume per min. The serum used in these studies was prepared by allowing the blood to clot in glass tubes for 2.5 hr at 37 °C. The clot was removed and the serum heat inactivated at 56 °C for one hr. The adhesion of ^{51}Cr-platelets to the vessel was determined by the method described. Results of the Studentized range test showed that the difference in platelet adherence between the groups is not significant.

unchanged following incubation in serum for 8 hours. Since incubation in either Eagle's medium, plasma or serum does not decrease the reactivity of injured vessels, it would appear that the absorption of plasma proteins, or other factors, are not responsible for the loss of reactivity of an injured vessel.

The results of these in vitro studies suggest that the loss of reactivity of injured vessels observed in vivo is not due to substances produced by the vessel wall, or the interaction of plasma factors with the surface of the vessel. However, it is possible that the in vitro culture system did not provide the optimal conditions necessary for the injured vessel wall to generate substances or undergo changes responsible for the loss of reactivity. Therefore, the lack of decrease in reactivity if incubated vessels does not necessarily indicate that substance generated by the vessel wall are not responsible for the loss of reactivity of injured vessel walls in vivo. Thus, it remains possible that the decrease in the reactivity of an injured vessel might depend on substances generated by the vessel.

3. Rotation with Suspension of Red Blood Cells: Effect on Subsequent Reactivity of Collagen-Coated Glass

Since it was considered possible that remnants of the red cell glycocalyx might be deposited on the surface of an injured vessel during contact with circulating red cells, and

that this material might decrease the availability of reactive sites on the surface of the vessel, the effect of exposure to flowing red cells on the reactivity of a collagen-coated surface was examined. Table XXXXII shows that, following the rotation of collagen-coated glass in suspensions of red blood cells, platelet adhesion is not significantly different from adhesion to freshly coated surfaces, indicating that red blood cell interaction with the surface of injured vessels is not responsible for the loss of reactivity of the injured surface.

TABLE XXXXII

EFFECT OF INCUBATING COLLAGEN-COATED GLASS IN SUSPENSIONS OF RED BLOOD CELLS FOR 2 HOURS ON THE ADHESION OF PLATELETS TO THE SURFACE

TREATMENT OF COLLAGEN-COATED SURFACE	PLATELET ADHERENCE NO. PER SQ. MM.
FRESHLY COATED	61,900 ± 2,800
ROTATED IN SUSPENSION OF RED BLOOD CELLS	71,200 ± 3,700

Values are mean ± standard error for 4 experiments. Collagen-coated glass segments were mounted on probes and rotated at 200 rpm for 2 hr in suspensions of 40 per cent red blood cells in Eagle's medium with 4 per cent albumin, apyrase, and Hepes buffer at 37 °C. Following the first hr of rotation the suspension of red cells was replaced with a fresh suspension. The adhesion of ^{51}Cr-platelets was then determined by the method described. The Studentized range test showed that the differnce in platelet adherence between the groups is not significant.

C. Discussion

The results of previous studies show that, although platelets rapidly accumulate on a vessel following removal of the endothelium, by 30 minutes following injury few additional platelets accumulate on the layer of platelets that cover the surface of the vessel (Groves et al., 1979). The results of these experiments show that 1) platelet interaction with injured vessels can be inhibited by drugs such as dipyridamole or PGI_2 that are known to inhibit platelet aggregation and the release of platelet granule contents; 2) damaged vessels can lose their reactivity to circulating platelets through mechanisms that are independent of the interactions of platelets with injured surfaces; 3) the loss of vessel wall reactivity takes 6 to 8 hours to manifest itself; 4) during this period the loss of reactivity does not appear to be due to substances elaborated by the vessel wall (at least in the in vitro studies); and 5) plasma proteins or the interaction of red blood cells with reactive sites, for example collagen, do not appear to be responsible for the loss of reactivity. Although these studies have lead us to conclude which factors do not contribute to the loss of reactivity of a damaged vessel wall, unfortunately they do not allow us to come to any conclusion concerning the factors that might be responsible for this effect. From the evidence it appears that platelets and plasma proteins are not crucial for the lack of attraction of fresh platelets to injury sites

with the passage of time. However, it is possible that reactive substances are lost from an injured vessel during this time. Thus, the evidence is inconclusive concerning whether or not materials elaborated by injured vessels might contribute to this effect.

With the experimental approaches used in the experiments described in this chapter a number of pathways can be implicated in interaction of platelets with vessel walls and the loss of vessel wall reactivity.

1. Inhibition of Platelet Accumulation with Drugs

Platelet accumulation on surfaces in vitro or in vivo was inhibited both by dipyridamole or PGI_2. However, the extent of inhibition of accumulation produced by PGI_2 was substantially greater than the inhibition produced by dipyridamaole.

Dipyridamole, in addition to inhibiting platelet accumulation on surfaces in vitro, an observation that confirms the findings of other investigators (Cazenave et al., 1978; Mustard and Packham, 1978), also reduced the extent of release of granule contents from adherent platelets. Although dipyridamole (at a dose of 12.5 mg/kg) inhibited platelet accumulation on the subendothelium in vivo by only 50 per cent, many of the adherent platelets retained their discoid shape, indicating that treatment with dipyridamole also inhibits the spreading of platelets.

Since PGI_2 was first recognized as a potent inhibitor of platelet function, it has been extensively investigated for its potential antithrombotic effects in a number of animal and clinical studies. PGI_2 reduced thrombosis or platelet consumption associated with vascular shunts (Woods et al., 1978), haemodialysis (Zusman et al., 1981), cardiopulmonary bypass (van der Dugen et al., 1980; Malpass et al., 1981; Koshal et al., 1981; Coppe et al.,1981) and peripheral vascular disease (Szczeklik et al., 1980). In the present studies in which a different technique was used to induce vessel injury, the finding that platelet accumulation on injured vessels is inhibited by a continuous infusion of PGI_2 (850 ng/kg/min), confirms the observations of others (Adelman et al., 1981; Adelman et al., 1983). The fact that a continuous infusion of a very high dose of PGI_2 was required to inhibit platelet accumulation on an injured vessel wall, provides additional evidence that it is unlikely that the generation of PGI_2 by the vessel wall is responsible for preventing platelet accumulation on the normal vascular endothelium or for limiting platelet accumulation on damaged vessel walls. It is unlikely that sufficiently high concentrations could be achieved in vivo for several reasons. 1) PGI_2 produced by endothelial cells would be rapidly diluted by flowing blood. 2) Several investigators have been unable to demonstrate significant amounts of PGI_2 or 6-Keto-PGF_1 alpha in circulating blood (Dollery et al., 1983;

Haslam and McClenaghen, 1981). 3) Endothelial cells require stimulation to generate significant amounts of PGI_2 (Weksler et al., 1978). However, it is possible that the vessel wall could generate sufficient PGI_2 locally to modify platelet responses associated with thrombus formation, particularly in regions where thrombin is generated and stimulates vessel wall production of PGI_2, and where stasis of blood flow allows for the accumulation of PGI_2.

Since dipyridamole inhibits cAMP phosphodiesterase, the enzyme that breaks down cAMP, and PGI_2 stimulates adenylate cyclase which increases the synthesis of cAMP, it was important to determine whether the effect of dipyridamole on platelets was dependent on synergism between dipyridamole and PGI_2. The results of the present studies failed to demonstrate synergism between these drugs even though cAMP phosphodiesterase would have been inhibited by the concentrations of dipyridamole used in these experiments. This lack of synergism in the in vitro studies could not be attributable to the degradation of PGI_2 to its metabolite 6-keto-PGF_{1alpha} since PGI_2 alone inhibits platelet adhesion and it would be expected that the metabolic breakdown of PGI_2 would not be altered by the presence of dipyridamole. Although de-endothelialized vessels are still capable of generating PGI_2, treatment of these vessels with aspirin did not decrease the inhibitory effect of dipyridamole on platelet accumulation. These results indicate that the inhibitory

effect of dipyridamole on platelet adhesion is not dependent on the production of PGI_2 by vascular cells. Although PGI_2 enhanced the inhibition of platelet aggregation by dipyridamole, since there was little evidence of thrombus formation on de-endothelialized vessels in the present studies, it is unlikely that the effect of PGI_2 on the action of dipyridamole on thrombosis would be demonstrable under these conditions.

Adelman and co-workers (1983) reported that the combination of PGI_2 and heparin had a greater inhibitory effect on platelet accumulation on the injured neointima that had formed 10 days after the removal of the endothelium, than it did on the subendothelium exposed by a single injury. These investigators also observed that, although the combination of PGI_2 and heparin reduced platelet accumulation on neointima injured 29 days following de-endothelialization, neither drug alone significantly reduced platelet accumulation on this surface. Thus, the surface exposed by neointimal injury might be more thrombogenic following injury of an older neointima than following injury of a neointima that formed at earlier times following injury.

In the present studies, the response to injury of a neointima that formed by 7 days following removal of the endothelium was examined. When either PGI_2 or dipyridamole was used in combination with heparin, the extent of inhibition of platelet accumulation on the injured neointima was

greater than when either drug was used alone. These observations provide additional evidence that 1) the properties of the injured neointima are different from the properties of the subendothelium exposed by a single injury, and 2) some of the platelet accumulation on the injured smooth muscle cell-rich neointima is dependent on the activation of coagulation with the generation of thrombin and the formation of fibrin; heparin inhibits the effects of thrombin, and PGI_2 prevents platelets from contributing to the activation of coagulation by maintaining the platelets in a disc shape and preventing the exposure of platelet factor 3 on their surface.

2. Role of Platelets, Plasma Proteins and Red Blood Cells in the Loss of Vessel Wall Reactivity

The previous observations that the vascular surface exposed by the loss of adherent platelets approximately 2 days following the removal of the endothelium, was morphologically similar to the subendothelium, but non-reactive to circulating platelets, indicated that the properties of the vascular surface were altered when compared to the properties of a freshly injured surface (Groves et al., 1979). This raised the possiblity that platelet interaction with subendothelial constituents might be responsible for the loss of vessel wall reactivity. However, the results of these experiments show that when platelet accumulation is inhibited by either dipyridamole or PGI_2, the vessel remains highly

reactive for a short time following injury but loses its reactivity with time; by 4 hours following injury the surface is less reactive than at 30 minutes, and by 8 hours following injury, the surface becomes virtually non-reactive to circulating platelets. Although the accumulation of platelets on a reinjured vessel involve both the generation of thrombin and the interaction of platelets with components of connective tissue, when the effect of PGI_2 on platelet accumulation on reinjured vessels was examined, it was found that the PGI_2-sensitive accumulation of platelets on the injured neointima followed a similar pattern to that observed for the subendothelium; following the the inhibition of platelet accumulation on the injured vessel by infusing PGI_2 for 8 hours following injury the injured neointima lost its reactivity to circulating platelets. Thus it appears reasonable to conclude that platelet interaction with freshly injured and reinjured surfaces is not responsible for the loss of vessel wall reactivity that develops by 8 hours following injury.

It also appears unlikely that the loss of vessel wall reactivity is due to plasma proteins or other plasma factor masking sites on the injured vessels with which platelets interact, since the deposition of plasma proteins would occur rapidly following injury. Similarily, since approximately 8 hours was required for damaged vessels to lose their reactivity (even though they were continuously exposed to circu-

lating red blood cells, and since rotation of everted aortae in suspensions of red cells failed to decrease the reactivity of injured vessel walls or collagen-coated glass surfaces), it is unlikely that the loss of reactivity of injured vessels can be attributed to the interaction of red blood cells with the subendothelial surface.

In conclusion, although the reactivity of injured vessels did not decrease during incubation in vitro in the present studies, since neither the interaction of platelets or red blood cells, nor the adsorption of plasma proteins or other plasma factors seem to be responsible for the loss of reactivity of damaged vessels by several hours after injury, it appears that the loss of reactivity of injured vessels might be due to changes in the surface properties produced by the vessel wall itself. Since proteoglycans can inhibit platelet function (Ts'ao et al., 1977), it is possible that glycosaminoglycans in the vessel wall or elaborated by vascular cells stimulated by injury might mask the sites on the vessel surface with which platelets interact. Since enzymes with fibrinolytic activity are elaborated by the vessel wall in some species it is possible that these enzymes could alter reactive sites on the vessel surface (Astrup and Buluk, 1963). However, since there is little evidence of fibrinolytic activity in layers of rabbit aorta (Astrup and Buluk, 1963) it is unlikely that this accounts for the loss of reactivity of the damaged vessels in the present studies.

CHAPTER VI

GENERAL COMMENTS AND FUTURE RESEARCH

GENERAL COMMENTS AND FUTURE RESEARCH

The purpose of this thesis was to examine how previously injured vessels respond to an additional injury. In these experiments a normal vessel was de-endothelialized and then reinjured after 7 days - at a time when there was substantial smooth muscle cell proliferation in response to the initial injury. This approach was used because previous studies, in which injury to a normal vessel had been used to determine the nature of the early events in atherosclerosis, have been criticized on the premise that atherosclerosis occurs as a consequence of repeated vessel injury, and the response to injury of a previously undamaged vessel in a young experimental animal may not represent the changes in more advanced atherosclerosis. The major findings of the studies that form the basis of this thesis are:

1. When previously damaged vessels are subjected to a second injury the number of platelets that accumulate at the injury site is similar to that observed following de-endothelialization. In contrast, to the findings after a single injury, there is an extensive coagulation component leading to thrombin generation and fibrin on a reinjured vessel.

2. Despite the extensive coagulation component, reinjured vessels rapidly lose their reactivity to circulating plate-

lets; few additional platelet accumulate after one hour.

3. The non-thrombogenicity of normal endothelium, the control of platelet accumulation on injured vessels, and the loss of reactivity of injured vessels cannot be attributed to the formation of PGI_2 by the vessels.

4. The loss of reactivity of injured vessels is not dependent on the interaction of platelets with reactive sites on the subendothelium or on injured neointimal smooth muscle cells, since surfaces still lose their reactivity when platelet adherence is inhibited by treatment with dipyridamole or blocked almost completely by the infusion of PGI_2.

5. Neither red blood cells nor plasma proteins appear to influence the reactivity of injured vessels.

6. Loss of vessel wall reactivity takes between 6 to 8 hours to develop following injury.

Several conclusions about the response of vessels to injury have been drawn from the results of these studies. They have shown that even vessels with complicated forms of response to injury lose their reactivity, implying that repeated or continuous injury must be required for the dev-development of severe atherosclerosis and its thromboembolic complications. The lack of inhibitory effect of aspirin on

platelet accumulation on exposed subendothelium or damaged neointimal smooth muscle cells accounts for its lack of inhibition of the development of the early lesions of atherosclerosis. Even in the presence of aspirin platelets that adhere to vessel wall constituents release the contents of their storage granules, including a factor that is mitogenic for smooth muscle cells. Furthermore, since thrombin generation plays a major role in the development of thrombi on previously damaged vessels and aspirin is a very weak inhibitor of the effects of thrombin on platelets, it is not surprising that aspirin has not been shown to be a clearcut inhibitor of the thrombo-embolic complications of atherosclerosis. In circumstances where aspirin has had a beneficial effect such as in transient attacks of cerebral ischemia or unstable angina, the clinical complications may have been, at least in part, secondary to the effects of thromboxane A_2 formed by platelet aggregates at the site of severe stenosis; thromboxane A_2 is a potent vasoconstrictor and inhibition of its formation by blocking platelet cyclooxygenase could prevent these effects of thromboxane A_2 at sites downstream from the stenotic lesions where platelet micro-emboli affect the micro-circulation. However, Chierchia and his colleagues (1982) showed that the generation of thromboxane A_2 is not responsible for initiating coronary artery vasospasm in patients with variant angina. In addition, Folts and his coworkers (1982) demonstrated that cyclic changes in blood flow

through stenosed coronary arteries in dogs are not due to vasospasm even though the changes in blood flow are inhibited by aspirin. They concluded that aspirin induced inhibition of platelet aggregate formation could be responsible for these cyclic changes in blood flow.

In contrast to aspirin, heparin has been shown to decrease the extent of accumulation of platelet-fibrin thrombi on the walls of carotid arteries in cats 4 hours following endarterectomy (Piepgras et al., 1976)(an observation that is confirmed in the present studies in rabbits).

These findings have implications for our understanding of the factors that contribute to the development of atherosclerosis and possibly have application in clinical settings. For example, a reduction in the accumulation of platelets at sites of vessel injury could theoretically lead to a decrease in the amount of the platelet-derived smooth muscle cell mitogen made available following injury and thereby reduce the extent of vessel wall thickening. In previous studies, antiplatelet therapy has been found to be ineffective in preventing smooth muscle cell proliferation in reponse to vascular surgery, possibly because treatment was delayed until after the surgery (Pantely et al., 1979). However, when antiplatelet therapy (dipyridamole) was started before coronary artery bypass surgery and supplemented by aspirin in the immediate post-operative period, obstruction of the vessels was substantially decreased during the first 6 months

(Chesebro et al., 1982). At later intervals after surgery there was some indication that the vessels had become thickened but the reasons for this have not been determined (Chesebro et al., 1982).

One aspect of the response of blood and vessels to injury that was not addressed during the course of this study was the role played by white blood cells, particularly monocytes. There is no doubt that while blood cells contribute to the response to vessel injury in the present studies since large numbers of white cells could be seen scattered on injured vessels and also localized at the lower margins of vessel orifices or branches. Monocytes have been reported to play a major role in the development of the early fatty lesions in atherosclerosis induced by hypercholesterolemia in pigs (Gerrity, 1981a; Gerrity, 1981b), a finding that has been confirmed by other investigators in monkeys (Faggiotto and Ross, 1984) and rats (Joris et al., 1979). Under these experimental conditions monocytes infiltrate vessels, incorporate lipid, become foam cells, cause endothelial cell disruption and subsequently return to the circulation. Since monocytes also produce a factor(s) that are mitogenic for smooth muscle cells they too may contribute significantly to the vessel wall thickening that occurs in response to injury. However, it has not been determined whether or not monocytes play a key role in atherogenesis in the absence of hypercholesterolemia.

Another aspect of this work that requires further definition is related to whether or not vessels that have been injured and from which platelet adhesion has been prevented for up to 8 hours (the time at which they appear to have lost their reactivity) subsequently become thickened. This is important to determine, particularly if some of these forms of treatment are to be used as adjuncts to surgical treatment of chronic vascular disease. Although inhibition of platelet adherence might reduce the extent of smooth muscle cell proliferation that occurs in response to vessel injury, other factors may contribute to vessel thickening that are unrelated to the elaboration of growth factors from platelets or monocytes and macrophages. For example, it is possible that mechanical stimulation of medial smooth muscle cells by the balloon catheters used to injure the blood vessels (and also in transluminal angioplasty) might change the synthetic capacity of the smooth muscle cells, causing them to undergo migration and proliferation independently of chemical growth factors.

Some of these possibilities are presently under investigation by myself and by some of my colleagues in our laboratory.

CHAPTER VII

REFERENCES

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Abrahamsen, A.F. Platelet survival studies in man. Scand. J. Haematol., (Suppl.) 3:7, 1968.

Adelstein, R.S., and Conti, M.A. Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. Nature, 256:597, 1975.

Adelman, B., Stemerman, M.B., and Handin, R.I. Interaction of platelets and fibrin with injured rabbit aortic neointima: effect of prostaglandin I_2 and heparin. Arteriosclerosis, 3:141, 1983.

Adelman, B., Stemerman, M.B., Mennell, D., and Handin, R.I. The interaction of platelets with aortic subendothelium: inhibition of adhesion and secretion by prostaglandin I_2. Blood, 58:198, 1981.

Agranoff, B.W., Murthy, P., and Seguin, E.B. Thrombin-induced phosphodiesteratic cleavage of phosphatidyl-inositol biphosphate in human platelets. J. Biol. Chem., 258:2076, 1983.

Aharony, D., Smith, J.B., and Silver, M.J. Inhibition of platelet aggregation, malondialdehyde and thromboxane formation by hydroperoxides of arachidonic acid. Thromb. Haemost(abstr)., 46:265, 1981.

Aiken, J.W., Shebuski, R.J., Miller, O.V., and Gorman, R.R. Endogenous prostacyclin contributes to the efficacy of a thromboxane synthetase inhibitor for preventing coronary artery thrombosis. J. Pharmacol. Exp. Ther., 219:299, 1981.

Aminoff, D., Bell, W.C., Fulton, I., and Ingebrigtsen, N. Effect of sialidase on the viability of erythrocytes in circulation. Am. J. Hematol., 1:419, 1976.

Aminoff, D., Bruegge, W.F.V., Bell, W.C., Sarpolis, K., and Williams, R. Role of sialic acid in survival of erythrocytes in the circulation: Interaction of neuraminidase-treated and untreated erythrocytes with spleen and liver at the cellular level. Proc. Natl. Acad. Sci. USA, 74:1521, 1978.

Ardlie, N.G., Packham, M.A., and Mustard, J.F. Adenosine diphosphate-induced platelet aggregation in suspensions of washed rabbit platelets. Br. J. Haematol., 19:7, 1970.

Armstrong, M.L., Megen, M.B., Warner, E.D. Intimal thickening in normocholesterolemic rhesus monkeys fed low supplements of dietary cholesterol. Circ. Res., 34:447, 1974.

Asch, A.S., Leung, L.L.K., Polley, M.J., and Nachman, R.L. Co-localization of thrombospondin, fibrinogen, and glycoproteins IIb/IIIa on the activated platelet surface. Circ. (Part II), 70:193, 1984.

Ashford, T.P. The role of endothelium in the resolution of a thrombus. In: Thrombosis. (eds.) Sherry, S. Brinkhouse, K., Genton, E., and Stengle, J.M. National Academy of Sciences, Washington D.C., p.447, 1969.

Aster, R.J., and Jandl, J.H. Platelet sequestration in man I. Methods. J. Clin. Invest., 43:843, 1964.

Astrup, T. Tissue activators of plasminogen. Fed. Proc., 25:42,1966.

Astrup, T, and Buluk, K. Thromboplastic and fibrinolytic activities in vessels of animals. Circ. Res., 13:253, 1963.

Astrup, T., and Permin, P.M. Fibrinolysis in animal organisms. Nature, 159:681, 1947.

Awbrey, B.J., Hoak, J.C., and Owen, W.G. Binding of human thrombin to cultured human endothelial cells. J. Biol. Chem., 254:4092, 1979.

Baenziger, N.L., Becherer, P.R. and Majerus, P.W. Characterization of prostacyclin synthesis in cultured human arterial smooth muscle cells, venous endothelial cells and skin fibroblasts. Cell, 16:967, 1979.

Balleisen, L., Gay, S., Marx, R., and Kuhn, K. Comparative investigations of the influence of human and bovine collagen types I, II and III on the aggregation of human platelets. Klin. Wochenschr., 53:903, 1975.

Balleisen, L., and Ratherberg,J. Platelet activation by basement membrane collagens. Thromb. Res., 18:725, 1980.

Barnes, M.J. Collagen polymerization in relation to the role of collagen induced platelet aggregation in haemostasis and thrombosis. In: Connective Tissue Research: Chemistry, Biology and Physiology, Alan R. Liss Inc., New York, New York, p.163, 1981.

Barnes, M.J., Gordon, J.L., and MacIntyre, D.E. Platelet-aggregating activity of type I and type III collagens form human aorta and chicken skin. Biochem. J., 160:647, 1976.

Barnett, H.M.J. The Canadian Cooperative Study of Platelet-Suppressive Drugs in Transient Cerebral Ischemia. In: Cerebrovasoular Disease, (eds.) Price, T.R., and Nelson, E. Raven Press, New York, p.221, 1979.

Barnhart, M.I. Platelet responses in health and disease. Mol. Cell Biochem., 22:113, 1978.

Barnhart, M.I., and Chen, S. Vessel wall models for studying interaction capabilities with blood platelets. Semin. Thromb. Hemosts., 5:112, 1978.

Barnhart, M.I., Wilkins, R.M., and Lusher, J.M. Platelet-vessel-wall interactions: Experience with von Willebrand platelets. Ann. New York Acad. Sci., 370:154, 1981.

Baumgartner, H.R. The subendothelial surface and thrombosis. In Thrombosis: Pathogenesis and Clinical Trials. Thromb. Diath. Haemorr.(Suppl) 59:91, 1973a.

Baumgartner, H.R. The role of blood flow in platelet adhesion, fibrin deposition, and formation of mural thrombi. Microvasc. Res., 5:167, 1973b.

Baumgartner, H.R., Muggli, R., Tschopp, T.B.,and Turitto, V.T. Platelet adhesion, release and aggregation in flowing blood: effects of surface properties and platelet function. Thrombos. Haemost., 35:124, 1976.

Baumgartner, H.R., and Spaet, T.H. Endothelial replacement in rabbit arteries. Fed. Proc. 29:710,1970.

Baumgartner, H.R., Stemerman, M.B., and Spaet, T.H. Adhesion of blood platelets to subendothelial surface: distinct from adhesion to collagen. Experientia, 27:283, 1971.

Baumgartner, H.R., Tschopp, T.P., and Weiss, H.J. Platelet interaction with collagen fibrils in flowing blood. I. Impaired adhesion-aggregation in bleeding disorders. A comparison with subendothelium. Thrombos. Haemostas., 37:17, 1977.

Becker, C.G., Dubin, T., and Weidemann, H. P. Hypersensitivity to tobacco antigen. Proc. Natl.

Acad. Sci. USA, 73:1712, 1976.

Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. Diglyceride lipase: a pathway for arachidonate release from human platelets. Proc. Natl. Acad. Sci. USA, 76:3238, 1979.

Belch, J.J.F., Greer, I., Saniabadi, A.R., McLaren, M., Sturrock, R., Forbes, C.D., and Prentice, C.R.M. Effect of ZK36374, a stable prostacyclin analogue in healthy volunteers. Thrombos. Heamosts., 50:279, 1983.

Benhke, O. Effects of some chemicals on blood platelet microtubules, platelet shape and some platelet function in vitro. Scand. J. Haematol., 7:123, 1970.

Bennett, J.S., and Vilaire, G. Exposure of platelet fibrinogen receptors by ADP and epinephrine: J. Clin. Invest., 64:1393, 1979.

Bensusan, H.B., Koh, T.L., Henry, K.G., Murray, B.A., and Culp, L.A. Evidence that fibronectin is the collagen receptor on platelet membranes. Prod. Natl. Acad. Sci. USA., 75:5864, 1978.

Bentz, H., Fietzek, P.P., and Kuhn, K. The covalent structure of calf skin type III collagen: III. The amino acid sequence of cyanogen bromide peptide-alpha-1(III)CB4(Position 403-551). Hoppe Seylers Z. Physiol. Chem., 360:833, 1979.

Bergstrom, S. Prostaglandins: members of a new hormonal system. Science 157:382, 1967.

Bernard, J., and Soulier, J.-P. Sur une nouvelle variete de dystrophie thrombocytaire-hemorragiapare congenitale. Sem. Hop. Paris, 24:3217, 1948.

Bertele, V., Cerletti, C., Schieppati, A., Di Minno, G., and De Gaetano, G. Inhibition of thromboxane synthetase does not necessarily prevent platelet aggregation. Lancet, I:1057, 1981.

Bertele, V., Falanga, A., Tomasiak, M., Chiabrando, C., Cerletti, C., and de Gaetano, G. Pharmacological inhibition of thromboxane synthetase and platelet aggregation: modulatory role of cyclooxygenase products. Blood, 63:1460, 1984.

Best, L.C., Martin T.J., Russell, R.G.G., and Preston, F.E., Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platlets. Nature, 267:850, 1977.

Bills, T.K., Smith, J.B., and Silver, M.J. Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. J. Clin. Invest., 60:1, 1977.

Bjorkerud, S., and Bondjers, G. Arterial repair and atherosclerosis after mechanical injury. I. Permeability and light microscopic characteristics of endothelium in non-atherosclerotic and atherosclerotic lesions. Atherosclerosis, 13:355, 1971.

Blair, I.A., Barrow, S.E., Waddell, K.A., Lewis, P.J., and Dollery, C.T. Prostacyclin is not a circulating hormone in man. Prostaglandins., 23:579, 1982.

Bloom, A.L. The von Willebrand syndrome. Sem. Hematol., 17:215, 1980.

Bocci, V. The role of sialic acid in determining the life-span of circulating cells and glycoproteins. Experientia, 32:135, 1976.

Bolhuis, P.A., Sakariassen, K.S., Sander, H.J., Bouma, B.N., and Sixma, J.J. Binding of factor VIII-von Willebrand factor to human arterial subendothelium precedes increased platelet adhesion and enhances platelet spreading. J. Lab. Clin. Med., 97:568, 1981.

Bonchek, L.I. Prevention of endothelial damage during preparation of saphenous veins for bypass grafting. J. Thorac. Cardiovasc. Surg., 79:911, 1980.

Bondjers, G., and Bjornheden, T. Experimental atherosclerosis induced by mechanical trauma in rats. Atherosclerosis, 12:301, 1970.

Boneu, B., Boneu, A., Raisson, C., Guiraud, R., and Bierme, R. Kinetics of platelet "populations" in the stationary state. Thromb. Res., 3:605, 1973.

Boneu, B., Corberand, J., Plante, J., and Bierme, R. Evidence that platelet density and volume are not related to aging. Thromb. Res., 10:475, 1977.

Booyse, F.M., Bell, S., Sedlak, B., and Rafelson, M.E. Development of an in vitro vessel wall model for

studying certain aspects of platelet-vessel (endothelial) interactions. Artery, 1:518, 1975.

Borchgrevink, C.F. A method for measuring platelet adhesiveness in vivo. Acta. Med. Scand., 168:157, 1960.

Bounameaux, V. L'accolement des plaquettes fibres sous-endotheliales. Comp. Rend. Saences Societe. Biol., Paris, 153:658;1959.

Bourgain, R.H. The inhibitory effect of PGI_2 (prostacyclin) on white platelet arterial thrombus formation. Haemostasis, 8:117, 1979.

Bowen-Pope, D.F. and Ross, R. Platelet-derived growth factor. II. Specific binding to cultured cells. J. Biol. Chem., 257:5161, 1982.

Broekman, M.J., Ward, J.W., and Marcus, A.J. Phospholipid metabolism in stimulated human platelets. Changes in phosphatidylinositol, phosphatidic acid, and lysophospholipids. J. Clin. Invest., 66:275, 1980.

Brownlee, M., and Cerami, A. The biochemistry of the complications of diabetes mellitus. Annu. Rev. Biochem., 50:385, 1981.

Buchanan, M.R., Butt, R.W., Magas, Z., Van Ryn, J., Hirsh, J., and Nazir, D.J. Endothelial cells produce an endothelial cell derived chemo-repellent which influences platelet/endothelial cell interactions - effect of aspirin and salycilate. Thrombos. Haemostas., 53:306, 1985.

Buchanan, M.R., Butt, R.W., Nazir, D., Turpie, A.G.G., and Hirsh, J. The regulation of platelet/endothelial cell adhesion by two arachidonic acid metabolites. Thromb. Haemost., 50:190, 1983.

Buchanan, M. R., Carter, C.J., and Hirsh, J. Decreased platelet thrombogenicity in association with increased platelet turnover and vascular damage. Blood, 54:1369, 1979.

Buchanan, M.R., Haas, T.A., Lagarde, M., and Guichardant, M. 13-hydroxyoctadecadienoic acid is the vessel wall chemorepellant factor, LOX. J. Biol. Chem., 260:16056, 1985b.

Buchanan, M.R., Rosenfeld, J., and Hirsh, J. The prolonged

effect of sulfinpyrazone on collagen induced platelet aggregation in vivo. Thrombos. Res., 13:883, 1978.

Buchanan, M.R., Vazquez, M.J., and Gimbrone, M.A.Jr. Arachidonic acid metabolism and the adhesion of human polymorphonuclear leukocytes to cultured vascular endothelial cells. Blood, 62:889, 1983.

Buchwald, H., Fitch, L., and Moore R.B. Overview of random atherosclerotic cardiovascular disease. Controlled Clinical Trials, 3:271, 1982.

Buonassisi, V. Sulfated mucopolysaccharids synthesis and secretion in endothelial cell cultures. Exp. Cell. Res., 76:363, 1973.

Brass, L.F., and Bensusan, H.B. The role of collagen quarternary structure in the platelet:collagen interactions. J. Clin. Invest., 54:1480, 1974.

Bunting, S., Gryglewski, R., Moncada, S., and Vane, J.R. Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation. Prostaglandins, 12:897, 1976.

Burch, J.W., Baenziger, N.L., Stanford, N., and Majerus, P.W. Sensitivity of fatty acid cyclooxygenase from human aorta to acetylation by aspirin. Proc. Natl. Acad. Sci., USA, 75:5181, 1978.

Busch, C., Dawes, J., Pepper, D.S., Wasteson, A. Binding of platelet factor 4 to cultured human umbilical vein endothelial cells. Thromb. Res., 19:129, 1980.

Butler, K.D., Wallis, R.B., and White, A.M. A study of the relationship between ex vivo and in vivo effects of sulphinpyrazone in the guinea pig. Haemostasis, 8:353, 1979.

Caen, J.P., Nurden, A.T., Jeanneau, C., Michel., H., Tobelem, G., Levy-Toledano, S., Sultan, Y., Valensi, F., and Bernard,J. Bernard-Soulier syndrome: a new platelet glycoprotein abnormality. Its relationship with platelet adhesion to subendothelium and with the factor VIII von Willebrand protein. J. Lab. Clin. Med., 87:586, 1976.

Cagguila, A.W., Christakis, G., Farrand, M., Hulley, S.B., Johnson, R., Lasser, N.L., Stamler, J., and Widdoson,

G. The multiple risk intervention trial (MRFIT). IV. Intervention on blood lipids. Prev. Med., 10:443, 1981.

Cairns, J.A., Gent, M., Singer, J., Finnie, K.J., Froggatt, G.M., Holder, D.A., Jablonsky, G., Kostuk, W.J., Melendez, L.J., Myers, M.G., Sackett, D.L., Sealey, B.J., and Tanser, P.H. Aspirin, sulfinpyrazone, or both in unstable angina. N.Engl.J.Med., 313:1369, 1985.

Canadian Cooperative Study Group. A randomized trial of aspirin and sulfinpyrazone in threatened stroke. N. Engl. J. Med., 299:53, 1978.

Castellot, J.J., Addonizio, M.L., Rosenberg, R., and Karnovsky, M.J. Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. J. Cell Biol., 90:372, 1981.

Cazenave, J.-P., Blondowska, D., Richardson, M., Kinlough-Rathbone, R.L., Packham. M.A., and Mustard, J.F. Quantitative isotopic measurement and scanning electron microscopic study of platelet adherence to collagen-coated surface and to subendothelium with a rotating probe device. J. Lab. Clin. Med., 93:60, 1979.

Cazenave,J.-P., Dejana, E., Kinlough-Rathbone, R.L., Richardson, M., Packham, M.A., and Mustard. J.F. Prostaglandins I_2 and E_1 reduce rabbit and human platelet adherence without inhibiting serotonin release from adherent platelets. Thromb. Res., 15:273, 1979.

Cazenave, J.-P., Guccione, M.A., Mustard, J.F., and Packham, M.A. Lack of effect of UDP, UDPG, and glucosamine on platelet reactions with collagen. Thromb. Diath. Haemorrh., 31:521, 1974.

Cazenave, J.-P., Guccione, M.A., Packham, M.A., and Mustard, J.F. Inhibition of platelet adherence to damaged surface of rabbit aorta. J. Lab. Clin. Med., 86:551, 1975.

Cazenave. J.-P., Guccione, M.A., Packham, M.A., and Mustard, J.F. Inhibition of platelet adherence to damaged vessel wall by drugs. In: Atherosclerosis, metabolic, morphologic and clinical aspects. (eds.) Manning, G.W., and Haust, M.D. Adv. Exp. Med. Biol., 82:228, 1977.

Cazenave, J.-P., Kinlough-Rathbone, R.L., Packham, M.A., and Mustard, J.F. The effect of acetylsalicyclic acid and indomethacin on rabbit platelet adherence to collagen and the subendothelium in the presence of a low or high hematocrit. Thromb. Res., 13:971, 1978.

Cazenave, J.-P., Packham, M.A., Guccione, M.A., and Mustard, J.F. Inhibition of platelet adherence to a collagen-coated surface by agents that inhibit platelet shape change and clot retraction. J. Lab. Clin. Med., 84:483, 1974.

Cazenave, J.-P., Packham, M.A., Guccione, M.A., and Mustard, J.F. Inhibition of platelet adherence to collagen-coated surface by nonsteroidal anti-inflammatory drugs, pyrimido-pyridimine and tricyclic compounds, and lidocaine. J. Lab. Clin. Med., 83:797, 1974.

Cazenave, J.-P., Packham, M.A., Guccione, M.A., and Mustard, J.F. Inhibition of platelet adherence to damaged surface of rabbit aorta. J. Lab. Clin. Med., 86:551, 1975.

Cazenave, J.-P., Packham, M.A., Davies, J.A., Kinlough-Rathbone, R.L., and Mustard, J.F. Studies of platelet adherence to collagen and the subendothelium. In: Platelet Function Testing. (eds.) Day, H.J., Holmsen, H., and Zucker, M.B., DNEW Publication No. (NIH)78-1087. U.S. Govt. Print. Off., Washington, D.C., p.181, 1978.

Cazenave, J.-P., Packham, M.A. Kinlough-Rathbone, R.L., and Mustard, J.F. Platelet adhesion to the vessel wall and to collagen-coated surfaces. In: Thrombosis - Animal and Clinical Models. (eds.) Day, H.J., Molony, B.A., Nishizawa, E.E., and Rynbrandt, R.H., Adv. Exp. Med. Biol., 102:31, 1978.

Cazenave, J.-P., Packham, M.A., and Mustard, J.F. Adherence of platelets to a collagen-coated surface: development of a quantitative method. J. Lab. Clin. Med., 82:978, 1973.

Cazenave, J.-P., Reimers, H.-J., Packham, M.A., and Mustard, J.F. Effects of reserpine on rabbit platelet aggregation and adherence to collagen or injured rabbit aorta. Biochem. Pharmacol., 26:149, 1977.

Cerilli, J., Holliday, J.E., Fesperman, D.P., and Folger, M.R. Antivascular endothelial cell antibody - its

role in transplantation. Surgery, 81:132, 1977.

Chao, F.C., Shepro, D., Tullis, J.L., Belamarich, F.A., and Curby, W.A. Similarities between platelet contraction and cellular mobility during mitosis: role of microtubules in clot retraction. J. Cell Sci., 20:569, 1976.

Charmatz, A., and Karpatkin, S. Heterogenity of rabbit platelets. I. Employment of an albumin density gradient for separation of a young platelet population identified with Se^{75}-selenomethionine. Thromb. Diath. Haemorrh., 31:485, 1974.

Chazov, E.I., Alexeev, A.V., Antonov, A.S., Koteliansky, V.E., Leytin, V.L., Lyubimova, E.V., Repin, V.S., Svirdov, D.D., Torchilin, V.P., and Smirnov, V.N. Endothelial cell culture on fibrillar collagen: model to study platelet adhesion and liposome targeting to intercellular collagen matrix. Proc. Natl. Acad. Sci. USA, 78:5603, 1981.

Chesbro, J.H., Clements, I.P., Fuster, V., Elveback, L.R., Smith, H.C., Bradsley, W.T., Frye, R.L., Holmes, D.R. Jr., Vlietstra, R.E., Pluth, J.R., Wallace., R.B., Puga, F.J., Orszulak, T.A., Piehler, J.M., Schaff., H.V., and Danielson, G.K. A platelet-inhibitor-drug trial in coronary-artery bypass operations. Benefit of perioperative dipyridamole and aspirin therapy on early postoperative vein-graft patency. N. Engl. J. Med., 307:73,1982.

Chierchia, S., De Caterina, R., Crea, F., Patrono, C., and Maseri, A., Failure of thromboxane A_2 blockade to prevent attacks of vasospastic angina. Circulation, 66:702, 1982.

Christensen, C.B., and Garbash, C. Repar in arterial tissue: scanning electron microscope (SEM) and light microscopic study on the endothelium of rabbit thoracic aorta following a single dilatation injury. Virchows Arch. (Pathol. Anat.), 360:93, 1973.

Christensen, B.C., Chemnitz, J., Tkocz, I., and Kim, C.M. Repair in arterial tissue. I. Endothelial regrowth, subendothelial tissue changes and permeability in the healing rabbit thoracic aorta. Acta. Path. Microbiol. Scand. [A], 87:265, 1979.

Cieslar, P., Greenberg, J.P., Rand, M.L. Packham, M.A., Kinlough-Rathbone, R.L., and Mustard, J.F.

Separation of thrombin-treated platelets from normal platelets by density-gradient centrifugation. Blood, 53:867, 1979.

Clark, J.M., and Glagov, S. Luminal surface of distended arteries by scanning electrom microscopy: eliminating configurational and technical artefacts. Br. J. Exp. Pathol., 57:129, 1976.

Clarkson, T.B., Bond, M.G., Bullock, B.C., and Marzetta, C.A. A study of atherosclerosis regression in Macaca mulatta. IV. Changes in coronary arteries from animals with atherosclerosis induced for 19 months and then regressed for 24 or 48 months at plasma cholesterol concentrations of 300 or 200 mg/dl. Exp. Molec. Pathol., 34:345, 1981.

Clowes, A.W., and Karnovsky, M.J. Failure of certain antiplatelet drugs to affect myointimal thickening following arterial endothelial injury in the rat. Lab. Invest., 36:452, 1977.

Collen, D. On the regulation and control of fibrinolysis. Thromb. Haemost., 43:77, 1980.

Colwell, J. A., Haluska, P.V., Sarji, K., Levine, J., Sagel, J., and Nair, R.M.G. Altered platelet function in diabetes mellitus. Diabetes, 25:826, 1976.

Colwell, J.A., Winocour, P.D., Lopes-Virella, M., and Haluska, P.V. New concepts about the pathogenesis of atherosclerosis in diabetes-mellitus. Am. J. Med., 75:67, 1983.

Coppe, D., Sobel, M., Seamans, L., Levine, F., and Salzman, E. Preservation of platelet function and number by prostacyclin during cardiopulmonary bypass. J. Thorac. Cardiovasc. Surg., 81:274, 1981

Corash, L., Shafer, B., and Perlow, M. Heterogeneity of human whole blood platelet subpopulations. II. Use of a subhuman primate model to analyze the relationship between density and platelet age. Blood, 52:726, 1978.

Corash, L., Tan, H., and Gralnick, H.R. Heterogenity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume, and ultrastructure. Blood, 49:71, 1977.

Cornu, P., Larrieu, M.J., Caen, J., and Bernard, J.

Transfusion studies in von Willebrand's disease: effect on bleeding time and factor VIII. Br. J. Haematol., 9:189, 1963.

Cowdry, E.V. Structure and physiology of blood vessels. In: Atherosclerosis, (ed.) Chowdry, E.V., Macmillian, New York, p.53, 1983.

Cucianu, M.P., Nishizawe, E.E., and Mustard, J.F. Effect of pyridamo-pyrimidine compounds on platelet function. J. Lab. Clin. Med., 77:958, 1971.

Cunningham, L.W., Santoro, S., and Davies, H. Collagen-platelet interaction-primary hemostasis: introduction. Thromb. Haemostas. Suppl., 63:205, 1978.

Curwen, K.D., Gimbrone, M.A., and Handin, R.I. In vitro studies of thromboresistance. The role of prostacyclin (PGI_2) in platelet adhesion to cultured normal and virally transformed human vascular endothelial cells. Lab. Invest., 42:366, 1980.

Cutler, L., Rodan, G.M., and Feinstein, M.B. Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPase in the dense tubular system of human blood platelets. Biochim. Biophys. Acta., 542:357, 1978.

Czervionke, R.L., Hoak, J.C., and Fry, G.L. Effect of aspirin on thrombin-induced adherence of platelets to cultured cells from the blood vessel wall. J. Clin. Invest., 62:847, 1978.

Czervionke, R.L., Smith, J.B., Fry, G.L., Hoak, J.C., and Haycraft, D.L. Inhibition of prostacyclin by treatment of endothelium with aspirin: correlation with platelet adherence. J. Clin. Invest., 63:1089, 1979.

Dale, J., Thaulow, E., Myhre, E., and Parry, J. The effect of a thromboxane synthetase inhibitor, dazoxiben, and acetylsalicylic acid on platelet function and prostaglandin metabolism. Thrombos. Haemos., 50:703, 1983.

Daoud, A.S., Jarmolych, J., Augustyn, J.M., and Fritz, K.E. Sequential morphological studies of regression of advanced atherosclerosis. Arch. Pathol. Lab. Med., 105:233, 1981.

Data, J.L., Molony, B.A., Meinzinger, M.M., and Gorman, R.R. Intravenous infusion of prostacyclin sodium in man:

clinical effects and influence on platelet adenosine diphosphate sensitivity and adenosine 3':5'-cyclic monophosphate levels. Circulation, 64:4, 1981.

Dawber, T.R., Moore, F.E., and Mann, G.V. Coronary heart disease in the Framingham study. Am J. Pub. Health, 47:4, 1957.

Davey, M.G., and Luscher, E.F. Release reactions of human platelets induced by thrombin and other agents. Biochim. Biophys. Acta, 11655:40, 1968.

Davies, J.A., and Menys, V.C. Effect of heparin on platelet monolayer adhesion, aggregation and production of malondialdehyde. Thrombos. Res., 26:31, 1982.

Davies, D.F., Rees, B.W.G., Johnson, A.P., Elwod, P.C., and Abernethy, M. Food antibodies and myocardial infarction. Lancet, 1:1012, 1974.

DeGowen, R.L., Lewis, L.J., Hoak, J.C., Mueller, A.L., and Gibson D.P. Radiosensitivity of human endothelial cells in culture. J. Lab. Clin. Med., 84:42, 1974.

Dejana, E., Cazenave, J.-P., Hatton, M.W.C., Richardson, M., Groves, H.M., Kinlough-Rathbone, R.L., Packham, R.L., and Mustard, J.F. The effect of thrombin on platelet accumulation on the vessel wall - influence of heparin and aspirin. Thromb. Haemost., 49:196, 1983.

deSa, D.J., and Moore, S. Thromboatherosclerotic lesions caused by neonatal umbilical catheterization. Am. Heart J., 50 (Suppl. III):262, 1975.

Detwiler, T.C., Odell, T.T., and MacDonald, T.P. Platelet size, ATP content and clot retraction in relation to platelet age. Am. J. Physiol., 203:107, 1962.

Deuel, T.F., and Huang, J.S. Roles of growth factor activities in oncogenesis. Blood, 64:951, 1984.

Diamon, T., Mizuhira, V., and Uchida, K. Ultrastructural localization of calcium around the membrane of the surface connected system in the human platelet. Histochemistry, 55:271, 1978.

DiMinno, G., DeGaetano, G., and Garattini, S. Dipyridamole and platelet function. Lancet, 2:1258, 1978.

Dollery, C.T., Barrow, S.E., Blair, I.A., Lewis, P.J., MacDermot, J., Orchard, M.A., Ritter, J.M., Robinson,

C., Shepherd, G.L., Waddell, K.A., and Allison, D.J. Role of prostacyclin. In: Atherosclerosis; Mechanisms and Approaches to Therapy, (ed.) Miller, N.E., Raven Press, New York, p.105, 1983.

Dollery, C.T., and Hensby, C.N. Is prostacyclin a circulating anti-coagulant? Nature, 273:706, 1978.

Doyle, D.J., Chesterman, C.N., Cade, J.F., McGready, J.R., Rennie, G.C., and Morgan, F.J. Plasma concentrations of platelet-specific proteins correlated with platelet survival. Blood, 55:82, 1980.

Dryjski, M., Olsson, P., and Swedenborg, J. Uptake and inhibition of thrombin by the vascular wall. Thromb. Res., 27:467, 1982.

Duguid, J.B., and Robertson, W.B. Mechanical factors in atherosclerosis. Lancet, I:1205, 1957.

Duncan, L.E. Jr., Buck, K., Lynch, A. Lipoprotein movement through canine aortic wall. Science, 142:972, 1963.

Dvorak, H.F., Van De Water, L., Dvorak, A.M., Harvey, V.S., Anderson, D., DeWolf, W., and Bach, R. Human tumor cells and guines pig macrophages shed plasma membrane derived vesicles with procoagulant activity (PCA). Fed. Proc., 41:270, 1982.

Ehrman, M., Jaffe, E.A., and Weksler, B.B. Prostaglandin (PGI_2) inhibits the development in platelets of ADP and arachidonate-mediated shape change and procoagulant activity. Clin. Res., 27:293A, 1979.

Ehrman, M.L., and Jaffe, E.A. Prostacyclin (PGI_2) inhibits the development in human platelets of ADP and arachinoinc acid-induced shape change and procoagulant activity. Prostaglandins, 20:1103,1980.

Eika, C. The platelet aggregating effect of eight commercial heparins. Scand. J. Haematol., 9:480, 1972.

Eika, C. Platelet refractory state induced by heparin. Scand. J. Haematol., 9:665, 1972

Eika, C. On the mechanisms of platelet aggregation induced by heparin, protamine and polybrene. Scand. J. Haematol., 9:248, 1972.

Eika, C., and Godal, H.C. Thrombocytopenia in rabbits induced by heparin, protamine and polybrene. Scand,

J. Haematol., 8:481, 1971.

Eldor, A., and Weksler, B.B. Heparin and dextran sulphate antagonize PGI_2 inhibition of platelet aggregation. Thromb. Res., 16:617, 1979.

Eldor, A., Falcone, D.J., Hajjar, D.P., Minick, C.R., and Weksler, B.B. Recovery of prostacyclin production by de-endothelialized rabbit aorta. J. Clin. Invest., 67:735, 1981

Emmons, P.R., Harrison, M.J.G., Honour, A.J., and Mitchell, J.R.A. Effects of dipyridamole on human platelet behavior. Lancet, 2:603, 1965.

Essien, E.M., Cazenave, J.-P., Moore, S., and Mustard, J.F. Effect of heparin and thrombin on platelet adherence to the surface of rabbit aorta. Thromb. Res., 13:69, 1978.

Evensen, S.A., Jeremic, M., and Hjort, P.F. Experimental thrombocytopenia induced by busulphan (myleran) in rabbits: extremely low platelet levels and intact plasma clotting system. Thrombos. Diathesis Haemorrh., 19:570, 1968.

Faggiotto, A., Ross, R., and Harker, L. Studies of hypercholesterolemia in the nonhuman primate I. Changes that lead to fatty streak formation, Arteriosclerosis, 4:323, 1984.

Fauvel, F., Grant, M.E., Legrand, Y.J., Soushon, H., Tobelem, G., Jackson, D.S., and Caen, J.P. Interaction of blood platelets with a microfibrillar extract from adult bovine aorta: requirement for von Willebrand factor. Proc. Natl. Acad. Sci., USA., 80:551, 1983.

Fauvel, F., Legrand, Y.J., Kuhn, K., Bentz, H., Fietzek, P.P., and Caen, J.P. Platelet adhesion to type III collagen: involvement of a sequence of nine aminoacids from alpha-1(III)CB4 peptide. Thromb. Res., 16:269, 1979.

Feinstein, M.B., and Walenga, R. The role of calcium in platelet activation. In: Biochemistry of the Acute Allergic Reactions. (eds.) Becker, E.L., Simon, A.S., and Austen, K.F., A.R. Liss Inc., New York, p.279, 1981.

Feuerstein, I.A., Brophy, J.M., and Brash, J.L. Platelet transport and adhesion to reconstituted collagen and

artifical surfaces. Trans. Am. Soc. Artif. Organs, 21:427, 1975.

Fields W.S., Lemak, N.A., Frankowski, R.F., and Hardy, R.J. Controlled trial of aspirin in cerebral ischemia. Stroke, 8:301, 1977.

Fishman, J.A., Ryan, G.B., and Karnovsky, M.J. Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myointimal thickening. Lab. Invest., 32:339, 1975.

Flower, R. Steroidal antiinflammatory drugs as inhibitors of phospholipase A_2. In: Advances in Prostaglandin and Thromboxane Research, vol 3. (eds.) Galli, C., Galli, G., and Porcellati, G. New York, Raven Press, p.105, 1978.

Folts, J.D., Gallagher, K., and Rowe, G.G. Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation. Circulation, 65:2, 1982.

Forgarty, T.J., Cranley, J.J., Krause, R.J., Strasser, E.S., and Hafner, C.D. A method for extraction of arterial emboli and thrombi. Surg. Gynecol. Obstet., 116:241, 1963.

Fox, J.E.B., and Phillips, D.R. Inhibition of actin polymerization in blood platelets by cytochalasins. Nature, 292:650, 1981.

French, J.E. Atherosclerosis in relation to the structure and function of the arterial intima, with special reference to the endothelium. Int. Rev. Exp. Pathol., 5:253, 1966.

Friedman, M., and Byers, S.O. Experimental thrombo-atherosclerosis. J. Clin. Invest., 40:1139, 1961.

Friedman, R.J., Animal models involving the thrombotic process. (discussion following workshop). In: The thrombotic process in atherogenesis. (eds.) Chandler, A.B., Eurenius, K., McMillan, G.C., Nelson, C.B., Schwartz, C.J., and Wessler, S. Adv. Exp. Med. Biol., Vol. 104, Plenum Press, New York, New York, p.401, 1978.

Friedman, R.J., Moore, S., and Singal, D.P. Repeated endothelial injury and induction of atherosclerosis in normolipemic rabbits by human serum. Lab. Invest., 30:404, 1975.

Friedman, R.J., Moore, S., Singal, D.P., and Gent, M. Regression of injury-induced atheromatous lesions in rabbits. Arch. Pathol. Lab. Med., 100:189, 1976.

Fry, D.L. Responses of the arterial wall to certain physical factors. Ciba Found. Symp., 12:93, 1973.

Fukami, M.H., and Salganicoff, L. Human platelet storage organelles. A review. Thrombos. Haemostas., 38:963, 1977.

Fuster, V. Badimon, L., Rosemark, J., and Bowie, E.J.W. Platelet-arterial wall interaction: quantitative and qualitative study following selective carotid endothelial injury in normal and von Willebrand pigs. Clin. Res., 31:459A, 1983.

Fuster, V., Bowie, E.J.W., Fass, D.N., and Owen, C.A., Jr. Long-term prospective study on spontaneous atherosclerosis in normal and von Willebrand pigs. Circulation 60(suppl.II):271, 1979.

Fuster, V., Bowie, E.J.W., Lewis, J.C., Owen, C.A., Jr., and Brown, A.L. Resistance to atherosclerosis in pigs with von Willebrand's disease: Spontaneous and high cholesterol diet-induced atherosclerosis. J. Clin. Invest. 61:722, 1978.

Fuster, V., Bowie, E.J.W., and Fass, D.N. Atherosclerosis in homozygous and heterozygous von Willebrand pigs fed a high cholesterol diet. Circulation 60 (suppl.II):272,1979.

Fuster, V., Chesebro, J.H., Frye, L.R., and Elveback, L.R. Elwood, P.C., Cochrane, A.L., Burr, M.L., Sweetnam, P.M., Williams, G., Welsby, E., Hughes, S.J., and Renton, R. A randomized controlled trial of acetylsalicylic acid in the secondary prevention of mortality from myocardial infarction. Br. Med. J., 1:436, 1974.

Fuster,V., Chesebro, J.H., Frye, R.L., and Elveback, L.R. Platelet survival and the development of coronary disease in the young adult; effects of cigarette smoking, strong family history and medical therapy. Circulation, 63:546, 1981.

Gabbiani, G., Badonnell, M.C., Mathewson, M.B., and Graeme, B.R. Acute cadmium intoxication. Lab. Invest. 30:686,1974.

Gahmberg, Q.G., and Hakamori, S.-I. External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem., 248:4311, 1973.

Gajdusek, C., DiCorleto, P., Ross, R., and Schwartz, S.M. An endothelial cell-derived growth factor. J. Cell Biol., 85:467, 1980.

Gartner, T.K., Gerrard, J.M. White, J.G., and Williams, D.C. Fibrinogen is the receptor for the endogenous lectin of human platelets. Nature, 289:688, 1981.

Gartner, T.K., Phillips, D.R. and Williams, D.C. Expression of thrombin-enhanced platelet lectin activity is controlled by secretion. FEBS Lett., 113:196, 1980.

Gay, S., Balleisen, L., Remberger, K., Fietzek, P.P., Adelmann, B.C., and Kuhn, K. Immunohistochemical evidence for the presence of collagen type III in human arterial walls, arterial thrombi, and in leukocytes incubated with collagen in vitro. Klin. Wochenschr., 53:899, 1975.

George, N.J. Studies on platelet plasma membranes. IV. Quantitative analysis of platelet membrane glycoproteins by (^{125}I)-diazotized diiodosulfanilic acid labeling and SDS-polyacrylamide gel electrophoresis. J. Lab. Clin. Med., 92:432, 1978.

George, J.N., Lewis, P.C., and Morgan, R.K. Studies on platelet plasma membranes. III. Membrane glycoprotein loss from circulating platelets in rabbits: inhibi- by aspirin-dipyridamole and accelerated by thrombin. J. Lab. Clin. Med., 91:301, 1978.

Gerrard, J.M., Peterson, D.A., and White, J.G. Calcium mobilization. In: Platelets in Biology and Pathology, Vol 2, (ed.) Gordon, J.L., p.407, 1981.

Gerrard, J.M., Phillips, D.R., Rao, G.H.R., Plow, E.F., Walz, D.A., Ross, R., Harker, L.A., and White, J.G. Biochemical studies of two patients with gray platelet syndrome. Selective deficiency of platelet alpha granules. J. Clin. Invest., 66:102, 1980.

Gerrard, J.M., Stuart, M.J., Rao, G.H.R., Steffes, M.W., Mauer, M.S., Brown, D.M., and White, J.G. Alterations in the balance of prostaglandin and thromboxane synthesis in diabetic rats. J. Lab. Clin. Med., 95:950, 1980.

Gerrity, R.G. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am. J. Pathol., 103:181, 1981a.

Gerrity, R.G. The roll of monocytes in atherogenesis II. Migration of foam cells from atherosclerotic lesions. Am. J. Pathol., 103:191, 1981.

Gerrity, R.G., Naito, H.K., Richardson, M., and Schwartz, C.J. Dietary induced atherogenesis in swine. Morphology of the intima in prelesion stages. Am J. Pathol., 95:775, 1979.

Giddings, J.C., Brookes, L.R., Piovella, F., and Bloom, A.L. Immunohistological comparison of platelet factor 4 (PF4), fibronectin (Fn) and factor VIII related antigen (VIIIR:Ag) in human platelet granules. Br. J. Haematol., 52:79, 1982.

Gimbrone, M.A., and Alexander, R.W. Angiotensin II stimulation of prostaglandin production in cultured human vascular endothelium. Science, 189:219, 1975.

Gimbrone, M.A., and Buchanan, M.R. Interactions of platelets and leukocytes with vascular endothelium: In vitro studies. Ann. N. Y. Acda. Sci., 401:171, 1982.

Ginsberg, M.H., Painter, R.G., and Birdwell, C. The detection, immunofluorescent localization, and thrombin induced release of human platelet-associated fibronectin antigen. J. Supramol. Struct., 11:167, 1979.

Ginsberg, M.H., Painter, R.G., Forsyth, J., Birdwell, C., and Plow, E.F. Thrombin increases expression of fibronectin antigen on the platelet surface. Proc. Natl. Acad. Sci. Biol. USA, 77:1049, 1980.

Ginsberg, M.H., Wencel, J.D., White, J.G., and Plow, E.F. Binding of fibronectin to alpha-granule-deficient platelets. J. Cell Biol., 97:571, 1983.

Goldberg, I.D., Stemerman, M.B., and Handin, R.I. Vascular permeation of platelet factor 4 after endothelial injury. Science, 209:611, 1980.

Goldsmith, H.L. The flow of model particles and blood cells and its relation to thrombogenesis. In: Progress in Hemostasis and Thrombosis, Vol. 1, (ed.) Spaet, T.H., New York, NY, p. 97, 1972.

Goldsmith, H.L., and Karino, T. Mechanically induced

thromboemboli. In: Hwang, N.H.C., Gross, D.R., and Patel, D.J. (eds.) Quantitative Cardiovascular Studies. Clinical and Research Applications of Engineering Principles. University Park Press. p.289, 19...

Goldsmith, H.L., and Karino, T. Microrehology and clinical medicine: unravelling some problems related to thrombosis. Clin. Hemorheol., 2:143, 1982.

Goode, T.B., Davies, P.F., Reidy, M.A., and Bowyer, D.E. Aorta endothelial cell morphology observed in situ by scanning electron microscopy during atherogenesis in the rabbit. Atherosclerosis, 27:235, 1977.

Gordon, T., Kannel, W.B., McGee, D., and Dwaber, T.R. Death and coronary attacks in men after giving up cigarette smoking. A report from the framingham study. Lancet, 1:1345, 1974.

Gore, I., Fujinami, T., and Shirahama, T. Endothelial changes produced by ascorbic acid deficiency in guinea pigs. Arch. Path., 80:371, 1965.

Gorman, R.R., Bunting, S., and Miller, O.V. Modulation of human platelet adenylate cyclase by prostacyclin (PGX). Prostaglandins, 13:377, 1977.

Gralnick, H.R., Williams, S.B., and Coller, B.S. Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. Blood, 64:797, 1984.

Greenberg, J, Packham, M.A., Cazenave, J.-P., Reimers, H.-J., and Mustard, J.F. Effects on platelet function of removal of platelet sialic acid by neuraminidase. Lab. Invest., 32:176, 1975.

Greenberg, J.P., Packham, M.A., Guccione, M.A., Rand, M.L., Reimers, H.-J., and Mustard, J.F. Survival of rabbit platelets treated in vitro with chymotrypsin, plasmin, trypsin, or neuraminidase. Blood, 53:916, 1979.

Grette, K. Studies on the mechanism of thrombin-catalyzed hemostatis reactions in the blood. Acta Physiol. Scand., 56(Suppl. 195):5, 1962.

Griggs, T.R., Reddick, R.L., Sultzer, D., and Brinkhous, K.M. Susceptibility to atherosclerosis in aortas and coronary arteries of swine with with von Willebrand's disease. Am. J. Pathol., 102:137, 1981.

Groves, H.M., Kinlough-Rathbone, R.L., Richardson, M., and

Groves, H.M., Kinlough-Rathbone, R.L., Richardson, M., and Mustard, J.F. Platelet survival and interaction with damaged rabbit aorta. Blood, 50(Suppl I):241, 1977.

Groves, H.M., Kinlough-Rathbone, R.L., Richardson,M., Moore, S., and Mustard, J.F. Platelet interaction with damaged rabbit aorta. Lab. Invest., 40:194, 1979.

Groves, H.M., Richardson, M., and Kinlough-Rathbone, R.L. Ultrastructural examination of the wall of the aorta after a single balloon catheter injury. Scan. Electron Microsc., 2:491, 1978.

Gryglewski, R.J., Bunting, S., Moncada, S., Flower, R.J., and Vane, J.R. Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. Prostaglandins, 12:685, 1976.

Gryglewski, R.J., Korbut, R., and Ocetkiewicz, A.C. Generation of prostacyclin by lungs in vivo and its release into the arterial circulation. Nature, 273:765, 1978.

Guccione, M.A., Kinlough-Rathbone, R.L., Packham, M.A., Harfenist, E.J., Rand, M.L., Greenberg, J.P., Perry, D.W., and Mustard, J.F. Effects of Plasmin on rabbit platelets. Thromb. Haemostas., 53:8, 1985.

Gutstein, W.H., and Parl, F. Ultrastructural changes of coronary endothelium associated with biliary obstruction in the rat. Am. J. Pathol. 71:49, 1973.

Halushka, P.V., Lurie, D., and Colwell, J.A. Increased synthesis of prostaglandin-E-like material by platelets from patients with diabetes mellitus. N. Engl. J. Med., 297:1306, 1977.

Hamberg, M., Svensson, J., Wakabayashi, T., and Samuelsson, B. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. Proc. Natl. Acad. Sci. USA., 71:345, 1974.

Hamberg, M., Svensson, J., Samuelsson, B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. Proc. Natl. Acad. Sci. USA., 72:2994, 1975.

Hammarstrom, S.,and Falardeau, P. Resolution of prostaglandin endoperoxide synthetase and thromboxane synthetase of human platelets. Proc.

Natl. Acad. Sci. USA., 74:3691, 1977.

Hanson, S.R., and Harker, L.A. Effect of dazoxiben on arterial graft thrombosis in the baboon. Br. J. Clin. Pharmacol., 15(Suppl.1):57S, 1983.

Hardin, N.J., Minick, C.R., and Murphy, G.E. Experimental induction of atherosclerosis by the synergy of allergic injury to arteries and lipid-rich diet. Am. J. Pathol., 73:301, 1973.

Hardisty, R.M. Disorders of platelet function. Br. Med. Bull., 33:207, 1977.

Hardisty, R.M., and Caen, J.P. Disorders of platelet function. In: Haemostasis and Thrombosis, (eds) Bloom, A.L., and Thomas, D.L., Churchill Livingstone, New York, NY, p.301) 1981.

Hardisty, R.M., and Hutton, R.A. The kaolin clotting time of platelet-rich plasma: a test of platelet factor-3 availability. Br. J. Haematol., 11:258, 1965.

Hardisty, R.M., and Hutton, R.A. Platelet aggregation and the availability of platelet factor 3. Br. J. Haematol., 12:764, 1966.

Harfenist, E.J., Izzotti, M.J., Packham, M.A., and Mustard, J.F. Plasma fibronectin is not involved in ADP-induced aggregation of rabbit platelets. Thromb. Haemost., 44:108, 1980.

Harfenist, E.J., Packham, M.A., Kinlough-Rathbone, R.L., and Mustard, J.F. Inhibitors of ADP-induced platelet aggregation prevent fibrinogen binding to rabbit platelets and cause rapid deaggregation and dissociatoin of bound fibrinogen. J. Lab. Clin. Med., 97:680, 1981.

Harsfalvi, J., Muszbek, L., Stadler, I., and Fesus, L. Inhibition of platelet factor 3 availability by prostacyclin. Prostaglandins, 20:935, 1980.

Harker, L.A., Hanson, S.R., and Kirkman, T.R. Experimental arterial thromboembolism in baboons. Mechanism, quantitation, and pharmacological prevention. J. Clin. Invest., 64:559, 1979.

Harker, L.A., Ross, R., Slichter, S.J., and Scott, C.R. Homocystine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. J. Clin. Invest., 58:731, 1976.

Harker, L.A., and Scott, C.R. Platelets in homocystinuria. N. Engl. J. Med., 296:818, 1977.

Harker, L.A., and Slichter, S.J. Studies of platelet and fibrinogen kinetics in patients with prosthetic heart valves. N. Engl. J. Med., 283:1302, 1970.

Harker, L.A., and Slichter, S.J. The bleeding time as a screening test for the evaluation of platelet function. N. Engl. J. Med., 287: 155, 1972a.

Harker, L.A., and Slichter, S.J. Platelets and fibrinogen consumption in man. N. Engl. J. Med., 287:999, 1972b.

Harker, L.A., and Slichter, S.J. Arterial and venous thromboembolism: kinetic characterization and evaluation of therapy. Thromb. Diath. Haemorrh., 31:188, 1974.

Harter, H.R., Burch, J.W., Majerus, P.W., Stanford, N., Delmez, J.A., Anderson, C.B., and Weerts, C.A. Prevention of thrombosis in patients on hemodialysis by low-dose aspirin. N. Engl. J. Med., 301:577, 1979.

Haslam, R.J. Inhibition of blood platelet function by cytochalasins: effects on thrombosthenin and on glucose metabolism. Biochem. J., 127:34, 1972.

Haslam, R.J. Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. Ser. Haematol., 6:333, 1973.

Haslam, R.J., Davidson, M.M.L., and Davies, T. Regulation of blood platelet function by cyclic nucleotides. In: Adv. Cyclic Nucleotide Res., Vol. 9. (eds.) George, W.J., and Ignarro, L.J. New York, Raven Press, p.533, 1978.

Haslam, J.R., Davidson, M.M.L., Davies, T., Lynham, J.A., and McClenaghan, M.D. Regulation of blood platelet function by cyclic nucleotides. Adv. Cyclic Nucleotide Res., 9:533, 1978.

Haslam, R.J., Davidson, M.M.L., and McClenaghan, M.D. Cytochalasin B, the blood platelet release reaction and cyclic GMP. Nature, 253:455, 1975.

Haslam, R.J., and Lyman, J.A. Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents. I.

Effects of different aggregating agents. Biochem. Biophys. Res. Commun., 77:714, 1977.

Haslam, R.J., Lyman, J.A., and Fox, J.E.B. Effects of collagen, ionophore A23187 and prostaglandin E_1 on the phosphorylation of specific proteins in blood platelets. Biochem. J., 178:397, 1979.

Haslam, R.J., and McClenaghen, M.D., Measurement of circulating prostacyclin. Nature, 292:364, 1981.

Hathaway, D.R., and Adelstein, R.S. Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity. Proc. Natl. Acad. Sci. USA, 76:1653, 1979.

Hatton, M.W.C., Dejana, E., Cazenave, J.-P., Regoeczi, E., and Mustard, J.F. Heparin inhibits thrombin binding to rabbit thoracic aorta endothelium. J. Lab. Clin. Med., 96:861, 1980.

Haudenschild, C.C. Growth and control of endothelial cells in atherogenesis and tumor angiogenesis. Adv. Microcirc., 9:226, 1980.

Haudenschild, C.C., Gould, K.E., Quist, W.C., and LoGerfo, F.W. Protection of endothelium in vessel segments excised for grafting. Circulation, 64 (Suppl. II):101, 1981.

Heath, H., Brigden, W.D., Canever, J.V., Pollock, J., Hunter, P.R., Kelsey, J., and Bloom A. Platelet adhesiveness and aggregation in relation to diabetic retinopathy. Diabetologia, 7:308, 1971.

Hemker, H.C., van Rijn, J.L.M.L., Rosing, J., van Dieijen, G., Bevers, E.M., and Zwaal, R.F.A. Platelet membrane involvement in blood coagulation. Blood Cells, 9:303, 1983.

Higgs, G.A., Cardinal, D.C., Moncada, S., and Vane, J.R. Micro circulatory effects of prostacyclin (PGI_2) in the hamster cheek pouch. Microvascular Res., 18:245, 1979.

Higgs, E.A., Higgs, G.A., Moncada, S., and Vave, J.R. Prostacyclin (PGI_2) inhibits the formation of platelet thrombi in arterioles and venules in the hamster cheak pouch. Br. J. Pharmacol., 63:5353, 1978.

Higgs, E.A., Moncada, S., Vane, J.R., Caen,J.P., Michel, H.,

and Tobelem, G. Effect of prostacyclin (PGI_2) on platelet adhesion to rabbit arterial subendothelium. Prostaglandins, 16:17, 1978.

Higgs, G.A., and Vane, J.R. Inhibition of cyclo-oxygenase and lipoxygenase. Br. Med. Bull., 39:265, 1983.

Hirsh, J., Glynn, M.F., and Mustard, J.F. The effect of platelet age on platelet adherence to collagen. J. Clin. Invest., 47:466, 1968.

Hoff, H.F., and Gottlob, R. A fine structure study of injury to the endothelial cells of rabbit abdominal aorta by various stimuli. Angiology, 18:440, 1967.

Holmsen, H. Collagen-induced release of adenosine diphosphate from blood platelets incubated with radioactive. phosphate in vivo. Scand. J. Lab. Clin. Invest. 17:239, 1965.

Holmsen, H. Prostaglandin endoperoxide-thromboxane synthesis and dense granule secretion as positive feedback loops in the propagation of platelet responses during "the basic platelet reaction", Thromb. Haemost., 38:1030, 1977.

Holmsen, H. and Weiss, H.J. Secretable storage pools in platelets. Ann. Rev. Med., 30:119,1979.

Hossman, V., Heinen, A., Auel, H., and Fitzgerald, G.A. A randomized, placebo controlled trial of prostacyclin (PGI_2) in peripheral arterial disease. Thrombos. Res., 22:481, 1981.

Houdijk, W.P.M., Sakariassen, K.S., Nievelstein, P.F.E.M., and Sixma, J.J. Role of factor VIII-von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. J. Clin. Invest., 75:531, 1985.

Hougie, C., Denson, K.W.E., and Biggs, R. A study of the reaction product of factor VIII and factor IX by gel filtration. Throm. Diath. Haemorrh., 18:211, 1967.

Hovig, T. Aggregation of rabbit blood platelets produced in vitro by saline "extract" of tendons. Thromb. Diath. Haemorrh., 9:248, 1963.

Hovig, T., Jorgensen, L., Packham, M.A., and Mustard, J.F. Platelet adherence to fibrin and collagen. J. Lab.

Clin. Med., 71:29, 1968.

Howard, M.A., and Firkin, B.G. Ristocetin - a new tool in the investigation of platelet aggregation. Thromb. Diath. Haemorrh., 26:362, 1971.

Howard, M.A., Hutton, R.A., and Hardisty, R.M. Hereditary giant platelet syndrome: a disorder of a new aspect of platelet function. Br. Med. J., 2:586, 1973.

Howard, M.A., Montgomery, D.C., and Hardisty, R.M. Factor VIII-related antigen in platelets. Thromb. Res. 4:617, 1974.

Howell, W.H. The nature and the action of the thromboplastin (zymoplastic) substance of the tissues. Am. J. Physiol., 31:1, 1912.

Huang, T.W., and Benditt, E.P. Mechanisms of platelet adhesion to the basal lamina. Am. J. Pathol., 92:99, 1978.

Hugod, G., Hawkins, L.H., Kjeldsen, K., Thompsen, H.K., and Astrup, P. Effect of carbon monoxide exposure on aortic and coronary intimal morphology in the rabbit. Atherosclerosis 30:333, 1978.

Huttner, I., Boutet, M., and More, R.H. Studies on protein passage through arterial endothelium. I. Structural correlates of permeability in rat arterial endothelium. Lab. Invest., 28:672, 1973.

Huttner, I., and Peters, H. Heterogeneity of cell junctions in rat aortic endothelium: a freeze-fracture study. J. Ultrastruct. Res., 64:303, 1978.

Hugues, J. Accolement des plaquettes au collagene. C. R. Soc. Biol.(Par), 154:866, 1960.

Hugues, J., Herion, F., Nusgens, B., and Lapiere, Ch.M. Type III collagen and probably not type I collagen aggregates platelets. Thromb. Res., 9:223, 1976.

Humphrey, J.H., and Jaques, R. The release of histamine and 5-hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions (in vitro). J. Physiol., 128:9, 1955.

Hynes, R.O., Ali, I.U., Destree, A.T., Mauter, V., Perkins, M.E., Senger, D.R., Wagner, D.D., and Smith, K.K. A large glycoprotein lost from the surfaces of

transformed cells. Ann. NY Acad. Sci., 312:317, 1978.

Hynes, R.O., and Yamada, K.M. Fibronectine: multifunctional modular glycoproteins. J. Cell Biol., 95:369, 1982.

Ibels, L.S., Stewart, J.H., Mahony, J.F., and Sheil, A.G.R. Deaths from occlusive arterial disease in renal allograft recipients. Br. Med. J., 3:552, 1974.

International Committee for Standardization in Hematology. Recommended method for radioisotope platelet survival studies. Blood, 50:1137, 1977.

Jaeger, S., and Bernsten, H. Deposition of human labelled platelets on damaged rabbit aorta before and after ingestion of acetylsalicylic acid. Haemostasis, 8:99, 1979.

Jaffe, R., and Deykin, D. Evidence for a structural requirement for the aggregation of platelets by collagen. J. Clin. Invest., 53:875, 1974.

Jaffe, E.A., and Weksler, B.B. Recovery of endothelial cell prostacyclin production after inhibition by low doses of aspirin. J. Clin. Invest., 63:532, 1979.

Jamieson, G.A., Okamura, T., Fishback, B., Johnson, M.M., Egan, J.J., and Weiss, H.J. Platelet membrane glycoproteins in thrombasthenia, Bernard-Soulier syndrome, and storage pool disease. J. Lab. Clin. Med., 93:652, 1979.

Jamieson, G.A., Urban, C.L., and Barber, A.J. Enzymatic basis for platelet:collagen adhesion as the primary step in haemostasis. Nature New Biol., 234:5, 1971.

Jenkins, C.S.P., Phillips, D.R., and Clemetson, K.J. Platelet membrane glycoproteins implicated in ristocetin-induced aggregation. Vth Congress of International Society on Thrombosis and Haemostasis, Paris, p. 453, 1975.

Jenkins, C.S.P., Phillips, D.R., Clemetson, K.J., and Meyer, D., Larrieu, M.-J., and Luscher, E.F. Platelet membrane glycoproteins implicated in ristocetin-induced aggregation. J. Clin. Invest., 57:112, 1976.

Joist, J.H., Dolelez, G., Lloyd, J.V., Kinlough-Rathbone, R.L., and Mustard, J.F. Platelet factor-3 availability and the platelet release reaction. J.

Lab. Clin. Med., 84:474, 1974.

Joist, J.H., Niewiarowski, S., and Mustard, J.F. Release of antiplasmin during thrombin or collagen induced platelet aggregation. In: Thrombolytic Therapy, Thromb. Diath. Haemorrh., 47 (Suppl.):113, 1971.

Jorgensen, L. Packham, M.A., Rowsell, H.C., and Mustard, J.F. Deposition of formed elements of blood on the intima and signs of intimal injury in the aorta of rabbit, pig, and man. Lab. Invest., 27:341, 1972.

Jorgensen, L., Packham,M.A., Rowsell, H.C., Buchanan, M.R., and Mustard, J.F. Focal arterial injury caused by cannulation: increased plasma protein accumulation and thrombosis. Acta. Pathol. Microbiol. Scand. [A], 82:637, 1974.

Jorgensen, L., Rowsell, H.C., Hovig, T., and Mustard, J.F. Resolution and organization of platelet-rich mural thrombi in carotid arteries of swine. Am. J. Pathol., 51:681, 1967.

Joris, I., Stetz, E., and Majno, G. Lymphocytes and monocytes in the aortic intima. Atherosclerosis, 34:221, 1979.

Josa, M., Lie, J.T., Bianco, R.L., and Kaye, M.P. Reduction of thrombosis in canine coronary bypass vein grafts with dipyridamole and aspirin. Am. J. Cardiol. 47:1248, 1981.

Kao, K.-J., Pizzo, S.V., and McKee, P.A. Platelet receptors for human factor VIII/von Willebrand protein: functional correlation of receptor occupancy and ristocetin-induced platelet aggregation. Proc. Natl. Acad. Sci., USA, 76:5317, 1979.

Kaplan, K.L. Platelet granule proteins: localization and secretion. In: Platelets in Biology and Pathology. Vol 2, (ed.) Gordon, J.L., Elsevier/North-Holland Biomedical Press, p.77, 1981.

Kaplan, K.L., Broekman, M.J. Chernoff, A., Lesznik, G.R., and Drillings, M. Platelet alpha-granule proteins: studies on release and subcellular localization. Blood, 53:604, 1979.

Kaplan, K.L., and Owen, J. Plasma levels of beta-thromboglibulin and platelet factor 4 as indices of platelet activation in vivo. Blood,

57:199, 1981.

Kaplan, K.L., Nossel, H.L., Drillings, M., and Lesznik, G. Radioimmunoassay of platelet factor 4 and beta-thromboglobulin: development and application to studies of platelet release in relation to fibrinopeptide A generation. Br. J. Haematol., 39:129, 1978.

Karino, T., and Goldsmith, H.L. Adhesion of human platelets to collagen on the walls distal to a tubular expansion. Microvas. Res., 17:238, 1979a.

Karino, T., and Goldsmith, H.L. Aggregation of human platelets in the annular vortex distal to a tubular expansion. Microvasc. Res., 17:217, 1979b.

Karnovsky, M.J. Endothelial-vascular smooth muscle cell interactions. Am. J. Pathol., 105:200, 1981.

Karniguian, A., Simmons, P., Legrand, Y.J., Moncada, S., and Caen, J.P. A comparison of the inhibitory effects of prostacyclin and carbacyclin on platelet adhesion to collagen. Prostaglandins, 24:827, 1982.

Karniguian, A., Legrand, Y.J., and Caen, J.P. Prostaglandins: specific inhibition of platelet adhesion to collagen and relationship with cAMP level. Prostaglandins, 23:437, 1982.

Karpatkin, S. Heterogeneity of rabbit platelets. IV. Further resolution of changes in platelet density, volume, and radioactivity following cohort labelling with ^{75}Se-selenomethione. Br. J. Haematol., 39:459, 1978.

Karpatkin, S.,and Charmatz, A. Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets. J. Clin. Invest., 48:1073, 1969.

Kelton, J.G., Antithrombotic therapy in coronary artery disease. In: Hemostasis and Thrombosis: Basic Principles and Clinical Practice, (eds.) Coleman, R.W., Hirsh, J., Marder, V.J., and Salzman, E.W., Lippincott Company, Philadelphia, p.1068, 1982.

Kernoff, L.M., Rose, A.G., Hughes, J., and Jacobs, P. Autopsy findings in elderly man suffering from severe von Willebrand's disease. Thromb. Haemost., 46:714, 1981.

Keyes, A. Coronary heart disease - the global picture. Atherosclerosis, 22:149, 1975.

Kinlough-Rathbone, R.L., Cazenave, J.-P., Packham, M.A., and Mustard, J.F. Effect of inhibitors of the arachidonate pathway on the release of granule contents from rabbit platlets adherent to collagen. Lab. Invest, 42:28, 1980.

Kinlough-Rathbone, R.L., Packham, M.A., and Mustard, J.F. Platelet survival in relation to vessel wall injury. Thrombos. Haemost., 46:248, 1981.

Kinlough-Rathbone, R.L., Packham, M.A., and Mustard, J.F. Vessel injury, platelet adherence, and platelet survival. Arteriosclerosis, 3:529, 1983a.

Kinlough-Rathbone, R.L., Packham, M.A., and Mustard, J.F. Platelet aggregation. In: Methods in Hematology. Measurements of Platelet Function. (eds.) Harker, L.A., and Zimmerman, T.S., Churchill Livingston, Edinburgh, p.64, 1983b.

Kinlough-Rathbone, R.L., Packham, M.A., Reimers, H.-J., Cazenave, J.-P., and Mustard, J.F. Mechanisms of platelet shape change, aggregation, and release induced by collagen, thrombin, or A23,187. J. Lab. Clin. Med., 90:707, 1977.

Kirk, J.E. Anticoagulant activity of human arterial mucopolysaccharides, Nature, 184:369, 1959.

Kisiel, W., Canfield, W.M., Ericsson, L.H., and Davie, E.W. Anticoagulant properties of bovine plasma protein C following activation by thrombin. Biochemistry, 16:5824, 1977.

Kjaerheim, A., and Hovig, T. The ultrastructure of haemostatic blood platelet plugs in rabbit mesenterium. Thromb. Diath. Haemorrh., 7:1, 1962.

Knicker, W.T., and Cochrane, C.G. The localization of circulating immune complexes in experimental serum sickness. J. Exp. Med., 127:119, 1968.

Korbut, R., and Moncada, S. Prostacyclin (PGI_2) and thromboxane A_2 interaction in vivo. Regulation by aspirin and relationship with anti-thrombotic therapy. Thrombos. Res., 13:489, 1978.

Koshal, A., Krausz, M.M., Utsunomiya, T., Hechtman, H.B., Collins, J.J., and Cohn, L.H. Preservation of platelets and their function in prolonged cardiopulmonary bypass using prostacyclin. Circulation, 64 (Suppl. II):44, 1981.

Klebe, R.J. Isolation of a collagen-dependent cell attachment factor. Nature, 250:248, 1974.

Krausz, M.M., Utsunomiya, T., Feuerstein, G., Wolfe, J.H.N., Shepro, D., and Hechtman, H.B. Prostacyclin reversal of lethal endotoxemia in dogs. J. Clin. Invest., 67:1118, 1981.

Kritchevsky, D., Tepper, S.A., Scott, D.A., Klurfeld, D.M., Vesselinovitch, D., and Wissler, R.W. Cholesterol vehicle in experimental atherosclerosis. Part 18. Comparison of North American, African and South American peanut oils. Atherosclerosis, 38:291, 1981.

Kritchevsky, D., Tepper, S.A., Vesselinovitch, D., and Wissler, R.W. Cholseterol vehicle in experimental atherocslerosis. Part 11. Peanut oil. Atherosclerosis, 14:53, 1971.

Kwaan, H.C., and Hatem, A. Effect of lung and gut heparin on experimental arterial thrombosis. In: Heparin: Structure, Function and Clinical Applications, (eds.) R.A. Bradshaw and S Wessler. Plenum, New York, Adv. Exp. Med. Biol., 52:263, 1975.

Lahav, J., Schwartz, M.A., and Hynes, R.O. Analysis of platelet adhesion with a radioactive chemical cross linking reagent: Interaction of thrombospondin with fibronectin and collagen. Cell, 31:253, 1982.

Langille, B.L., and Adamson, S.L. Relationship between blood flow direction and endothelial cell orientation at arterial branch sites in rabbits and mice. Cir. Res., 48:481, 1981.

Langley., P.G., Hughes, R.D., Ton, H.Y., Silk, D.B.A., and Williams, R. The use of prostacyclin, PGI_2, in the prevention of platelet aggregation during charcoal hemoperfusion. Int. J. Artif. Organs, 2:207, 1979.

Le Pape, A., Gutman, N., Guitton, J.D., Legrand, Y., and Muh, J.P. Non enzymatic glycosylation increases platelet aggregating potency of collagen from placenta of diabetic human beings. Biochem. Biophys. Res.

Commun., 111:602, 1983.

Lebowitz, E.A., and Cooke, R. Contractile properties of actomyosin form human blood platelets. J. Biol. Chem., 253:5443, 1978.

Legrand, Y.J., Rodriguez-Zeballos, A., Kartalis, G., Fauvel, F., and Caen, J.P. Adsorption of factor VIII antigen-activity complex by collagen. Thromb. Res., 13:909, 1978.

Levy, G.A., Schwartz, B.S., Curtiss, L.K., and Edgington, T.S. Plasma lipoprotein induction and suppression of the generation of cellular procoagulant activity in vitro. Requirements for cellular collaboration. J. Clin. Invest., 67:1614, 1981.

Lewis, H.D., Davis, J.W., and Archibald, D.G. Protective effect of aspirin against acute myocardial infarction and death in men with unstable angina: results of a Veterans Administration Cooperative Study. N. Engl. J. Med., 309:396, 1983.

Lindner, A., Charra, B., Sherrard, D.J., and Scribner, B.H. Accelerated atherosclerosis in prolonged maintenance of hemodialysis. N. Engl. J. Med., 290:697, 1974.

Lindner, A., and Curtis, K. Morbidity and mortality associated with long-term hemodialysis. Hosp. Pract., 9:143, 1974.

Linos, A., Worthington, J.W., O'Fallon, W., Fuster, V., Whisnant, J.P., and Kurland, L.T. Effect of aspirin on prevention of coronary and cerebrovascular disease in patients with rheumatoid arthritis. A long-term follow-up study. May Clin. Proc., 53:581, 1978.

Longmore, D.B., Hoyle, P.M., Gregory, A., Bennett, J.G., Smith, M.A., Osivand, T., and Jones, W.A. Prostacyclin administration during cardiopulmonary bypass in man. Lancet, 1:800, 1981.

Lollar, P., and Owen, W.G. Clearence of thrombin from circulation in rabbits by high-affinity binding sites on endothelium. J. Clin. Invest., 66:1222, 1980.

Lough, J., and Moore, S. Endothelial injury induced by thrombin or thrombi. Lab. Invest., 33:130, 1975.

Lullmann, H., and Wehling, M. The binding of drugs to

different polar lipids in vitro. Biochem. Pharmacol., 28:3409, 1979.

Lundblad, R.L., and Davie, E.W. The activation of antihemophilic factor (factor VIII) by activated Christmas factor (activated factor IX). Biochemistry, 3:1720, 1964.

Lusher, J.M., and Barnhart, M.I. Congenital disorders affecting platelets. Semin. Thromb. Hemost., 4:123, 1977.

Lyman, B., Rosenberg, L., and Karpatkin, S. Biochemical and biophysical aspects of human platelet adhesion to collagen fibers. J. Clin. Invest., 50:1854, 1971.

Macfarlane, R.G., Briggs, R., Ash, B.J., and Denson, K.W.E. The interaction of factors VIII and IX. Br. J. Haematol., 10:530, 1964.

Machin, S.J., Chamone, D.A.F., Defreyn, G., and Vermylen, J. The effect of clinical prostacyclin infusions in advanced arterial disease on platelet function and plasma 6-keto $PGF_{1\text{-alpha}}$ levels. Br. J. Haematol., 47:413, 1981.

MacIntyre, D.E., Pearson, J.D., and Gordon, J.L. Localization and stimulation of prostaglandin production in vascular cells. Nature, 271:549, 1978.

Mahley, R.W., Weisgraber, K.H., Innerarity, T., Brewer, H.B., and Assmann, G. Swine lipoproteins and atherosclerosis. Changes in the plasma lipoproteins and apoproteins induced by cholesterol feeding. Biochemistry, 14:2817, 1975.

Malmros, H. Dietary prevention of atherosclerosis. Lancet, II:479, 1969.

Malmsten, C., Hamberg, M., Svensson, J., and Samuelsson, B. Physiological role of an endoperoxide in human platelets: hemostatic defect due to platelet cyclo-oxygenase deficiency. Proc, Natl. Acad. Sci. USA., 72:1446, 1975.

Malpass, T.W., Hanson, S.R., Savage, B., Hessel, E.A. II, and Harker, L.A. Prevention of acquired transient defect in platelet plug formation by infused prostacyclin. Blood, 57:736, 1981.

Manilow, M.R. Introduction: the role of nonhuman primates in

research on atherosclerosis regression - hypothetical mechanisms implicated in regression. Artery, 9:2, 1981.

Marchesi, V.T., Jackson, R.L., Segrest, J.P., and Kahane, I. Molecular features of the major glycoprotein of the human erythrocyte membrane. Fed. Proc., 32:1833, 1973.

Marciniak, E. Factor-Xa inactivation by antithrombin III: evidence for biological stabilization of factor Xa by a factor V-phospholipid complex. Br. J. Haematol., 24:391, 1973.

Marcus, A.J., Weksler, B.B., Jaffe, E.A., and Broekman, M.J. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J. Clin. Invest., 66:979, 1980.

Marguerie, G.A., Plow, E.F., and Edgington, T.S. Human platelets possess an inducible and saturable receptor specific for fibrinogen. J. Biol. Chem., 254:5357, 1979.

Mason, R.G. The interaction of blood hemostatic elements with artificial surfaces. In: Progress in Hemostasis and Thrombosis, (ed.) Spaet, T.H., p.141, 1972.

Maynard, J.R., Dreyer, B.E., Stemerman, M.B., and Pitlick, F.A. Tissue-factor coagulant activity of cultured human endothelial and smooth muscle cells and fibroblasts. Blood, 50:387, 1977.

Maynard, J.R., Fintel, D.J., Pitlick, F.A., and Nemerson, Y. Tissue factor in cultured cells. Metabolic control. Lab. Invest., 35:542, 1976.

Maynard, J.R., Heckman, C.A., Pitlick, F.A., and Nemerson, Y. Association of tissue factor activity with the surface of cultured cells. J. Clin. Invest., 55:814, 1975.

McCullagh, K.G., and Balian, G. Collagen characterization and cell transformation in human atherosclerosis. Nature, 258:73, 1975.

McCullagh, K.G., Duance, V.C., and Bishop, K.A. The distribution of collagen types I, III and V (AB) in normal and atherosclerotic human aorta. J. Pathol., 130:45, 1980.

McCully, K.S. Homocysteine theory of atherosclerosis: development and current status. Atherosclerosis Reviews, 11:157, 1983.

McDonald, J.W.D., Dupre, J., Rodger, N.W., Champion, M.C., Webb, C.D., and Ali, M. Comparison of platelet thromboxane synthesis in diabetic patients on conventional insulin therapy and continuous insulin infusions. Thromb. Res., 28:705, 1982.

Mehta, P., and Mehta, J. Potentiation of endoperoxide analog-induced platelet aggregation by heparin. Thrombos. Res., 25:91, 1982.

Menys, V.C., and Davies, J.A. Selective inhibition of thromboxane synthetase with dazoxiben - basis of its inhibitory effect on platelet adhesion. Thrombos. Hemostas., 49:96, 1983.

Meuleman, D.G., Vogel, G.M.T., and van Delft, A.M.L. Effects of intra-arterial cannulation on blood platelet consumption in rats. Thrombos. Res., 20:45, 1980.

Meyer, D., and Baumgartner, H.R. Role of von Willebrand factor in platelet adhesion to the subendothelium. Br. J. Haematol., 54:1, 1983.

Michaeli, D., and Orloff, K.G. Molecular considerations of platelet adhesion. Prog. Haemostas. Thromb. 3:29, 1976.

Miletich, J.P., Jackson, C.M., and Majerus, P.W. Interaction of coagulation factor X_a with human platelets. Proc. Natl. Acad. Sci. USA., 74:4033, 1977.

Miletich, J.P., Jackson, C.M., and Majerus, P.W. Properties of factor X_a binding site on human platelets. J. Biol. Chem., 253:6908, 1978a.

Miletich, J.P., Majerus, D.W., and Majerus, P.W. Patients with congenital factor V deficiency have decreased factor X_a binding sites on their platelets. J. Clin. Invest., 62:824, 1978b.

Mills, D.C.B. Drugs that affect platelet behavior. Clinics in Haematology, 1:295, 1972.

Mills, D.C.B., and Smith, J.B. The infulence on platelet aggregation of drugs that affect the accumulation of adenosine 3':5'-cyclic monophosphate in platelets.

Biochem. J., 121:185, 1971.

Minick, C.R., Alonzo, D.R., And Starek, P.K. The role of endothelial injury in coronary atherosclerosis induced by graft rejection. Circulation, 50 (Suppl.III):92 1974.

Minick, C.R., and Murphy, G.E. Experimental induction of atheroarteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet. II. Effect of repeatedly injected foreign protein in rabbits fed a lipid-rich, cholesterol-poor diet. Am. J. Pathol., 73:265, 1973.

Minick, C.R., Murphy, G.E., and Campbell, W.G.Jr. Experimental induction of athero-arteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet. I. Effect of repeated injections of horse serum in rabbits fed a dietary cholesterol supplement. J. Exp. Med., 124:635, 1966.

Mohammad, S.F., Anderson, W.H., Smith, J.B., Chuang, H.Y.K., and Mason, R.G. Effects of heparin on platelet aggregation and release and thromboxane A_2 production. Am. J. Pathol., 104:132, 1981.

Molnar, J., and Lorand, L. Studies on apyrases. Arch. Biochem. Biophys., 93:353, 1961.

Moncada, S., Gryglewski, R., Bunting, S., and Vane, J.R. An enzyne isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature, 263:663, 1976a.

Moncada, S., Gryglewski, R.J., Bunting, S., and Vane, J.R. A lipid peroxide inhibits the enzyme in blood vessel microsomes that generates from prostaglandin endoperoxides the substance (prostaglandin X) which prevents platelet aggregation. Prostaglandins, 12:715, 1976b.

Moncada, S., Herman, A.G., Higgs, E.A., and Vane, J.R. Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the antithrombotic properties of vascular endothelium. Thromb. Res., 11:323, 1977.

Moncada, S., Higgs, E.A., and Vane, J.R. Human arterial and venous tissues generate prostacyclin (prostaglandin X), a potent inhibitor of platelet aggregation.

Lancet, 1:18, 1977.

Moncada, S., and Korbut, R. Dipyridamole and other phosphodiesterase inhibitors act as antithrombotic agents by potentiating endogenous prostacyclin. Lancet, 1:1286, 1978.

Moncada, S., Korbut, R., Bunting, S., and Vane, J.R. Prostacyclin is a circulating hormone. Nature, 273:767, 1978.

Moncada, S., and Vane, J.R. Pharmacology and endogenous roles of prostacyclin endoperoxides, thromboxane A_2, and prostacyclin. Pharmacol. Rev., 30:293,1979a.

Moncada, S., and Vane, J.R. The role of prostacyclin in vascular tissue. Fed. Proc., 38:66, 1979b.

Moon, D.G., and Kaplan, J.E. Inhibition of collagen and ADP-induced platelet aggregation by plasma fibronectin. Thromb. Haemost., 46:84, 1981.

Moore, S. Plaque lipid: an expression of endothelial damage. Circulation, 44(Suppl. II):23, 1971.

Moore, S. Platelets and organ injury. In: Platelets, Drugs, and Thrombosis. (ed.) Hirsh, J., Symposium, Hamilton, Karger, Basel, Switzerland, p.158, 1972.

Moore, S. Thromboatherosclerosis in normolipemic rabbits. A result of continued endothelial damage. Lab. Invest., 29:478, 1973.

Moore, S. Vascular disease in patients with renal failure. Lancet, I:967, 1974.

Moore, S. Atherosclerosis. In: Animal Models of Thrombosis and Haemorrhagic Diseases. U.S. Department of Health, Education, and Welfare, DHEW Publication No (NIH) 76-982, p.132, 1975.

Moore, S., Belbeck, L.W., Richardson, M., and Taylor, W. Lipid accumulation in the neointima formed in normally fed rabbits in response to one or six removals of the aortic endothelium. Lab. Invest., 47:37, 1982.

Moore, S., Friedman, R.J., Singal, D.P., Gauldie, J., Blachman, M.A., and Roberts, R.S. Inhibition of injury induced thromboatherosclerotic lesions by anti-platelet serum in rabbits. Thromb. Haemost., 35:70, 1976.

Morita, I., Takashi, R., Ito, H., Orimo, H., and Murota, S. Increased arachidonic acid content in platelet phospholipids from diabetic patients. Prostaglandins Leukotrienes Med., 11:33, 1983.

Morrison, A.D., Orci, L., Berwick, L., Perrelet, A., and Winegrad, A.I. The effect of anoxia on the morphology and composite metabolism of the intact aortic intima-media preparation. J. Clin. Invest., 59:1027, 1977.

Mosher, D.F. Fibronectin. In: Progress in Hemostasis and Thrombosis, Vol 5, (ed.) Spaet, T.H., Grune and Stratton, New York, p.111, 1980.

Mosher, D.F., Doyle, M.J., and Jaffe, E.A. Synthesis and secretion of thrombospondin by cultured human endothelial cells. J. Cell Biol., 93:343, 1982.

Movat, H.Z., Uriuhara, T., Taichman, N.S., Rowsell, H.C., and Mustard, J.F. The role of PMN-leukocyte lysosomes in tissue injury, inflammation and hypersensitivity. IV. The participation of the PMN-leukocyte and the blood platelet in systemic aggregate anaphylaxis. Immunology, 14:637, 1968.

Muggli, R., and Baumgartner, H.R. Collagen induced platelet aggregation: requirement for tropocollagen multimers. Thromb. Res., 3:715, 1973.

Murata, K., Nakazawa, K., and Hamai, A. Distribution of acidic glycosaminoglycans in the intima, media and adventitia of bovine aorta and their anti-coagulant properties. Atherosclerosis, 21:93, 1975.

Murphy, E.A., and Bolling, D.R. Platelet survival. In: Platelet Function Testing. (eds.) Day, H.J., Holmsen, H., and Zucker, M.B., DHEW Publication No. (NIH) 78-1087, p.561, 1978.

Murphy, E.A., Francis, M.E., and Bolling, D.R. Estimation of blood platelet survival. - V. A method for the analysis of population data. J. Chron. Dis., 26:797, 1973.

Mustard, J.F. Platelets, endothelium and vascular disease. J. Assoc. Physicians India, 11:201, 1963.

Mustard, J.F., Kinlough-Rathbone, R.L., and Packham, M.A. Aspirin in the treatment of cardiovascular disease: a review. Am. J. Med., 74:43, 1983.

Mustard, J.F., and Packham, M.A. Factors influencing platelet function: adhesion, release, and aggregation. Pharmacol. Rev., 22:97, 1970.

Mustard, J.F. and Packham, M.A. Platelets, thrombosis and drugs. In: Drugs, Vol 9, (ed.) Avery, G.S., Australial Drug Information Services Pty., Ltd., Sydney, p.19, 1975a.

Mustard, J.F., and Packham, M.A. The role of blood and platelets in atherosclerosis and the complications of atherosclerosis. Thromb. Diath. Haemorrh., 33:444, 1975b.

Mustard, J.F., and Packham, M.A. Platelets and diabetes mellitus. N. Engl. J. Med., 297:1345, 1977.

Mustard, J.F., and Packham, M.A. Platelets, thrombosis and drugs. In: Antithrombotic Drugs, Vol 3 (ed.) Avery, G.S., Australial Drug Information Services, Sydney, Australia, p.1, 1978.

Mustard, J.F., and Packham, M.A. Platelets and diabetes mellitus. N. Engl. J. Med., 311:665, 1984.

Mustard, J.F., Packham, M.A., and Kinlough-Rathbone, R.L. Platelet survival. In: Platelet Function Testing. (eds.) Day, H.J., Holmsen, H., and Zucker, M.B., Proc. of International Workshop on Platelets, Philadelphia, p.545, 1978a.

Mustard, J.F., Packham, M.A., and Kinlough-Rathbone, R.L. Platelets, thrombosis and atherosclerosis. In: The Thrombotic Process in Atherogenesis (eds.) Chandler, A.B., Eurenius, K., McMillan, G.C., Nelson, C.B., Schwartz, C.J. and Wessler, S. Adv. Exp. Med. Biol., 104:127, 1978b.

Mustard, J.F., and Murphy, E.A. Effect of smoking on blood platelet survival in man. Br. Med. J., 1:846, 1963.

Mustard, J.F., Rowsell, H.C., and Murphy, E.A. Platelet economy (platelet survival and turnover). Br. J. Haematol., 12:1, 1966.

Nachman, R.L., Jaffe, E.A., and Weksler, B.B. Immunoinhibition of ristocetin-induced platelet aggregation. J. Clin. Invest., 59:143, 1977.

Neame, P.B., Hirsh, J., Browman, G, Denberg, J., D'Sousa, T.J., Gallus, A., and Brain, M.C. Thrombotic thrombo-

cytopenia purpura: a syndrome of intravascular platelet consumption. Can. Med. Assoc. J., 114:1108, 1976.

Nelson, E., Gertz, S.D., Forbes, M.S., Rennels, M.L., Heald, F.P., Kahn, M.A., Farber, T.M., Miller, E., Husain, M.M. and Earl, F.L. Endothelial lesions in the aorta of egg-yolk-fed miniature swine: a study by scanning and transmission electron microscopy. Exp. Mol. Pathol., 25:208, 1976.

Nemerson, Y., and Bach, R. Tissue factor revisited. In: Progress in Hemostasis and Thrombosis, Vol 6. (ed.) Spaet, T.H.,Grune and Stratton, New York, p.198, 1982.

Nemerson, Y., Pitlick, F.A. The tissue factor pathway of blood coagulation. Prog. Hemostasis Thromb., 1:1, 1972

Nerem, R.M. Arterial fluid dymanics and interactions with the vessel walls. In: Structure and function of the circulation, Vol. 2, (eds.) Schwartz, C.J., Werthessen, N.T., Wolf, S., Plenum Press, New York and London, p.719, 1981.

Niemetz, J. Coagulation activity of leukocytes. Tissue factor activity. J. Clin. Invest., 51:307, 1972.

Niewiarowski, S., Bankowski, E., and Rogowicka, I. Studies on the adsorption and activation of the Hageman factor (fXII) by collagen and elastin. Throm. Diath. Haemorrh., 14:387, 1965.

Niewiarowski, S., Lukasiewicz, H., Nath, N., and Sha, A.T. Inhibition of human platelet aggregation by dipyridamole and two related compounds and its modification by acid glycoproteins of human plasma. J. Lab. Clin. Med., 86:64, 1975.

Niewiarowski, S., Regoeczi, E., Stewart, G.J., Senyi, A.F., and Mustard, J.F. Platelet interaction with polymerizing fibrin. J. Clin. Invest., 51:685, 1972a.

Niewiarowski, S., Regoeczi, E., and Mustard, J.F. Platelet interaction with fibrinogen and fibrin: comparison of the interaction of platelets with that of fibroblasts, leukocytes, and erythrocytes. Ann. NY Acad. Sci., 201:72. 1972b.

Niewiarowski, S., Stuart, R.K., and Thomas, D.P. Activation of intravascular coagulation by collagen. Proc. Soc. Exp. Biol. Med., 123:196, 1966.

Nilsson, I.M. Report of the working party on factor VIII-related antigens. Thromb. Haemost., 39:511, 1978.

Nilsson, I.M., Blomback, M., and Blomback, B. V. Willebrand's disease in Sweden. Its pathogenesis and treatment. Acta. Med. Scand., 164:263, 1959.

Nordoy, A., Svensson, B., Schroeder, C., and Hoak, J.C. The inhibitory effect of aspirin on human endothelial cells. Thromb. Haemost., 40:103, 1978.

Nurden, A.T. and Caen, J.P. Specific roles for platelet glycoproteins in platelet function. Nature, 255:720, 1975.

Nurden, A.T., and Caen, J.P. The different glycoprotein abnormalities in thrombasthenic and Bernard-Soulier platelets. Sem. Haematol., 16:234, 1979.

Nurden, A.T., Dupuis, D., Kunicki, T.J., and Caen, J.P. Analysis of the glycoprotein and protein composition of Bernard-Soulier platelets by single and two-dimensional sodium dodecyl sulfate-polyacrylaminde gel electrophoresis. J. Clin. Invest., 67:1431, 1981.

Oblath, R.W., Buckley, F.O., Green, R.M., Schwartz, S.I., and DeWeese, J.A. Prevention of platelet aggregation and adherence to prosthetic vascular grafts by aspirin and dipyridamole. Surgery, 84:37, 1978.

Ogston, D., and Bennett, B. Surface-mediated reactions in the formation of thrombin, plasmin and kallikrein. Br. Med. Bull., 34:107, 1978.

Okumura, T., and Jamieson, G.A. Platelet glycocalicin: a single receptor for platelet aggregation induced by thrombin and ristocetin. Thromb. Res., 8:701, 1976a.

Okumura, T., and Jamieson, G.A. Platelet glycocalicin. I. Orientation of glycoproteins of the human platelet surface. J. Biol. Chem., 251:5944, 1976b.

O'Malley, B.C., Timperley, W.R., Ward, J.D., Porter, N.R., and Preston, F.E. Platelet abnormalities in diabetic peripheral neuropathy. Lancet, 2:1274, 1975.

Osterud, B., and Rapaport, S.I. Activation of factor IX by the reaction product of tissue factor and factor

VII: additional pathway for initiating blood coagulation. Proc. Natl. Acad. Sci. USA., 74:5260, 1977.

Owen, W.G. The control of hemostasis. Arch, Pathol. Lab. Med., 106:209, 1982.

Owen, W.G., and Esmon, C.T. Functional properties of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem., 256:5532, 1981.

Packham, M.A., Cazenave, J.-P., Kinlough-Rathbone, R.L., and Mustard, J.F. Drug effects on platelet adherence to collagen and damaged vessel walls. In: Drugs, Kipid Metabolism, and Atherosclerosis, (eds.) Kritchevsky, D., Paoletti, R., and Holmes, W.L., Adv. Exp. Med. Biol., 109:253, 1978.

Packham, M.A., Evans, G., Glynn, M.F., and Mustard, J.F. The effect of plasma proteins on the interaction of platelets with glass surfaces. J. Lab. Clin. Med., 73:686, 1969.

Packham, M.A., Guccione, M.A., Greenberg, J.P., Kinlough-Rathbone, R.L., and Mustard, J.F. Release of ^{14}C-serotonin during initial platelet changes induced by thrombin, collagen, or A23187. Blood, 50:915, 1977.

Packham, M.A., Guccione, M.A., Kinlough-Rathbone, R.L., and Mustard, J.F. Platelet sialic acid and platelet survival after aggregation by ADP. Blood, 56:876, 1980.

Packham, M.A., Kinlough-Rathbone, R.L., Reimers, H.-J., Scott, S., and Mustard, J.F. Mechanisms of platelet aggregation independent of adenosine diphosphate. In: Prostaglandins in Hematology., (eds.) Silver, M.J., Smith, J.B., and Kocsis, J.J., Spectrum Publications Inc., New York, p.247, 1977.

Packham, M.A., and Mustard, J.F. Pharmacology of platelet-affecting drugs. Circulation, 62(Suppl. V):26, 1980.

Packham, M.A., and Mustard, J.F. Pharmacology of antiplatelet agents and their role in coronary disease. In: Myocardial Infarction and Cardiac Death. (ed.) Margulies, E., Academic Press, New York, p.93, 1983.

Packham, M.A., and Mustard, J.F. Normal and abnormal

platelet activity. In: Blood Platelet Function and Medicinal Chemistry. (ed.) Laslo, A., New York, Elsevier Biomedical, p.61, 1984a.

Packham. M.A., and Mustard, J.F. Platelet adhesion. In: Progress in Hemostasis and Thrombosis. Vol. 7, (ed.) Spaet, T.H., Grune and Stratton, New York, New York, p. 211, 1984b.

Packham, M.A., Mustard, J.F., Guccione, M.A., Winocour, P.D., Groves, H.M., Rand, M.L., Kinlough-Rathbone, R.L., Greenberg, J.P., and Cieslar, P. Investigation of in vivo factors that may influence platelet survival and density. Thromb. Haemostas., 42:328, 1979(abstr).

Packham, M.A., Nishizawa, E.E., and Mustard, J.F. Response of platelets to tissue injury. Biochem. Pharmacol., (Suppl.):171, 1968.

Packham, M.A., Perry, D.W., Kinlough-Rathbone, R.L., Rand, M.L., Guccione, M.A., Evans, R.M., and Mustard, J.F. Effects on the bouyant density of rabbit platelets of ADP and agents that increase the concentration of cyclic AMP. Blood, 65:564, 1985.

Packham, M.A., Rowsell, H.C., Jorgensen, L., and Mustard, J.F. Localized protein accumulation in the wall of the aorta. Exp. Mol. Pathol., 7:214, 1967.

Pantely, G.A., Goodnight, S.H.Jr., Rahimtoola, S.H., Harlan, B.J., DeMots, H., Calvin, L. and Rosch, J. Failure of antiplatelet and anticoagulant therapy to improve patency of grafts after coronary-artery bypass. A controlled, randomized study. N. Engl. J. Med., 301:962, 1979.

Parbtani, A., Chahil, A., Kinlough-Rathbone, R.L., and Mustard, J.F. Survival of rabbit platelets exposed to immune complexes. Fed. Proc., 43:978, 1984.

Paulus, J.-M. Platelet size in man. Blood, 46:321, 1975.

Paulus, J.M., Bury, J., and Grosdent, J.C. Control of platelet territory development in megakaryocytes. Blood Cells, 5:59, 1979.

Pedersen, A.K. and Jakobsen, P. Sulphinpyrazone metabolism during long-term therapy. Brit. J. Clin. Pharmacol., 11:597, 1981.

Pennington, D.G., Lee, N.L.Y., Roxburgh, A.E., and McGready, J.R. Platelet density and size: the interpretation of heterogeneity. Br. J. Haematol., 34:365, 1976.

Peto, R. Clinical trial methodology. Biomedicine, 28:24, 1978.

Pfueller, S.L., and Luscher, E.F. The effect of aggregated immunoglobulins on human blood platelets in relation to their complement-fixing abilities. I. Studies of immunoglobulins of different types. J. Immunol., 109:517, 1972.

Phillips, D.R., Jennings, L.K., and Berndt, M.C. Studies of inherited bleeding disorders to identify platelet membrane glycoproteins involved in adhesion and aggregation. In: Membranes and Genetic Disease. Progress in Clinical and Biological Research. Sheppard, J.R., Anderson, V.E., and Eaton, J.W. (eds.), Alan R. Liss, New York, vol. 97, p.151, 1982.

Phillips, D.R., Jennings, L.K., and Prasanna, H.R. Ca^{2+}-mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. J. Biol. Chem., 255:11629, 1980.

Philp, R.B. Effects of intravenous administration in rabbits of dipyridamole and five related agents on platelet aggregation, blood pressure and heart rate. Thromb. Res., 4:361, 1974.

Piepgras, D.G., Sundt, T.M., and Didisheim, P. Effect of anticoagulants and inhibitors of platelet aggregation on thrombotic occlusion of endarterectomized cat carotid arteries. Stroke, 7:248, 1976.

Pietu, G., Cherel, G., Marguerie, G., and Meyer, D. Inhibition of von Willebrand factor-platelet interaction by fibrinogen. Nature, 308:648, 1984.

Pittilo, R.M., Mackie, I.J., Rowles, P.M., Machin, S.J., and Woolf, N. Effects of cigarette smoking on the ultrastructure of rat thoracic aorta and its ability to produce prostacyclin. Thrombos. Haemost., 48:173, 1982.

Plachetka,J.R., Salomon, N.W., Larson, D.F., and Copeland, J.G. Platelet loss during experimental cardiopulmonary bypass and its prevention by prostacyclin. Ann. Thorac. Surg., 30:58, 1979.

Plow, E.F., Birdwell, C., and Ginsberg, M.H. Identification and quantitation of platelet-associated fibronectin antigen. J. Clin. Invest., 63:540., 1979.

Plow, E.F., and Collen, D. The presence and release of alpha$_2$-antiplasmin from human platelets. Blood, 58:1069, 1981.

Porter, K.A., Rendall, J.M., Stolinski, C., Terasaki,P.I., Marchioro, T.L., and Starzl, T.E. Light and electron microscopic study of biopsies from thirty-three human renal allografts and an isograft 1 3/4 -2 1/2 years after transplantaton. Ann. N.Y. Acad. Sci., 129:615, 1966.

Poston, R.N., and Davies, D.F. Immunity and inflammation in the pathogenesis of atherosclerosis. Atherosclerosis, 19:353, 1974.

Radcliff, R., and Heinze, T. Isolation of plasminogen activator from human plasma by chromatography on lysine-sepharose. Arch. Biochem. Biophys., 189:185, 1978.

Raines, E.W., and Ross, R. Platelet-derived growth factor. 1. High yield purification and evidence for multiple forms. J. Biol. Chem., 257:5154, 1982.

Rajah, S.M., Crow, M.J., Penny, A.F., Ahmad, R., and Watson, D.A. The effect of dipyridamole on platelet function: correlation with blood levels in man. Br. J. Clin. Pharmacol., 4:129, 1977.

Rand, J.H., Sussman, I.I., Gordon, R.E., Chu, S.V., and Solomon, V. Localization of factor VIII-related antigen in human vascular subendothelium. Blood 55:752, 1980.

Rand, M.L. Studies of rabbit platelets as they age in vivo. PhD Thesis, University of Toronto, 1983.

Rand, M.L., Greenberg, J.P., Packham, M.A., and Mustard, J.F. Density subpopulations of rabbit platelets: size, protein, and sialic acid content, and specific radioactivity changes following labeling with ^{35}S-sulfate in vivo. Blood, 57:741, 1981.

Randall. M.J., and Wilding, R.I.R. Acute arterial thrombosis in rabbits: reduced platelet accumulatiton after treatment with thromboxane synthetase inhibitor

dazoxiben hydrochloride (UK-37, 248-01). Thromb. Res., 28:607, 1982.

Rathbone, R.L., Ardlie, N.G., and Schwartz, C.J. Platelet aggregation and thrombus formation in diabetes mellitus: an in vitro study. Pathol., 2:307, 1970.

Ratnoff, O.D. The surface-mediated initiation of blood coagulation and related phenomena. In: Haemostasis: Biochemistry, Physiology and Pathology. (eds.) Ogston, D., and Bennett, B., John Wiley and Sons, London, p.25, 1977.

Ratnoff, O.D., and Rosenblum, J.M. The role of Hageman factor in the initiation of clotting by glass. J. Lab. Clin. Med., 50:941, 1957.

Reches, A., Fleldor, A., and Salomon, Y. Heparin inhibits PGE_1 sensitive adenlyate cyclase and antagonizes PGE_1 anti-aggregating effect in human platelets. J. Lab. Clin. Med., 93:638, 1979.

Reddick, R.L., Griggs, T.R., Lamb, M.A., and Brinkhous, K.M. Platelet adhesion to damaged coronary arteries: comparison in normal and von Willebrand disease swine. Proc. Natl. Acad. Sci., USA, 79:5076, 1982.

Reidy, M.A., Standaert, D., and Schwartz, S.M. Inhibition of endothelial cell regrowth: Cessation of aortic endothelial cell replication after balloon catheter denudation. Arteriosclerosis, 2:216, 1982.

Reimers, H.-J., Kinlough-Rathbone, R.L., Cazenave, J.-P., Seyni, A.F., Hirsh, J., Packham, M.A., and Mustard, J.F. In vitro and in vivo functions of thrombin-treated platelets. Thromb. Haemost., 35:151, 1976.

Richardson, M., and Moore, S. Preparation of large curved biological surfaces for SEM. Artery, 6:409, 1980.

Rittenhouse-Simmons, S. Production of diglyceride from phosphatidylinositol in activated human platelets. J. Clin. Invest., 63:580, 1979.

Robison, R.L., Hayes, K.C., McCombs, H.L, and Faherty, T.P. Effect of dietary fat and cholesterol on circulating lipids and aortic ultrastructure of squirrel monkeys. Exp. Mol. Pathol., 15:281, 1971.

Rodgers, G.M., Greenberg, C.S., and Shuman, M.A. Characterization of the effect of cultured vascular

cells on the activation of coagulation. Blood, 61:1155, 1983.

Rosenberg, R.D. The function of heparin. In: Heparin: Chemistry and clinical usage, (eds.) Kakker, V.V., and Thomas, D.P., Academic Press, London, p.101, 1977.

Rosenkranz, B., Fischer, C., Weimer, K.E., and Frolich, J.C. Metabolism of prostacyclin and 6-keto-prostaglandin F_{1alpha} in man. J. Biol. Chem., 255:10194, 1980.

Ross, R., Faggiotto, A., Bowen-Pope, D., and Raines, E. The role of endothelial injury and platelet and macrophage interactions in atherosclerosis. Circulation, 70:77, 1984.

Ross, R., and Glomset, J.A. Atherosclerosis and the arterial smooth muscle cell. Science, 180:1332, 1973.

Ross, R., and Glomset, J.A. The pathogenesis of atherosclerosis (First of two parts). N. Engl. J. Med., 295:369, 1976.

Ross, R., Glomset, J., Kariya, B., and Harker, L. A. A platelet-dependent serum factor that stimulates the proliferaton of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. USA., 71:1207, 1974.

Ross, R., and Harker, L. Hyperlipidemia and atherosclerosis. Chronic hyperlipidemia initiates and maintains lesions by endothelial cell desquamation and lipid accumulation. Science, 193:1094, 1976a.

Ross, R., and Harker, L.A. Hyperlipidemia and atherosclerosis. Science, 193:1094, 1976b.

Ross, R., Nist, C., Kariya, B., Rivest, M.J., Raines, E., and Callis, J. Physiological quiescence in plasma-derived serum: influence of platelet-derived growth factor on cell growth in culture. J. Cell. Physiol., . 97:497, 1978.

Ross, R., and Vogel, A. The platelet-derived growth factor. Cell, 14:203, 1978.

Roth, J.G., Stanford, N., and Majerus, P.W. Aceytlation of prostaglandin synthestase by aspirin. Proc. Natl. Acad. Sci. USA., 72:3073, 1975.

Rouslahti, E, and Vaheri, A. Interaction of soluble fibroblast surface antigen with fibrinogen and

fibrin. J. Exp. Med., 141:497, 1975.

Rowsell, H.C., Downie, H.G., and Mustard, J.F. The experimental production of atherosclerosis in swine following the feeding of butter and margarine. Can. Med. Assoc. J., 79:647, 1958.

Ruf, W., McNamara, J.J., Suehiro, A., Suehiro, G., and Wickline, S.A. Platelet trapping in myocardial infarct in baboons: therapeutic effect of aspirin. Am. J. Cardiol., 46:405, 1980.

Rugerri, Z.M., Bader, R., and De Marco, L. Glanzmann thrombosthenia: deficient binding of von Willebrand factor to thrombin stimulated platelets. Proc. Natl. Acad. Sci., USA, 79:6038, 1982.

Saba, H.I., Saba, S.R., Blackburn, C.A., Hartmann, R.C., and Mason, R.G., Heparin neutralization of PGI_2: Effects on platelets. Science, 205:499, 1979.

Sacks, T., Moldow, C.F., Craddock, P.R., Bowers, T.K., and Jacobs, H.S. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. An in vitro model of immune vascular damage. J. Clin. Invest., 61:1161, 1978.

Sakariassen, K.S., Bolhuis, P.A., and Sixma, J.J. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-Von Willebrand factor bound to the subendothelium. Nature, 279:636, 1979.

Sakariassen, K.S., Fressinaud, E., Grima, J.-P., Baumgartner, H.R., and Meyer, D. Mediation of platelet adhesion to fibrillar collagen in flowing blood by a proteolytic fragment of human von Willebrand factor. Blood, 5:1515, 1986.

Salzman, E.W. Measurement of platlet adhesiveness. A simple in vitro technique demonstrating an abnormality in von Willebrand's disease. J. Lab. Clin. Med., 62:724, 1963.

Salzman, E.W. Nonthrombogenic surfaces: critical review. Blood, 38:501, 1971.

Salzman, E.W. Prostaglandins and platelet function. In: Advances in Prostaglandin and Thromboxane Research, (eds.) Samuelsson, B., and Paoletti, R. Vol 2., New York, Raven Press, p.767, 1976.

Salzman, E.W., Merrill, E.W., Binder, A., Wolf, C.F.W., Ashford, T.P., and Austen, W.G. Protein-platelet interaction on heparinized surfaces. J. Biomed. Mater. Res., 3:69, 1969.

Salzman, E.W., Rosenberg, R.D., Smith, M.H., Lindon, J.N., and Favreau, L. Effect of heparin and heparin fractions on platelet aggregation. J. Clin. Invest., 65:64, 1980.

Santoro, S.A. Preferential binding of high molecular weight forms of von Willebrand factor to fibrillar collagen. Biochim. Biophys. Acta., 756:123, 1983.

Santoro, S.A., and Cunningham, L.W. Collagen-mediated platelet aggregation. Evidence for multivalent interactions of intermediate specificity between collagen and platelets. J. Clin. Invest., 60:1054, 1977.

Santoro, S.A., and Cunningham, L.W. Fibronectin and the multiple interaction model for platelet-collagen adhesion. Proc. Natl.Acad. Sci. USA, 76:2644, 1979.

Schaub, R.G., Rawlings, C.A., and Keith, J.C. Effect of long-term aspirin treatment on platelet adhesion to chroncially damaged canine pulmonary arteries. Thromb. Haemost., 46:680, 1981.

Scheffel, U., McIntyre, P.A., Evatt, B., Dvornicky, J.A. Jr., Natarajan, T.K., Bolling, D.R., and Murphy, E.A. Evaluation of Indium-111 as a new high photon yield gamma-emitting "physiological" platelet label. Johns Hopkins Med. J., 140:285, 1977.

Schwartz, S.M., and Benditt, E.P. Studies on aortic intima. I. Structure and permeability of rat thoracic aortic intima. Am. J. Pathol., 66:241, 1972.

Schwartz, S.M., Gajdusek, C.M., Reidy, M.A., Selden, S.C.III, and Haudenschild, C.C. Maintenance of intergity in aortic endothelium. Fed. Proc., 39:2618, 1980.

Schwartz, S.M., Haudenschild, C.C., and Eddy, E.M. Endothelial regeneration. I. Quantitative analysis of initial stages of endothelial regeneration in rat aortic intima. Lab. Invest., 38:568, 1978.

Schwartz, S.M., Stemerman, M.B., and Benditt, E.P. The aortic intima. II. Repair of the aortic lining after mechanical denudation. Am. J. Pathol., 81:15, 1975.

Scott, G.B.D. Atherosclerosis, hyperlipaemia and hypocoagulability in the rat. An experimantal study. Br. J. Exp. Pathol., 45:102, 1964.

Shattil, S.J., Anaya-Galindo, R., Bennett, J., Coleman, R.W., and Cooper, R.A. Platelet hypersensitivity induced by cholseterol incorporation. J. Clin. Invest., 55:636, 1975.

Sheppard, G., and Marlow, C.G. The simultaneous measurement of ^{51}Cr and ^{14}C by liquid sintillation counting. Int. J. Appl. Radiat. Isot., 22:125, 1971.

Shepro, D., Belamarich, F.A., Robblee,L. and Chao, F.C. Antimotility effect of cytochalasin B observed in mammalian clot retraction. J. Cell. Biol., 47:544, 1970.

Shimamoto, T. The relationship of edematous reaction in arteries to atherosclerosis and thrombosis. J. Atheroscler. Res., 3:87, 1963.

Shimamoto, T., Yamashita, Y., Numano, F.,and Sunaga, T. Scanning and transmission electron microscopic observation of endothelial cells in the normal condition and in initial stages of atherosclerosis. Acta. Pathol. Jpn., 21:93, 1971.

Shuman, M.A., Isaacs, J.D., Maerowitz, N., Savion, N., and Gospodarowicz, D. Endothelial cell and platelet binding sites for thrombin. Ann. NY Acad. Sci., 370:57, 1981.

Siegel, M.I., McConnell, R.T., Abrahams, S.L. Porter, N.A., and Cuatrecasas, P. Regulation of arachidonate metabolism via lipoxygenase and cyclo-oxygenase by 12-HPETE, the product of human platelet lipoxygenase. Biochem. Biophys. Res. Commun., 89: 1273, 1979.

Siegel, M.I., McConnell, R.T., and Cuatrecasas, P. Aspirin-like drugs interfere with arachidonate metabolism by inhibition of the 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid peroxidase activity of the lipoxygenase pathway. Proc. Soc. Natl. Acad. Sci. USA, 76:3774, 1979.

Silkworth, J.B., and Stehbens, W.E. The shape of endothelial cells in en face preparations of rabbit blood vessels. Angiology, 26:474, 1975.

Silwer, J. Cronberg, S., and Nilsson, I.M. Occurrence of

arteriosclerosis in von Willebrand's disease. Acta. Med. Scand., 180:475, 1966.

Simionescu, M., Simonescu, N., and Palade, G.E. Segmental differentiations of intercellular junctions in the vascular endothelium. Arteries and veins. J. Cell Biol.(Suppl.)67:401a, 1975.

Simionescu, M., Simonescu, N., and Palade, G.E. Segmental differentiations of cell junctions in the vascular endothelium. Arteries and veins. J. Cell Biol., 68:705, 1976.

Simionescu, M., Simonescu, N., Silbert, J.E., and Palade, G.E. Differentiated microdomains on the luminal surface of the capillary endothelium. II. Partial characterization of their anionic sites. J. Cell Biol., 90:614, 1981.

Simionescu, N., Simonescu, M., and Palade, G.E. Permeability of muscle capillaries to small heme-peptide. Evidence for the existence of patent transendothelial chanels. J. Cell Biol., 64:586, 1975.

Sinzinger, H., Silberbauer, K., Horsch, A.K., and Gall, A. Decreased sensitivity of human platelets to PGI_2 during long-term intraarterial prostacyclin infusion in patients with peripheral vascular disease - a rebound phenomenon? Prostaglandins, 21:49, 1981.

Sinha, A.K., and Coleman, R.W. Persistence of increased platelet cyclic AMP induced by prostaglandin E_1 after removal of the hormone. Proc. Natl. Acad. Sci. USA, 77:2946, 1980.

Skubitz, K.M., and Craddock, P.R. Reversal of hemodialysis granulocytopenia and pulmonary leukostasis. A clinical manifestation of selective down-regulation of granulocyte responses to $C5a_{desarg}$. J. Clin. Invest., 67:1383, 1981.

Smith, J.B., Alam, I, Ingerman-Wojensky, C.M., Siegl, A.M., and Silver, M.J. Prostaglandins as modulators of platelet function. In: Cardiovascular Pharmacology of the Prostaglandins, (eds.) Herman, A.G., Vanhoutte, P.M., Denolin, H., and Goossens, A., Raven Press, New York, p.115, 1982.

Smith, J.B., Ingerman, C., Kocsis, J.J., and Silver, M.J. Formation of an intermediate in prostaglandin biosynthesis and its association with the platelet

release reaction. J. Clin. Invest., 53:1468, 1974.

Smith, J.B., and Jubiz, W. OKY-1481: A selective inhibitor of thromboxane synthesis in vivo and in vitro. Prostaglandins, 22:353, 1981.

Smith, J.B., Silver, M.J., Ingerman, C.M., and Kocsis, J.J. Prostaglandin D_2 inhibits the aggregation of human platelets. Thromb. Res., 5:291, 1974.

Smythe, H.A., Orgryzlo, M.A., Murphy, E.A., and Mustard, J.F. The effect of sulfinpyrazole (Anturan) on platelet economy and blood coagulation in man. Can. Med. Assoc. J., 92:818, 1965.

Smoking and health: Report of the Advisory Committee to the Surgeon General of the Public Health Service, United States Department of Health, Education and Welfare, 1964.

Snyderman, R., and Goetzl, E.J. Molecular and cellular mechanisms of leukocyte chemotaxis. Science, 213:830, 1981.

Sochynsky, R.A., Boughton, B.J., Burns, J., Sykes, B.C., and McGee, J.O'D. The effect of human fibronectin on platelet - collagen adhesion. Thromb. Res., 18:521, 1980.

Somer, J.B., and Schwartz, C.J. Focal [^{3}H]cholesterol uptake in the aorta. Part 2. Distribution of [^{3}H]cholesterol across the aortic wall in areas of high and low uptake in vivo. Atherosclerosis, 16:377, 1972.

Somers, D., Kinlough-Rathbone, R.L., Groves, H.M., Packham, M.A., and Mustard, J.F. Platelet incorporation into thrombi and platelet survival in rabbits. Fed. Proc., 39:629, 1980.

Somers, D.A., Winocour, P.D., Kinlough-Rathbone, R.L., and Mustard, J.F. Relation among platelet survival, vessel injury, thrombosis and platelet density. Fed. Proc., 42:1027, 1983.

Soppitt, G.D.,and Mitchell, J.R.A. The effect of colchicine on human platelet behavior. J. Atheroscler. Res., 10:247, 1969.

Spaet, T.H., and Cintron, J. Studies on platelet factor-3 availibility. Br. J. Haematol., 11:269, 1965.

Spaet, T.H., Drouet, L., Efremides, A., and Won, J. Platelet turnover on exposed subendothelium of balloon de-endothelialized rabbits. Blood (Suppl. I), 52:173, 1978.

Spurr, A.R. Low-viscosity embedding media. J. Ultrastruct. Res., 26:31, 1969.

Stamler, J. Nutrition-related risk factors for the atherosclerotic diseases - present status. Prog. Biochem. Pharmacol., 19:245, 1983.

Stary, H.C. Regression of atherosclerosis in primates. Virchows Arch. A Path. Anat. and Histol., 383:117, 1979.

Steele, P., Rainwater, J., and Vogel, R. Abnormal platelet survival time in men with myocardial infarction and normal coronary arteriogram. Am. J. Cardiol., 41:60, 1978.

Steele, P. Rainwater, J., Vogel, R., and Genton, E. Platelet suppressant therapy in patients with coronary artery disease. J. Am. Med. Assoc., 240:228, 1978.

Steele, P.P., Weily, H.S., and Genton, E. Platelet survival and adhesiveness in recurrent venous thrombosis. N. Engl. J. Med. 288:1148, 1973.

Stein, O., and Stein, Y. Lipid synthesis and degradation and lipoprotein transport in mammalian aorta. Ciba Found. Symp., 12:165, 1973.

Stein, O., Stein, Y., and Eisenberg, S. A radioautographic study of the transport of ^{125}I-labelled serum lipoproteins in rat aorta. Z. Zellforsch. Mikrosk. Anat., 138:223, 1973.

Stemerman, M.B. Thrombogenesis of the rabbit arterial plaque. An electron microscopic study. Am. J. Pathol., 73:7, 1973.

Stemerman, M.B. Platelet and smooth muscle cell responses after endothelial desquamation. Thromb. Haemostas., 45:248, 1981.

Stemerman, M.B., Baumgartner, H.R.,and Spaet, T.H. The subendothelial microfibril and platelet adhesion. Lab. Invest., 24:179, 1971.

Stemerman, M.B., and Ross, R. Experimental atherosclerosis. I. Fibrous plaque formation in primates, an electron

microscope study. J. Exp. Med., 136:769, 1972.

Stratton, J.R., Malpass, T.W., Ritchie, J.L., Pfeifer, M.A., and Harker, L.A. Studies of platelet factor 4 and beta thromboglobulin release during exercise: lack of relationship to myocardial ischemia. Circulation, 66:33, 1982.

Sun, F.F., McGuire, J.C., Morton, D.R., Pike, J.E., Sprecher, H., and Kunau, W.H. Inhibition of platelet arachidonic acid 12-lipoxygenase by acetylenic acid compounds. Prostaglandins, 21:333, 1981.

Szczeklik, A., Gryglewski, R.J., Nizankowski, R., Szcseklik, J., Skawinski, S., and Gluszko, P. Prostacyclin in the therapy of peripheral arterial disease. In: Advances in Prostaglandin and Thromboxane Research, Vol. 7. (eds.) Samuelsson, B., Ramwell, P.W., and Paoletti, R., Raven Press, New York, p. 687, 1980.

Tamborlane, W.V., Sherwin, R.S., Genel, M., and Felig, P. Restoration of normal lipid and aminoacid metabolism in diabetic patients treated with a portable insulin-infusion pump. Lancet, 1:1258, 1979.

The Canadian Cooperative Study Group. A randomized trial of aspirin and sulfinpyrazone in threatened stroke. N. Engl. J. Med., 299:53, 1978.

The Antruane Reinfarction Trial Research Group. Sulfinpyrazone in the prevention of sudden death after myocardial infarction. N. Engl. J. Med., 302:250, 1980.

The Persantine-Aspirin Reinfarction Study Research Group. Persantine and aspirin in coronary heart disease. Circ., 62:449, 1980.

Thomas, D.P., Merton, R.E., Hiller, K.F., and Hockley, D. Resistance of normal endothelium to damage by thrombin. Br. J. Haematol., 51:25, 1982.

Thomas, D.P., Merton, R.E., Wood, R.D., and Hockley, D.J. The relationship between vessel wall injury and venous thrombosis: an experimental study. Br. J. Haematol., 59:449, 1985.

Thompson, C., Forbes, C.D., and Prentice, C.R.M. The potentiation of platelet aggregation and adhesion by heparin in vitro and in vivo. Clin. Sci. Mol. Med., 45:485, 1973.

Tranzer, J.P., and Baumgartner, H.R. Filling gaps in the vascular endothelium with blood platelets. Nature 216:1126, 1967.

Triplett, D.A. Platelet Function. Laboratory Evaluation and Clinical Application. Appendix B, American Society of Clinical Pathologists, Chicago. p.264, 1978.

Ts'ao, C.-H. Eisenstein, R., and Schumacher, B. Effect of an aortic proteoglycan on platelet aggregation and thrombin time: plasma requirement and active moieties (39898), Proc. Soc. Exp. Biol. Med., 156:162, 1977.

Tschopp, T.B., Weiss, H.J., and Baumgartner, H.R. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. J. Lab. Clin. Med., 83:296, 1974.

Tschopp, T.B., Weiss, H.J., and Baumgartner, H.R. Interaction of thrombasthenic platelets with subendothelium: normal adhesion, absent aggregation. Experientia, 31:113, 1975.

Turitto, V.T. Blood viscosity, mass transport, and thrombogenisis. In: Progress in Hemostasis and Thrombosis, Vol 6, (ed.) Spaet, T.H. Grune and Stratton, New York, p.139, 1982.

Turitto, V.T., and Baumgartner, H.R. Platelet interaction with subendothelium in flowing rabbit blood: effect of blood shear rate. Microvasc. Res., 17:38, 1979.

Turitto, V.T., and Baumgartner, H.R. Platelet surface interactions. In: Hemostasis and Thrombosis. Basic principles and clinical practise. (eds.) Coleman, R.W. Hirsh, J., and Marder, V.J., Lippincott, Philadelphia, p.364, 1982.

Turrito, V.T., Muggli, R., and Baumgartner, H.R. Physical factors influencing platelet deposition on subendothelium: importance of blood shear rate. Ann. N.Y. Acad. Sci., 283:284, 1977.

Tyson, J.E., deSa, D.J., and Moore, S. Thromboatheromatous complications of umbilical arterial catheterization in the newborn period. Arch. Dis. Child., 51:744, 1976.

van der Dungen, J.J., Velders, A.J., Karliczek, G.F., Homan van der Heide, N.J., and Wildevuur, Ch.R.H. Platelet

preservation during cardiopulmonary bypass (CPB) with prostaglandin (PGE_1) and prostacyclin (PGI_2). Trans. A. Soc. Artif. Int. Organs, 26:481, 1980.

Vanderhoek, J.Y., Bryant, R.W., and Bailey, J.M. 15-hydroxy-5,8,11,13-eicosatetraenoic acid. A potent and selective inhibitor of platelet lipoxygenase. J. Biol. Chem., 255:5996, 1980.

Van Oost, B.A., Timmermans, A.M.P., and Sixma, J.J. Evidence that platelet density depends on the alpha-granule content in platelets. Blood, 63:482, 1984.

Vehar, G.A., and Davie, E.W. Preparation and properties of bovine factor VIII (antihemophilic factor). Biochemistry, 19:401, 1980.

Venable, J.H., and Coggeshall, R.A. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol., 25:407, 1965.

Vesselinovitch, D., and Fischer-Dzoga, K. Techniques in pathology and atherosclerosis research. Adv. Lipid Res., 18:1, 1981.

Vessilinovitch, D., Getz, G.S., Hughes, R.H., and Wissler, R.W. Atherosclerosis in the rhesus monkey fed three food fats. Atherosclerosis, 20:303, 1974.

Vicic, W.J., and Weiss, H.J. Evidence that platelet alpha-granules are a major determinant of platelet density: studies in storage pool deficiency. Thrombos. Haemostas., 50:878, 1983.

Vigdahl, R.L., Mongin, J.Jr., and Marquis, N.R. Platelet aggregation. IV. Platelet phosphodiesterase and its inhibition by vasodilators. Biochem. Biophys. Res. Commun., 42:1088, 1971.

Vital Statistics of the United States, Vol 2, Part A (DHEW Publication No. (HRA) 76-1102). National Centre for Health Statistics, Rockville Maryland, 1975.

Von Rokitansky, C. Hambuch der Patholischen Anatomie Braumuller. Vienna, Braumuller, Siedel, 1841-1846.

Von Rokitansky, C. A Manual of Pathological Anatomy. Translated by G.E. Day, London, p.261, 1852.

Wagner, W.D., St. Clair, R.W., Clarkson, T.B., and Connor, J.R. A study of atherosclerosis regression in Macaca

mulatta. III. Chemical changes in arteries from animals with atherosclerosis induced for 19 months and regressed for 48 months at plasma cholesterol concentrations of 300 or 200 mg/dl. Am. J. Pathol., 100:633, 1980.

Walker, F.J., Sexton, P.W., and Esmon, C.T. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. Biochem. Biophys. Acta., 571:333, 1979.

Walker, I.D., Davidson, J.F., Faichney, A., Wheatly, D.J., and Davidson, K.G. A double blind study of prostacyclin in cardiopulmonary bypass surgery. Br. J. Haematol., 49:415, 1981.

Walker, W.J. Changing United States life-style and declining vascular mortality: cause or coincidence? N. Engl. J. Med., 297:163, 1977.

Wall, R.T., Harlan, J.M., Harker, L.A., and Striker, G.E. Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. Thromb. Res., 18:113, 1980.

Walsh, P.N. The effects of collagen and kaolin on the intrinsic coagulant activity of platelets. Evidence for an alternative pathway in intrinsic coagulation not requiring factor XII. Br. J. Haematol., 2:393, 1972.

Walsh, P.N. Platelets and coagulation proteins. Fed. Proc., 40:2086, 1981.

Walsh, P.N., and Briggs, R. The role of platelets in intrinsic factor-Xa formation. Br. J. Haematol., 22:743, 1972.

Walsh, P.N., and Griffin, J.H. Contributions of human platelets to the proteolytic activation of blood coagulation factors XII and XI. Blood, 57:106, 1981.

Weber, G., Fabbrini, P., and Resi, L. Scanning and transmission microscopy observations on the surface lining of aortic intimal plaques in rabbits on hypercholesterolemic diet. Virchows Arch. (Pathol. Anat.), 364:325, 1974.

Wechezak, A.R., Mansfield, P.B., and Way, S.A. Platelet interaction with cultured endothelial cells following

in vitro injury. Artery, 1:507, 1975.

Weiss, H.J., Tschopp, T.B., Baumgartner, H.R., Sussman, I.I., Johnson, M.M. and Egan, J.J. Decreased adhesion of giant (Bernard-Soulier) platelets to subendothelium. Further implications on the role of the von Willebrand factor in hemostasis. Am. J. Med., 57:920, 1974.

Weiss, H.J., Tschopp, T.B., and Baumgartner, H.R. Impaired interaction (adhesion-aggregation) of platelets with the subendothelium in storage-pool disease and after aspirin ingestion. N. Engl. J. Med., 293:619, 1975.

Weiss, H.J., Turitto, V.T., and Baumgartner, H.R. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear rate-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. J. Lab. Clin. Med., 92:750, 1978.

Weiss, H.J., and Turitto, V.T. Prostacyclin (prostaglandin I_2) PGI_2) inhibits platelet adhesion and thrombus formation on subendothelium. Blood, 53:244, 1979.

Weksler, B.B. Prostacyclin. In: Progress in Hemostasis and Thrombosis, vol 6, (ed) Spaet, T.H., Grune and Stratton, New York, NY, p.113, 1982.

Weksler, B.B., Gillick, M., and Pink, J. Effect of propanolol on platelet function. Blood, 49:185, 1977.

Weksler, B.B., Ley, C.W., and Jaffe, E.A. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and the ionophore A 23187. J. Clin. Invest., 62:923, 1978.

Weksler, B.B., Marcus, A.J., and Jaffe, E.A. Synthesis of prostaglandin I_2 (prostacyclin) by cultured human and bovine endothelial cells. Proc. Natl. Acad. Sci. USA, 74:3922, 1977.

Weston, M.J., Woods, H.F., Ash, G., Bunting, S., Moncada, S., and Vane, J.R. Prostacyclin as an alternative to heparin for hemodialysis in dogs. In: Prostacyclin. (eds.) Vane, J.R., and Bergstrom, S., Raven Press, New Yrok, p.349, 1979.

White, J.G. Effect of colchicine and vinca alkaloids on human platelets. III. Influence on primary internal contraction and secondary aggregation. Am. J.

Pathol., 54:467, 1969.

White, J.G. Platelet microtubules and microfilaments: Effects of cytochalasin B on structure and function. In: Platelet Aggregation. (ed.) Caen, J., Mason et Cie, Paris, p.15, 1971.

White, J.G. Platelet morphology. In: The circulating Platelet. (ed.) Johnson, S.A., Academic Press, New York, p.45, 1971.

White, J.G., Shape change. Thromb. Diath. Haemorrh., 60(Suppl.):159, 1974.

White, J.G., Clawson, C.C., and Gerrard, J.M. Platelet ultrastructure. In: Haemostasis and Thrombosis. (eds.) Bloom, A.L., and Thomas, D.P., Churchill Livingston, New York, p.2, 1981.

Wight, T.N. Differences in the synthesis and secretion of sulfated glycosaminoglycans by aorta explant monolayers cultured from atherosclerosis-susceptible and -resistant pigeons. Am. J. Pathol., 101:127, 1980.

Wilkins, R.M. Von Willebrand platelet-vessel wall interactions. M.Sc. dissertation:1-100. Wayne State University, Detroit, Michigan, (referred to in Barnhart et al., Ann. N.Y. Acad. Sci., 370:154, 1981.)

Williams, W.J., and Norris, D.G. Purification of a bovine protein (Factor VII) which is required for the activity of lung microsomes in blood coagulation. J. Biol. Chem., 241:1847, 1966.

Wilner, G.D., Nossel, H.L., and LeRoy, E.C. Aggregation of of platelets by collagen: polar active sites of insoluble human collagen. Am. J. Physiol., 220:1074, 1971.

Winocour, P.D., Cattaneo, M., Somers, D., Richardson, M. Kinlough-Rathbone, R.L., and Mustard, J.F, Platelet survival and thrombosis. Arteriosclerosis, 2:458, 1982.

Winocour, P.D., Kinlough-Rathbone, R.L., and Mustard, P.D. Effect of the phospholipae A_2 inhibitor mepacrine on aggregation of washed rabbit platelets. Fed. Proc., 38:1271, 1979.

Winocour, P.D., Kinlough-Rathbone, R.L., Richardson, M. and

Mustard, J.F. Reversal of shortened platelet survival in rats by the antifibrinolytic agent, epsilon aminocaproic acid. J. Clin. Invest., 71:159, 1983.

Wissler, R.W., and Vesselinovitch, D. Evidence for prevention and regression of atherosclerosis in man and experimental animals at the arterial level. Proc. IIIrd International Symposium on Atherosclerosis III. (eds.) Schettler, G., and Weizel, A. Berlin, Springer, New York, p. 747, 1973.

Wolfe, S.M., and Schulman, N.R. Adenylate cyclase activity in human platelets. Biochem. Biophys. Res. Commun., 35:265, 1969.

Wong, A.J., Pollard, T.D., and Herman, I.M. Actin filament stress fibers in vascular endothelial cells in vivo. Science, 219:869, 1983.

Wong, P.Y.-K., and Cheung, W.Y. Calmodulin stimulates human platelet phospholipase A_2. Biochim. Biophys. Res. Commu., 90:473, 1979.

Woods, H.F., Ash, G., Weston, M.J., Bunting, S., Moncada, S., and Vane, J.R. Prostacyclin can replace heparin in haemodialysis in dogs. Lancet, 2:1075, 1978.

Woolf, N., Bradley, J.W.P., Crawford, T., and Carstairs, K.C. Experimental mural thrombi in the pig aorta. The early natural history. Br. J. Exp. Pathol., 49:22, 1968.

Wu, K.K., Chen, Y.-C., Fordham, E., Ts'ao, C.-H., Rayudu, G., and Matayoshi, D. Differential effects of two doses of aspirin on platelet-vessel wall interaction in vivo. J. Clin. Invest., 68:382, 1981.

Zetter, B.R., Johnson, L.K., Shuman, M.A., and Gospodarowicz, D. The isolation of vascular endothelial cell lines with altered cell surface and platelet-binding properties. Cell, 14:501, 1978.

Zimmerman, T.S., Ratnoff, O.D., and Powell, A.E. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. J. Clin. Invest., 50:244, 1971.

Zahler, P. Blood group antigens in relation to chemical and structural properties of the red cell membrane. Vox. Sang., 15:81, 1968.

Zucker, M.B. Session II- Biological aspects of heparin action. Heparin and platelet function. Fed. Proc., 36:47, 1977.

Zucker, M.B., and Borrelli, J. Platelet clumping produced by connective tissue suspensions and by collagen. Proc. Soc. Exp. Biol. Med., 109:779, 1962.

Zucker, M.B., Mosesson, M.W., Broekman, M.J., and Kaplan, K.L. Release of platelet fibronectin (cold-insoluble globulin) from alpha granules induced by thrombin or collagen; lack of requirement for plasma fibronectin in ADP-induced platelet aggregation. Blood, 54:8, 1979.

Zusman, R.M., Rubin, R.H., Cato, A.E., Cocchetto, D.M., Crow, J.W., and Tolkoff-Rubin, N. Hemodialysis using prostacyclin instead of heparin as the sole antithrombotic agent. N. Engl. J. Med., 304:934, 1981.

Zwaal, R.F.A. Membrane and lipid involvement in blood coagulation. Biochim. Biophys. Acta., 515:163, 1978.

APPENDIX I

PUBLICATIONS

During the period when these investigations were carried out several papers on the results of the studies were published. Papers on work presented in the following chapters include:

Chapter III

Packham, M.A., Kinlough-Rathbone, R.L., Cazenave, J.-P., Groves, H.M., Dejana, E., and Mustard, J.F. The interaction of platelets with the vessel wall. In: "Perspectives in Hemostasis", Fareed, J., Mesmore, J.W., Fenton, J.W., and Brinkhouse, K.M. (eds.). Permagon Press, New York, pp.90-103, 1981.

Groves, H.M., Kinlough-Rathbone, R.L., Richardson, M., Jørgensen, L., Moore, S., and Mustard, J.F. Thrombin generation and fibrin formation following injury to rabbit neointima: studies of vessel wall reactivity and platelet survival. Lab. Invest., 46:605, 1982.

Richardson, M., Kinlough-Rathbone, R.L., Groves, H.M., Jørgensen, L., Mustard, J.F. and Moore, S. Ultrastructural changes in the re-endothelialized and non-endothelialized rabbit aortic neointima following reinjury with a balloon catheter. Br. J. Exp. Pathol., 65:597, 1984.

Chapter IV

Dejana, E., Cazenave, J.-P., Groves, H.M., Kinlough-Rathbone, R.L., Richardson, M., Packham, M.A., and Mustard, J.F. The effect of aspirin inhibition of PGI_2 production on platelet adherence to normal and damaged rabbit aortae. Thrombos. Res., 17:453, 1980.

Groves, H.M., Kinlough-Rathbone, R.L., Cazenave, J.-P., Dejana, E., Richardson, M., and Mustard, J.F. Effect of dipyridamole and prostacyclin on rabbit platelet adherence in vitro and in vivo. J. Lab. Clin. Med., 99:548, 1982.

Dejana, E., Cazenave, J.-P., Hatton, M.W.C., Richardson, M., Groves, H.M., Kinlough-Rathbone, R.L., Packham, M.A., and Mustard, J.F. The effect of thrombin on platelet accumulation on the vessel wall: influence of heparin and aspirin. Thrombos. Haemostas., 50:567, 1983.

Chapter V

Groves, H.M., Kinlough-Rathbone, R.L., and Mustard, J.F. Development of nonthrombogenicity of rabbit aortas despite inhibition of platelet adherence. Arteriosclerosis, 6:189, 1986.

ABSTRACTS

In addition to these publications the following abstracts were published and presented at national and international meetings and congresses.

Chapter III

Kinlough-Rathbone, R.L., Groves, H.M., Maric, S., Packham, M.A., and Mustard, J.F. Quantitation of platelet adherence to rabbit aortae and platelet survival after two injuries with a balloon catheter. Thrombos. Haemostas., 42:92, 1979.

Somers, D., Kinlough-Rathbone, R.L., Groves, H.M., Packham, M.A., and Mustard, J.F. Platelet incorporation into thrombi and platelet survival in rabbits. Fed. Proc., 39:629, 1980.

Kinlough-Rathbone, R.L., Groves, H.M., Jorgensen, L., Richardson, M., Moore, S., Packham, M.A., and Mustard, J.F. The role of thrombin in the response of platelets to injury of the rabbit aorta. Clin. Res., 28:528A, 1980.

Groves, H.M., Kinlough-Rathbone, R.L., Jorgensen, L., Richardson, M., Moore, S., and Mustard, J.F. The role of thrombin and fibrin in platelet interaction with re-injured rabbit aortae. Circulation, 61:III-273, 1980.

Chapter IV

Groves, H.M., Kinlough-Rathbone, R.L., and Mustard, J.F. Comparison of the effects of aspirin and heparin on platelet accumulation on the injured neointima of rabbit aortae. Thrombos. Haemostas., 46:200, 1981.

Chapter V

Groves, H.M., Kinlough-Rathbone, R.L., and Mustard, J.F. Effect on vessel wall reactivity of inhibiting platelet accumulation with dipyridamole. Circulation, 66:II-53, 1982.

Kinlough-Rathbone, R.L., Groves, H.M., and Mustard, J.F. Factors influencing vessel wall reactivity following injury. Thrombos. Haemostas., 50:283, 1983.

Groves, H.M., Kinlough-Rathbone, R.L., and Mustard, J.F. Lack of reactivity of injured rabbit aortae following inhibition of platelet accumulation by PGI_2. Circulation, 70:93, 1984.