THE USE OF THROMBIN INHIBITORS TO EXAMINE THE ROLE OF THROMBIN AS A MEDIATOR OF (a) REOCCLUSION AFTER SUCCESSFUL THROMBOLYSIS, AND (b) SMOOTH MUSCLE HYPERPLASIA AFTER VESSEL WALL INJURY IN ANIMAL MODEL SYSTEMS

BY

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ABSTRACT

Using antithrombin III-dependent and independent thrombin inhibitors, we evaluated the role of thrombin in (a) reocclusion after successful thrombolysis, and (b) intimal thickening and stenosis induced by injury to a vessel wall. To accomplish this, two animal models were developed in the rat. The first was a model of tissue plasminogen activator (tPA) induced-thrombolysis, whereas the second was a carotid model of injury-induced stenosis.

In the rat model of tPA-induced thrombolysis, we compared the effects of heparin, hirudin, hirulog (a synthetic hirudin-derived peptide), and D-Phe-Pro-ArgCH_2Cl (PPACK) in doses that produced a 4-fold prolongation of the baseline activated partial thromboplastin time (APTT) with saline, in terms of their ability to accelerate thrombolysis and to prevent reocclusion. A thrombus rich in red cells and fibrin was formed in the distal aorta by a combination of vessel injury and stasis. Thrombolysis was induced with tPA (1 mg/kg bolus, followed by 1 mg/kg/hr over 30 min.) and the rats were randomized to receive a concomitant 80 min. infusion of a thrombin inhibitor or saline. Blood flow and pre- and post-stenotic blood pressures were monitored continuously, and the time to clot lysis, the duration of vessel patency, and the number of reocclusions were determined. Compared to saline, heparin had no significant effect on these variables. In contrast, hirudin, hirulog and PPACK significantly ($p<0.01$) increased the percentage of time that the vessel remained patent from 63.9 ± 7.7 to 90.7 ± 2.2, 94.0 ± 0.9, and 94.7 ± 1.0% respectively, by significantly ($p<0.01$) decreasing the number of reocclusions. The superiority of the ATIII-independent inhibitors over heparin supports the hypothesis that the limited effectiveness of heparin in this setting reflects its inability to inactivate clot-bound thrombin. Compared to saline,

hirulog and PPACK also significantly ($p<0.02$) accelerated the time to thrombolysis from 10.5 ± 2.3 to 4.4 ± 0.6, and 4.2 ± 0.8 min., respectively, whereas heparin and hirudin did not. The ability of the lower molecular weight inhibitors of thrombin to accelerate lysis may reflect their greater accessibility to clot-bound thrombin.

Hirudin, standard heparin, and low molecular weight heparin were compared with saline in terms of their ability to prevent injury-induced stenosis in the rat common carotid artery. After balloon injury, animals were randomized to receive a one week course of subcutaneous treatment with a thrombin inhibitor or saline. All of the thrombin inhibitors were given in concentrations that produced a continuous 6-fold prolongation of the baseline thrombin clotting times, and 2-fold prolongation of the APTT. Two weeks after injury, the animals were euthanized and the extent of stenosis was assessed by a combination of quantitative angiography, resin casting, and planimetry.

Compared to saline, only low molecular weight heparin significantly ($p<0.01$) inhibited injury-induced stenosis as assessed by angiography (from 22.9 ± 3.0% to 8.8 ± 7.8%), resin casting (from 46.7 ± 4.8% to 22.5 ± 8.3%), and by planimetry (from 21.8 ± 3.4% to 11.3 ± 2.1%).

These findings suggest that the inhibitory effects of low molecular weight heparin are independent of its anticoagulant activity and do not reflect its ability to inhibit thrombin. Instead, low molecular weight heparin may compete with surface-bound heparan sulfate for degradation by heparitinase released from activated platelets at sites of vascular injury. By maintaining the levels of heparan sulfate on smooth muscle cells, low molecular weight heparin has an antiproliferative effect because heparan sulfate keeps these cells in a resting,

nonsynthetic state. Although standard heparin can also compete with heparan sulfate for heparitinase, we used considerable lower concentrations of standard heparin than low molecular weight heparin which may explain its failure to block stenosis.

In summary, whereas thrombin appears to play a pivotal role in rethrombosis after thrombolysis, our results suggest that it is not a major mediator of injury-induced stenosis.

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I dedicate this work to my wife, Giannoula, and my children, Nicole and Christos. I am grateful to them for their care, love, and patience during the preparation of this thesis.

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ABBREVIATIONS

Ao.....	Area of lumen
A_1.....	Area of intima
A_2.....	Area of media
ACE.....	Angiotension converting enzyme
ADP.....	Adenosine diphosphate
AGII.....	Angiotensin II
AMI.....	Acute myocardial infarction
ANOVA.....	Analysis of variance
APC.....	Activator of protein C pathway
APTT.....	Activated partial thromboplastin time
ATA.....	Aurin tricarboxylic acid
ATS.....	Leech anticoagulant peptide (Antistasin)
ATIII.....	Antithrombin III
AV.....	Arterio-venous
B.....	Boundary perimeter
bFGF.....	Basic fibroblast growth factor
beta-TGF....	Beta transforming growth factor
CyA.....	Cyclosporin A
DIC.....	Disseminated intravascular coagulation
DS.....	Dermatan sulfate

DNA.....	Deoxyribonucleic acid
EDRF.....	Endothelium derived relaxing factor
ePTFE.....	Expanded polytetrafluoroethylene
EGF.....	Epidermal growth factor
E.E.L.....	External elastic lamina
FPA.....	Fibrinopeptide A
GAGs.....	Glycosaminoglycans
GISSI-2.....	Gruppo italiano per lo studio della streptochinasi nell' infarcto miocardico
GP.....	Glycoprotein
HDL.....	High density lipoprotein
HMWK.....	High molecular weight kininogen
inj.....	Injured
I.E.L.....	Internal elastic lamina
ISIS-3.....	Third International Study of Infarct Survival Collaborative Group
kV.....	Kilovolt
LDL.....	Low density lipoprotein
Lmwh.....	Low molecular weigh heparin
mg.....	Milligram
mm.....	Millimetre
µm.....	Micrometer
P.....	Perimeter
PDGF.....	Platelet derived growth factor

PGI_2.....	Prostaglandin I_2 (prostacyclin)
PE#.....	Polyethylene tubing #
PPACK.....	D-phenylalanyl-L-propyl-L-arginyl Chloromethyl Ketone
PTCA.....	Percutaneous transluminal coronary angioplasty
%.....	Percentage
π.....	Pi (3.14)
r.....	Radius or recombinant depending on context
RES.....	Reticuloendothelial system
RGD.....	Cysteine rich single chain polypeptide
RNA.....	Ribonucleic acid
SE.....	Standard error
SK.....	Streptokinase
stdh.....	Standard heparin
TAP.....	Tick anticoagulant peptide
TCT.....	Thrombin clotting time
tPA.....	Tissue plasminogen activator
TPI.....	Tissue factor pathway inhibitor
TRAPs.....	Thrombin receptor antagonist peptides
TXA_2.....	Thromboxane A_2
unj.....	Uninjured
UK.....	Urokinase
VLDL.....	Very low density lipoprotein

VSMC.....	Vascular smooth muscle cell
vWf.....	Von Willebrand factor
b.wt.....	Body weight
wt.....	Weight
II.....	Prothrombin
IIa.....	Thrombin
Va.....	Activated factor V
VII.....	Factor VII
VIIIa.....	Activated factor VIII
IX.....	Factor IX
Xa.....	Activated factor X
XI.....	Factor XI
XII.....	Factor XII
XIII.....	Factor XIII

CHAPTER 1. GENERAL INTRODUCTION

1.1 Reocclusion of the Coronary Artery following (A) Thrombolytic Therapy in Acute Myocardial Infarction (AMI) and (B) Percutaneous Transluminal Coronary Angioplasty (PTCA)

Acute myocardial infarction (AMI) is the major cause of death in Western societies. In a pivotal study, DeWood and associates (DeWood 1980) demonstrated that AMI is usually caused by a coronary thrombus which occludes the infarct-related artery. The occlusive thrombus usually occurs as a consequence of activation of the coagulation system and platelets by exposure of blood to contents of the ruptured atherosclerotic plaque which is rich in tissue factor and other thrombogenic material (Weiss 1989; Wilcox 1989; Zeldis 1972). Occlusive thrombosis in this setting occurs as a consequence of activation of these blood constituents in combination with altered blood flow as a result of vascular narrowing (DeWood 1980).

The goal of treatment of patients with AMI is to relieve the obstruction in the infarct-related coronary artery as rapidly as possible to prevent the sequelae of irreversible ischemia (GISSI 1986). If rapid patency is achieved and maintained, there is improvement in left ventricular function (Magnani 1989; White 1989) and in short-term (Ohman 1990; Hanson 1991; GISSI-2 1990; ISIS-2 1988) and long-term (ISIS-3 1992; The TIMI Research Group 1988) mortality.

The occluded coronary artery can be opened pharmacologically using thrombolytic agents (Hanson 1991; ISIS-3 1992; Magnani 1989; White 1989). Residual stenosis can also

be relieved mechanically using percutaneous transluminal coronary angioplasty (PTCA) (Hanson 1992). Although both techniques rapidly restore blood flow, their ultimate success depends on maintaining patency of the re-opened artery. Acute reocclusion can occur after patency is achieved by either pharmacologic (Ohman 1990; Ellis 1989; The TIMI Study Group 1985; Topol 1987) or mechanical methods (Hanson 1992), thereby limiting the success of these procedures. In addition, mechanical dilatation of the narrowed arterial lumen is complicated by re-stenosis which occurs in 30% to 40% of cases within 3 to 6 months of the procedure, thereby limiting the long-term success of this treatment (Liu 1988; Leimgruber 1986; Mata 1985).

There is mounting evidence that thrombin plays a major role in both acute and chronic reocclusion after pharmacologic thrombolysis and PTCA (Weitz 1990; Bar-Shavit 1989; Bar-Shavit 1990; Wilcox 1992; Fuster 1992; Fuster 1992; Ip 1991). Thrombin is bound to fibrin in the mural thrombus (Weitz 1990) or to exposed matrix proteins in the ruptured atherosclerotic plaque (Bar-Shavit 1989) after thrombolysis or PTCA. This bound thrombin remains enzymatically active and is protected from inactivation by blood-based thrombin inhibitors. As a result, the surface-bound thrombin can locally amplify coagulation by activating platelets and factors V and VIII, thereby promoting acute rethrombosis (Fuster 1992).

Re-stenosis after PTCA may also be mediated by thrombin (Bar-Shavit 1990; Wilcox 1992; Fuster 1992; Fuster 1992; Ip 1991). Injury to the intima and media caused by PTCA triggers a complex sequence of events, including vascular smooth muscle cell (VSMC) proliferation, which results in intimal thickening and progressive stenosis (Fuster 1992; Ip

1991). After deep vessel wall injury, thrombin binds to the exposed protein matrix and to overlying fibrin (Bar-Shavit 1989; Bar-Shavit 1990; Wilcox 1992). Thrombin bound to these sites is protected from inactivation by its inhibitors and remains mitogenic for VSMCs (Bar-Shavit 1990; Wilcox 1992; McNamara 1992). It is likely that its mitogenic activity is mediated by binding to the thrombin receptor on VSMCs (Bar-Shavit 1990; Wilcox 1992; McNamara 1992). In both rat and baboon experimental models, expression of the thrombin receptor on VSMCs (Wilcox 1992) is increased after PTCA and treatment with thrombin inhibitors can prevent or limit the development of late re-stenosis after PTCA (Clowes 1985; Clowes 1986; Hanson 1992; Sarembock 1991).

Thrombin bound to fibrin (Weitz 1990) or to the extracellular matrix (Bar-Shavit 1989) is protected from inactivation by heparin-ATIII. This may explain why heparin is limited in its ability to prevent both acute reocclusion after thrombolytic therapy (Ohman 1990; Ellis 1989; The TIMI Study Group 1985; Topol 1987) or late re-stenosis after PTCA (Ellis 1989). Although resistant to inactivation by heparin, surface-bound thrombin is inhibited by ATIII-independent inhibitors such as hirudin and D-Phe-Pro-ArgCH_2Cl (Weitz 1990). These findings raise the possibility that the ATIII-independent inhibitors might be more effective than heparin in preventing acute reocclusion after thrombolytic therapy and PTCA and in preventing late re-stenosis after PTCA. To test this possibility, we developed animal models of thrombolysis and angioplasty in rats, and compared ATIII-dependent (heparin and lmwh) with ATIII-independent inhibitors (hirudin, hirudin fragments and D-Phe-Pro-ArgCH_2Cl) in terms of their ability to prevent acute reocclusion and late re-stenosis.

1.2 Mechanism of Acute Reocclusion after Successful Thrombolysis

The thrombogenicity of a ruptured atherosclerotic coronary artery is affected by several factors, including: the degree of plaque disruption and extent of exposure of blood to thrombogenic substances such as collagen and tissue factor, the severity of stenosis, and disturbed blood flow, all of which contribute to activation of coagulation and platelets (Fuster 1992; Fuster 1992). After a mural thrombus forms it perpetuates the thrombogenic process because it contains active thrombin which is protected from inactivation by fluid-phase inhibitors (Weitz 1990). A variety of systemic risk factors also contribute to thrombogenesis (Fuster 1992; Fuster 1992). These include: high circulating levels of epinephrine induced by stress or smoking; increased levels of cholesterol and low density lipoprotein (LDL) which result in the impaired release of endothelial-derived relaxing factor (EDRF); increased concentrations of type 1 plasminogen-activator inhibitor (PAI 1) which inhibits fibrinolysis; and increased levels of fibrinogen or factor VII which may contribute to accelerated clotting. Several factors are considered to be important in the pathogenesis of rethrombosis after successful thrombolysis in patients with AMI (Fuster 1992; Fuster 1992). First, the residual mural thrombus narrows the vessel lumen causing increased shear stresses which in turn facilitate the local activation and deposition of platelets (Badimon 1989; Lassila 1990). The mural thrombus also provides a powerful thrombogenic surface because thrombin that is bound to fibrin within the clot is enzymatically active and protected from inactivation by the heparin-ATIII complex. This would explain the experimental observation that heparin is limited in its ability to inhibit thrombus growth. In contrast, direct thrombin inhibitors (i.e. hirudin, hirudin fragments and PPACK) may be better than

heparin in certain clinical settings because, unlike heparin, these agents can access and inactivate clot-bound thrombin (Weitz 1990). Second, thrombolytic agents may themselves contribute to rethrombosis by enhancing platelet activation and stimulating thrombin formation (Owen 1988; Fitzgerald 1988).

Based on these considerations thrombin appears to play an important role in reocclusion after pharmacologic thrombolysis, and its inactivation by effective thrombin inhibitors may prevent this event. This concept is supported by recent clinical studies which have demonstrated that hirudin or hirulog are better than heparin at accelerating thrombolysis and preventing reocclusion when used in conjunction with t-PA or streptokinase (TIMI 5 Trial Group 1993, Lidon, 1993).

1.3 Mechanism of Re-Stenosis after PTCA

Since its first introduction by Gruentzig in 1977, PTCA has gained wide acceptance as an alternative to surgical revascularization in selected patients with symptomatic coronary artery disease (Gruentzig 1977). Compared to surgical revascularization (aorto-coronary bypass), PTCA is not as invasive, has high patient acceptance, costs less and is relatively easier to perform. More than 90% of stenotic lesions can be successfully dilated with low (2-5%) risk of acute thrombosis (Detre 1988). However, the procedure is limited by re-stenosis which occurs in 30 to 40% of patients within 6 months (Leimgruber 1986; Mata 1985). Human (Lee 1980; Block 1981; Waller 1983) and animal (Faxon 1982; Block 1980) studies have demonstrated that PTCA causes stretching of the stenotic lesion, splitting of the plaque, endothelial desquamation, and dissection into the media. As a result, there is

localized vasoconstriction, and subsequent platelet adhesion and aggregation which can lead to thrombus formation (Chesebro 1987). The reasons why restenosis occurs in only 30 to 40% of patients after PTCA are not known (Detre 1988).

VSMC growth is regulated by a large array of biologic agents including: platelet derived growth factor (PDGF), endothelial cell growth factor (ECGF), fibroblast growth factor (FGF), smooth muscle cell derived growth factor (SMCDGF), interleukins-1 and -6, transforming growth factor beta (TGF-beta), low density lipoproteins (LDL), angiotensin II (AGII), epinephrine, norepinephrine, serotonin, neuropeptide substances P and K, endothelins, thrombin, leukotrines B_4, C_4, and D_4 interacting with growth inhibitors such as transforming growth factor beta (TGF-beta), heparin-like factors, EDRF, prostaglandin E_2, prostacyclin, and interferon gamma. These mediators are released by a variety of cells such as platelets, macrophages, fibroblasts, VSMCs, and lymphocytes (Fuster 1992; Fuster 1992; Ip 1991).

Thrombin, which is generated at the site of angioplasty is a potent mitogen for VSMCs (Bar-Shavit 1990; Wilcox 1992; McNamara 1992) and fibroblasts (Carney 1986; Glenn 1980; Perdue 1981). Given the pivotal role of thrombin in coagulation, the role of thrombin in the regulation of mitogenesis and vascular tone, and the fact that thrombin bound to fibrin or subendothelial matrix is protected from inactivation by heparin but not by the direct thrombin inhibitors, it is reasonable to hypothesize that ATIII-independent thrombin inhibitors (such as hirudin and PPACK) may be better than heparin at reducing or preventing post-PTCA re-stenosis.

1.4 VSMC Phenotypic Modulation and Its Role in Post-PTCA Re-Stenosis

Normal VSMCs exist in a growth-arrested "quiescent" state, which is associated with low levels of growth factors and a high content of contractile proteins (i.e. alpha-actin, myosin) (Owens 1992). Following vascular injury (such as that caused by PTCA), alterations in gene expression (e.g. c-myc) take place, resulting in conversion of VSMCs from a contractile to a synthetic (excretory) phenotype characterized by an increase in excretory organelles (Golgi apparatus, endoplasmic reticulum, ribosomes) and a reduction of contractile proteins (Owens 1992). These stimulated smooth muscle cells migrate from the media into the intima where they proliferate and produce extracellular matrix. This, in turn, leads to the formation of a neointima which narrows the lumen of the vessel. Analysis of atherosclerotic plaques has revealed increased concentrations of PDGF and PDGF-like molecules (Libby 1988; Wilcox 1988). Since PDGF is produced by VSMC, these findings suggest that the VSMCs in plaques are in a synthetic stage (Simons 1993).

1.5 VSMC Proliferation

Angioplasty produces moderate to severe vessel wall injury which stimulates VSMC migration and proliferation (Roubin 1988). Within a few minutes of injury, platelets and white cells adhere to the vascular subendothelium (Stemerman 1973) and release (Libby 1988; Gerrity 1981) mitogens and mitogenic promoters such as PDGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), serotonin, prostanoids (such as thromboxane A_2), endothelins, and other biologically active molecules (i.e. leukotrines) (Fuster 1992). In addition, thrombin is generated on the surface of activated cells such as

platelets and endothelial cells. Furthermore, white cells (circulating and/or in the atherosclerotic plaque) (Liebovich 1976) also release mitogens such as macrophage-derived growth factor, interleukin-1 (Libby 1988), cyclooxygenase, lipoxygenase pathway products and oxygen-derived free radicals (Rao 1990) which also stimulate VSMC growth.

1.6 VSMC Migration

Following balloon injury to the rat carotid artery, up to 50% of VSMCs migrate from the media into the intima within 2 weeks (Clowes 1986; Clowes 1985). Migrating VSMCs exhibit a secretory phenotype. The precise mechanism of VSMC phenotypic modulation and migration is not understood; however, PDGF has been implicated in both processes (Owens 1992).

1.7 Hypotheses

We hypothesized that: a) thrombin is a major mediator of reocclusion after thrombolysis and triggers re-stenosis following PTCA; b) inactivation of thrombin will prevent both of these complications; and c) ATIII-independent inhibitors will be better then heparin at blocking these processes.

To test these hypotheses we developed two rat models: (a) a model of tPA-induced thrombolysis in the aorta, and (b) a model of balloon injury-induced stenosis in the common carotid artery.

CHAPTER 2. BACKGROUND ON THROMBIN

2.1 Thrombin Structure

Thrombin is a serine protease, that cleaves peptide bonds at arginine residues. The clotting enzyme is generated from its precursor prothrombin, a plasma protein synthesized in the liver in a vitamin K-dependent process. Human prothrombin has three carbohydrate side chains, contains 308 amino acid residues, and has a half-life time of 3 days. The plasma concentration of prothrombin is approximately 1.4 μM, and about 10 % is converted to alpha-thrombin during blood coagulation (Fenton 1988).

Factor Xa first cleaves prothrombin at residue 49, forming a two-chain, highly unstable enzyme composed of an alpha-chain (49 residues) and a beta-chain (259 residues), linked by a single disulphide bond. This enzyme recognizes its own alpha-chain as a substrate for further cleavage at the arginine-13 position. The stable human alpha-thrombin consists of only 36 residues in the alpha-chain and 259 residues in the beta-chain (Downing 1975). The chains are connected by a disulphide bridge (Fenton 1977; Gorman 1976).

Thrombin (which is also known as α-thrombin) undergoes autoproteolytic cleavage, thereby forming beta- and gamma-thrombin, both of which have markedly reduced clotting activity because they are missing an exosite essential for fibrinogen recognition (Sonder 1986). Thrombin can also be cleaved by elastase or cathepsin, enzymes released from activated neutrophils, to produce epsilon and zeta forms with markedly reduced clotting activity (Brover 1987). Although these modified thrombins have decreased clotting activity, they retain mitogenic activity and may play a role in a variety of post-thrombotic events (Fenton 1988).

2.2 Thrombin Function in Coagulation and Mitogenesis

Thrombin converts fibrinogen to fibrin and activates factor XIII which then crosslinks the fibrin to form a stable clot (Fenton 1988; Fuster 1992). In addition, thrombin also amplifies coagulation by activating platelets and factors V and VIII (Fuster 1992).

Thrombin is regulated by a number of fluid-phase antiproteinases including ATIII, heparin cofactor II, and α_2-macroglobulin. In addition, thrombin also is inhibited by binding to thrombomodulin, a receptor on the endothelial cell surface (Fenton 1988). Once bound to thrombomodulin, thrombin undergoes a conformational change which converts the enzyme from a procoagulant to anticoagulant. Thus, when bound to thrombomodulin, thrombin loses its ability to convert fibrinogen to fibrin and to activate platelets, factor V and VIII. Instead, it acquires anticoagulant activity by increasing its ability to activate protein C, which together with protein S (its cofactor) acts as an anticoagulant by inactivating factors Va and VIIIa (Fenton 1988, Hanson 1993).

Thrombin also has a potential role in the regulation of vascular tone by stimulating synthesis and release of EDRF (Ignarro 1989; Furchgott 1983; Vanhoutte 1989) and the vasoconstricting endothelins (Moon 1989; Inoue 1989; Yanagisawa 1988; Sakurai 1990; Anggard 1990; Spinella 1991) from endothelial cells, and thromboxane A_2 from activated platelets (Coller 1990). Thrombin generated locally may be responsible for the vasoconstriction that occurs in denuded dog coronary arteries (Brum 1984) and atherosclerotic coronary arteries in miniature swine (Shimokawa 1983). Thrombin alone or in conjunction with other growth factors has been reported to stimulate VSMC proliferation (Carney 1986; Bar-Shavit 1990; Wilcox 1992) and the proliferation of chick embryo

fibroblasts in serum-free culture media (Chen 1975; Carney 1978). Thrombin also interacts with platelet membrane glycoprotein Ib (GPIb) (Hirsh 1993), although it is now clear that GPIb is not the platelet thrombin receptor.

The interaction of thrombin with components of the complement system has not yet been well established. There is some evidence that thrombin cleaves both C_3 and C_5 (Hugh 1977) and that the cleaved C_5 fragment is chemotactic for polymorphonuclear leukocytes.

2.3.1 Regulation of Free Thrombin in Circulation

Thrombin generated from prothrombin during blood coagulation is inactivated by a number of parallel inhibitory mechanisms (Figure 1) (Fuster 1992). Free thrombin is complexed and inactivated by ATIII, heparin cofactor II, alpha$_2$-macroglobulin, and alpha$_1$-antitrypsin. Binding to ATIII, heparin cofactor II, and alpha$_1$-antitrypsin is through covalent complexes, while binding to alpha$_2$-macroglobulin is non-covalent (Lanchantin 1966; Matheson 1976; Rosenberg 1973; Lollar 1980). ATIII is the most important inhibitor of thrombin (Hirsh 1993; Lollar 1980), while the other thrombin inhibitors play a relatively more minor role. However, there is evidence that ATIII is less effective at inhibiting thrombin that is bound to fibrin than free thrombin (Weitz 1990).

The presence of heparan sulphate on the endothelial cell surface further accelerates the inhibitory activity of ATIII (Lollar 1980). Thrombin also interacts with thrombomodulin located on the endothelial cell surface resulting not only in its inactivation (Esmon 1981) but also its enhanced ability to active protein C, which together with protein S (its cofactor), proteolytically inactivates factors Va and VIIIa (Hanson 1993).

2.3.2 Regulation of Thrombin Bound to Fibrin or Subendothelial Matrix

Although Seegers (Seegers 1945) demonstrated that thrombin binds to fibrin many years ago, confirmation of this phenomenon was delayed until 1979 (Liu 1979). More recent studies indicate that thrombin binds to fibrin through a site distinct from its catalytic centre (Kaminsky 1987; Kaminski 1983; Berliner 1985). Clot-bound thrombin has been shown to be protected from fluid-phase inhibitors (Weitz 1990; Hogg 1989). In buffer systems, the ATIII-heparin complex inactivates thrombin bound to fibrin monomer 300-fold less than free thrombin (Hogg 1989). More recently, Weitz et al. (Weitz 1990) have demonstrated that in a plasma system, thrombin bound to fibrin is enzymatically active despite the presence of physiologic concentrations of antithrombins, and that clot-bound thrombin is not inactivated by heparin-ATIII, but is sensitive to inactivation by antithrombin ATIII-independent thrombin inhibitors such as hirudin, hirugen, hirulog, or PPACK.

In addition to fibrin, thrombin also binds to subendothelial matrix where it is protected from inhibition by physiologic concentrations of antithrombin III (Bar-Shavit 1989). Thrombin bound to subendothelial matrix can activate platelets and convert fibrinogen to fibrin. These findings suggest that like its binding to fibrin, thrombin binds to subendothelial matrix through a site distinct from its catalytic centre (Bar-Shavit 1989).

The observations that thrombin bound to fibrin and to matrix is protected from inactivation by antithrombin III provide support for an important role for thrombin in rethrombosis after successful coronary thrombolysis and in restenosis after percutaneous coronary angioplasty.

CHAPTER 3. ADJUNCTIVE THERAPY FOR THE PREVENTION OF REOCCLUSION AFTER PHARMACOLOGIC FIBRINOLYSIS AND RE-STENOSIS AFTER PTCA

A number of agents have been evaluated in terms of their ability to inhibit both reocclusion after fibrinolysis and re-stenosis after PTCA. In this section, these agents will be discussed focusing on their structure, mechanism of action, and results from experimental and clinical studies.

3.1 Heparin

Heparin was discovered in 1916 (McLean 1916), and after over 50 years of controversy the chemical structure responsible for its anticoagulant activity was clarified (Casu 1989; Casu 1985). Heparin is a sulfated polysaccharide consisting of 1-4 linked residues of uronic acid and D-glucosamine (Casu 1989). Commercial heparin is a heterogeneous mixture of molecules with a molecular weight which ranges from 3,000 to 30,000 (Hirsh 1991) and a mean molecular weight of 15,000. Only one-third of heparin molecules have the unique pentasaccharide which is a necessary requirement for binding to anti-thrombin III (Lam 1976).

3.1.2 Mechanism of Action of Heparin on Coagulation

Heparin acts as an anticoagulant by catalyzing the inactivation of the coagulation enzymes, thrombin, activated factor X (factor Xa) and activated factor IX (factor IXa) by antithrombin III (ATIII) (Rosenberg 1987). Upon binding to lysine sites on ATIII, heparin

produces a conformational change at the active site which markedly accelerates the rate at which ATIII inhibits the coagulation enzymes (Rosenberg 1987). This conformationally induced change is sufficient to explain how heparin accelerates the inhibition of factor Xa by ATIII. In addition to its effect on ATIII, heparin also serves a template role as it increases the rate of thrombin inactivation by ATIII. Thus, by binding both thrombin and ATIII, heparin promotes their interaction. Heparin then dissociates from the enzyme-inhibitor complex and can catalyze other ATIII molecules (Rosenberg 1987).

Although the heparin-ATIII complex can inactivate a number of coagulation enzymes (Rosenberg 1987), recent studies in plasma suggest that the major mechanism by which heparin blocks coagulation is by catalyzing the inhibition of thrombin (Ofosu 1989; Beguin 1988). Thrombin plays a central role in hemostasis and thrombogenesis. It activates platelets and so induces their aggregation, it converts fibrinogen to fibrin, and activates factor XIII which then stabilizes the fibrin strands. Thrombin also amplifies coagulation by activating factors V and VIII, thereby accelerating both the generation of prothrombinase and the subsequent activation of prothrombin. By preventing thrombin-mediated activation of factors V and VIII, heparin blocks the feedback amplification of coagulation that is initiated by thrombin (Ofosu 1987). This amplification step is important in thrombogenesis because one thrombin molecule is able to catalyze the generation of many thousand thrombin molecules. Thus, the inhibition of thrombin generation and thrombin activity is the key to the prevention and treatment of both fibrin-dependent and platelet-dependent thromboembolic disorders.

3.1.3 Limitations of Heparin

The limitations of heparin are based on its pharmacokinetic, biophysical and antihemostatic properties. The pharmacokinetic limitations are based on its binding to plasma proteins and endothelial cells which result in reduced recovery and a complicated mechanism of clearance. Biophysical limitations occur because the heparin-ATIII complex is unable to access and inactivate a) factor Xa in the prothrombinase complex, b) thrombin bound to fibrin, and c) thrombin bound to subendothelial surfaces. The antihemostatic limitations are caused by a poorly defined inhibitory effect of heparin on platelet function.

The limitations related to the pharmacokinetic and antihemostatic properties of heparin are not shared by the low molecular weight heparins (lmwhs) and heparinoids, while the limitations caused by the lack of accessibility of the heparin-ATIII complex to fibrin-bound thrombin and factor Xa are overcome by several new classes of ATIII-independent thrombin and factor Xa inhibitors.

3.1.4 Heparin Pharmacokinetics

The mechanisms of heparin clearance are complex. Heparin binds to a number of plasma proteins other than ATIII (Lindahl 1978). Thus, these proteins compete with ATIII for heparin binding, thereby limiting the recovery of anticoagulantly active heparin. There is evidence that binding of heparin to plasma proteins contributes to the variability of anticoagulant response between patients and to the heparin resistance seen in some patients with thromboembolic disorders (Hirsh 1976).

Heparin is cleared by a rapid phase of elimination followed by a more gradual

disappearance which can best be explained by a combination of a saturable and a non-saturable, first-order mechanisms of clearance (DeSwart 1982). The saturable phase of heparin clearance is thought to be the result of heparin binding to receptors on endothelial cells and macrophages. Once bound, the heparin is internalized, depolymerized, and metabolized into smaller, less sulfated derivatives (Glimelius 1978; Mahadoo 1977). When heparin binds to platelets, it displaces platelet factor 4 which can then inactivate circulating heparin (Dawes 1978). The slower, non-saturable mechanism of heparin clearance probably reflects renal excretion. At therapeutic concentrations, a major proportion of the heparin is cleared by the more rapid saturable mechanism (DeSwart 1982).

This complex mechanism of heparin clearance explains why the apparent biologic half-lives of heparin increases from 60 to 100 to 150 minutes with intravenous boluses of 100, 200, and 400 units/kg of heparin, respectively (Olsson 1963). Heparin has decreased bioavailability when administered subcutaneously in low doses, but has approximately 90% bioavailability when administered in high therapeutic doses (eg. 35,000 units/24 hours) (Walker 1987). The reduced bioavailability of heparin, when it is administered subcutaneously, occurs because as heparin gradually enters the circulation from the subcutaneous depot site, it binds to plasma proteins and is rapidly cleared by the saturable mechanism resulting in reduced plasma levels and a delay in achieving a steady state.

The other potential limitations of heparin relate to the observations that the anticoagulant effect of heparin is modified by platelets, fibrin, vascular surfaces as well as plasma proteins. Platelets limit the anticoagulant effect of heparin in two ways. First, factor

Xa that is generated on the platelet surface is protected from inhibition by heparin-ATIII (Marciniak 1973; Walker 1979). Second, platelets release the heparin-neutralizing protein, platelet factor 4 (Lindahl 1978). Fibrin and its degradation products (monomers) binds thrombin and protects it from inactivation by heparin-ATIII (Hogg 1989; Weitz 1990). As a consequence, much higher concentrations of heparin are needed to inhibit thrombin bound to fibrin than is required to inactivate the free enzyme (Weitz 1990). Thrombin also binds to subendothelial matrix proteins where it is also protected from inhibition by heparin (Bar-Shavit 1989). These observations may explain why heparin is less effective than the ATIII-independent thrombin and factor Xa inhibitors at preventing thrombosis in experimental animals (Heras 1989; Agnelli 1990).

Heparin also inhibits platelet function, increases vascular permeability and increases experimental microvascular bleeding (Casu 1985; Ofosu 1986; Blajchman 1989; Fernandez 1986).

3.1.5 Mechanisms of Action of Heparin on Smooth Muscle Cell Proliferation

VSMC accumulate in the arterial intima and form an important component of naturally occurring atherosclerosis and of post-PTCA re-stenosis (Dartsch 1989). Over the last two decades, a number of in vitro (Hoover 1980; Benitz 1986; Cocchran 1985; Castellot 1984; Castellot 1985; Reilly 1986) and in vivo (Clowes 1985; Clowes 1986; Wilson 1991; Dryjski 1988; Guyton 1980) studies have demonstrated that heparin and its low molecular weight fragments modulate the pathogenesis of intimal thickening by inhibiting VSMC proliferation. Heparin and heparan sulphate arrest VSMCs proliferation between the G_0/G_1 and S phase

of the cell cycle (Castellot 1985; Wright 1989; Castellot 1987). A number of mechanisms for the antiproliferative effect of heparin on VSMCs have been proposed. These include: the inactivation of mitogens in plasma or the prevention of the interaction of mitogens with their receptors on the cell surface (Reilly 1986); inhibition of platelet function and PDGF release; inhibition of the mitogenic effect of thrombin on VSMCs; displacement of thrombospondin from pericellular matrix (bound thrombospondin regulates PDGF function while nonbound thrombospondin does not) (Majack 1985; Majack 1988; Majack 1986); inhibition of DNA synthesis (Reilly 1986; Wright 1989); stimulation of VSMCs to synthetize a protein which inhibits VSMC proliferation (Cocchran 1985; Castellot 1985); and competitive inhibition of degradation of heparan sulphate by heparitinase released from platelets (Castellot 1987; Wright 1989; Fritz 1985). This latter proposed mechanism is based on the following observations: 1) heparin and heparan sulphate have similar inhibitory effects on VSMC proliferation (Castellot 1981; Castellot 1987; Castellot 1990; Benitz 1990) and both endothelial (Benitz 1990; Castellot 1982) and VSMCs (Gamse 1978; Wight 1975) synthesize heparan sulphate; 2) confluent VSMC synthesize more heparan sulphate than exponentially growing cells (Fritz 1985); and 3) heparan sulphate-containing media conditioned with flavobacterium heparitinase display heightened sensitivity of VSMCs to mitogens and growth promoters (Castellot 1981). Furthermore, activated platelets and monocytes release heparitinase (Wright 1989; Oldberg 1980; Castellot 1982).

During vascular injury, adherent platelets and white cells at the site of injury release heparitinase which in turn degrades heparan sulphate on the surface of VSMC. As a result, the VSMC are more susceptible to mitogens and growth promoters which stimulate the cells

to proliferate and migrate. It may be that administration of exogenous heparin during vascular injury competes with heparan sulphate as a substrate for heparitinase degradation and therefore acts to maintain VSMCs in the "quiescent", nonproliferative state (Bar-Ner 1987).

Thrombin is mitogenic for a variety of cells including VSMCs (Chen 1975; Bar-Shavit 1990; Wilcox 1992) but its involvement in VSMC proliferation following angioplasty is uncertain (Hoover 1980; Castellot 1984; Guyton 1980). The observation that non-anticoagulant heparin fragments are as effective as heparin with anticoagulant activity in inhibiting VSMC proliferation argues against a role for thrombin as a inducer of smooth muscle proliferation (Hoover 1980; Castellot 1984; Guyton 1980; Wright 1989; Pukac 1991). In addition, Hoover and associates (Hoover 1980) reported that plasma depleted of ATIII and supplemented with heparin prevented VSMC proliferation. Heparin fragments which contain at least five sugar units but which lack anticoagulant activity have antiproliferative properties (Castellot 1984; Wright 1989). O- and N-sulfation are important for this property, since totally desulfated heparin loses most of its VSMC antiproliferative activity (Castellot 1984). Other glycosaminoglycans including dermatan sulphate, hyaluronic acid and chondroitin 4- and 6-sulphate failed to inhibit VSMC proliferation when compared to heparan sulphate (Castellot 1981; Fritz 1985).

In contrast to the observations listed above, recent experimental studies re-examined the role of alpha-thrombin in VSMC mitogenesis and the results suggest that when alpha thrombin is bound to its receptor on VSMCs it stimulates their proliferation (Bar-Shavit 1990; Wilcox 1992). Whether the catalytic activity of thrombin is necessary for its stimulatory

effect on VSMC mitogenesis is still debated. One group of investigators provided results which suggest that the mitogenic signal is produced by an interaction of a distinct nonenzymatic domain on thrombin with its receptor on VSMC and even catalytically inactive thrombin stimulates VSMC proliferation (Bar-Shavit 1990). On the other hand, results of other studies suggest that thrombin must be catalytically active in order to interact with its receptor (Coughlin 1992; Glenn 1988) and initiate the mitogenic signal. It is possible that, depending on the experimental conditions, both forms of thrombin can induce VSMC proliferation. The second messenger involved in the mitogenic response when thrombin binds to its receptor on VSMC is unknown.

To date none of the suggested mechanisms have been proven to be important in clinical studies. Results of studies evaluating the effect of heparin and other glycosaminoglycans on the incidence of post-PTCA re-stenosis have not been encouraging. One clinical trial (Ellis 1989) assessed the effect of a combination of heparin (24 hours) and aspirin (6 months) on post-PTCA re-stenosis and reported no difference in rates of re-stenosis between control and aspirin/heparin treatment groups at 180 days (Ellis 1989). The failure of heparin in this clinical study and its success in experimental animal studies could be due to a dose effect since the heparin dose used in the clinical study was much lower than those used in experimental animal studies (Clowes 1985; Clowes 1986; Wilson 1991; Dryjski 1988; Guyton 1980; Edelman 1994).

Other approaches including: heparin plus prostacyclin, coumadin and aspirin and dipyridamole have been evaluated in small clinical trials, but they have not been effective (Thornton 1984; Knudtson 1990).

3.2 Aspirin

Aspirin is an effective antithrombotic agent which acts by irreversibly inhibiting the enzyme cyclo-oxygenase (Burch 1978; Majerus 1983; Roth 1975) thereby blocking the conversion of arachidonic acid in platelets to prostaglandin-endoperoxide precursors of thromboxane-A_2 (TXA_2). In vascular wall cells, aspirin inhibits the conversion of arachidonic acid to prostaglandin I_2 (PGI_2) (Majerus, 1983; Moncada 1978; Moncada 1979; Moncada 1979; Weksler 1983; Patignani 1982; Fitzgerald 1983; Preston 1981; Kyrle 1987). TXA_2 induces platelet aggregation and vasoconstriction while PGI_2 inhibits platelet aggregation and induces vasodilation (Moncada 1978). Thus, aspirin has the potential to be antithrombotic by blocking formation of TXA_2, and to be thrombogenic by blocking formation of PGI_2. Evidence from clinical trials indicates that interruption of formation of PGI_2 is unlikely to be a sufficient stimulus to initiate the thrombotic process or to interfere with the antithrombotic effect of aspirin (ISIS-3 1992, Antiplatelet Trialists Collaboration 1988).

Aspirin is rapidly absorbed in the stomach and upper intestine. Peak plasma levels occur 15 to 20 minutes after aspirin ingestion and inhibition of platelet function is evident by one hour. The plasma concentration of aspirin decays with a half-life of 15 to 20 minutes. Despite the rapid clearance of aspirin from the circulation, the platelet-inhibitory effect lasts for the life-span of the platelet because of the inactivation of platelet cyclo-oxygenase is irreversible (Burch 1978; Majerus 1983). Aspirin also acetylates cyclo-oxygenase in megakaryocytes before new platelets are released into circulation (Burch 1979; O'Brien 1968). The mean life-span of the human platelet is approximately 10 days.

Therefore, approximately 10% of circulating platelets are replaced every 24 hours (Cerskus 1980; O'Brien 1968) and after 5 to 6 days, approximately 50% of the platelets function normally.

Aspirin is generally well tolerated but some patients suffer side-effects. The side-effects of aspirin are mainly gastrointestinal, dose related, and reduced by using low doses (325 mg/day or less) (UK-TIA Study Group 1988; Levy 1974). Aspirin-induced injury to the gastrointestinal tract can be acute or chronic. Acute aspirin use produces gastric erosions and gastric hemorrhage, while chronic use can produce gastric ulcers, anemia and major gastrointestinal hemorrhage (Graham 1986). Aspirin produces a dose-related increase in acute gastric bleeding (Prichard 1989; Hawkey 1988; Prichard 1987; Pierson 1961).

The exact cause of aspirin-induced gastric injury has not been elucidated. Inhibition by aspirin of prostaglandin synthesis in the gastric mucosa (Ali 1977) has been proposed as an important mechanism, but there is evidence that the gastric side effects of aspirin can be reduced by treatment with cimetidine (MacKercher 1977), by antacids (Hawkey 1988; Bowen 1977), and by the use of enteric coated or highly buffered aspirin (Graham 1986; Mielants 1979; Croft 1967).

Aspirin is effective in reducing the incidence of thrombotic complications of atherosclerosis. It has been shown to reduce the incidence of myocardial infarction and/or death in the following groups; males over the age of 50 (Hennekens 1988), asymptomatic females over the age of 50 (Manson 1991), subjects with stable angina (Ridker 1991), patients with unstable angina and non-Q wave infarction in which it appeared to be more effective than a short course of intravenous heparin (The RISC Group 1990), patients with

acute myocardial infarction (30 day incidence) (ISIS-3 1992) and long term incidence (Antiplatelet Trialists' Collaboration 1988), and in patients with cerebrovascular disease (UK-TIA Study Group 1988; Antiplatelet Trialists' Collaboration 1988; DeClerck 1989).

Since platelets play an active role in hemostasis and thrombosis (i.e. they adhere to the injured vessel wall, their surface is a part of the prothrombinase complex, and they release ADP and TXA_2 which promote aggregation) and in vessel wall repair (i.e. platelets release PDGF which stimulates the migration and proliferation of VSMCs) their inactivation may reduce post-PTCA re-stenosis. There are studies both supporting and disputing this hypothesis.

Three weeks of aspirin therapy significantly inhibited intimal thickening secondary to arterial injury in rats (Vockler 1990) and in canine coronary bypass vein grafts (Metke 1979). On the other hand, in dogs, aspirin did not prevent intimal thickening at the distal anastomosis of aorto-iliac bypass grafts (PTFE) (Brothers 1989) or in implanted vein grafts (Landymore 1991). Finally, in humans, several studies suggest that aspirin therapy does not prevent re-stenosis after angioplasty (Thornton 1984; Finci 1988; Schwartz 1988; White 1991; Ohman 1990; Chesebro 1989).

3.3 Oral Anticoagulants (Warfarin)

Oral anticoagulants are vitamin K antagonists which produce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its 2,3 epoxide (vitamin K epoxide). Vitamin K is a cofactor for the post-translational carboxylation of glutamate residues to gamma-carboxyglutamates (Gla) on the N-terminal regions of vitamin K

dependent proteins (Whitlon 1978; Fasco 1982; Choonara 1988; Stenflo 1974; Nelsestuen 1974; Nelsestuen 1976). The process of gamma-carboxylation permits the coagulation proteins to undergo a conformational change (Nelsestuen 1976; Prendergast 1977; Borowski 1986) which is necessary for calcium dependent complexing of vitamin K dependent proteins to their cofactors on phospholipid surfaces and for their biologic activity.

Carboxylation of vitamin K dependent coagulation factors is catalyzed by a carboxylase which requires the reduced form of vitamin K (vitamin KH_2), molecular oxygen, and carbon dioxide. During this reaction, the vitamin KH_2 is oxidized to vitamin K epoxide which is recycled to vitamin K by vitamin K epoxide reductase which in turn is reduced to vitamin KH_2 by vitamin K reductase. The vitamin K antagonists exert their anticoagulant effect by inhibiting vitamin K epoxide reductase (Whitlon 1978; Fasco 1982; Choonara 1988) and possibly vitamin K reductase (Fasco 1982). This process leads to the depletion of vitamin KH_2 and limits the gamma-carboxylation of the vitamin K dependent coagulant proteins (prothrombin, factor VII, factor IX and factor X). In addition, the vitamin K antagonists limit the carboxylation of the regulatory proteins (protein C and protein S), and as a result impair the function of these anticoagulant proteins. By inhibiting the cyclic conversion of vitamin K, oral anticoagulants result in the hepatic production and secretion of partially carboxylated and decarboxylated proteins (Friedman 1977; Malhotra 1985).

3.3.1 Pharmacokinetics and Pharmacodynamics of Warfarin

Warfarin (a 4-hydroxycoumarin compound) is the most widely used oral anticoagulant in North America. Warfarin is rapidly absorbed from the gastrointestinal tract, reaches

maximal blood concentrations in healthy volunteers in 90 minutes (Breckenridge 1978) and has a half-life of 10 to 45 hours (Breckenridge 1978). It circulates bound to plasma proteins and rapidly accumulates in the liver (O'Reilly 1970).

The dose response relationship of warfarin differs between healthy subjects (O'Reilly 1970) and can vary to a much greater extent among sick patients. Because of the variations in dose response in individual patients during the course of anticoagulant therapy their anticoagulant dosage must be monitored closely to prevent overdosing or underdosing.

The dose response to warfarin is influenced by both pharmacokinetic factors (due to differences in absorption or metabolic clearance of warfarin) and pharmacodynamic factors (due to differences in the hemostatic response to given concentrations of warfarin). Technical factors also contribute to the variability in dose-response, including inaccuracies in laboratory testing and reporting, poor patient compliance, and poor communication between patient and physician.

Drugs can influence the pharmacokinetics of warfarin by reducing its absorption from the intestine or by altering its metabolic clearance. The pharmacodynamics of warfarin are affected by many factors which can influence its anticoagulant effect. Hereditary resistance to warfarin has been described in humans (Alving 1985); they require doses which are 5 to 20-fold higher than average to achieve an anticoagulant effect. This disorder is thought to be caused by an altered affinity of the receptor for warfarin since the plasma warfarin levels required to achieve an anticoagulant effect are much higher than average.

Subjects receiving long-term warfarin therapy are sensitive to fluctuating levels of dietary vitamin K (O'Reilly 1970) which is obtained predominantly from phylloquinone in

plant material (Suttie 1988).

Important fluctuations in vitamin K intake occur in both apparently healthy and sick subjects. Increased intake of dietary vitamin K sufficient to reduce the anticoagulant response to warfarin (Lader 1980) occurs in patients on weight reduction diets (rich in green vegetables) and those treated with intravenous nutritional fluid supplements rich in vitamin K. The effects of warfarin can be potentiated in sick patients with poor vitamin K intake (particularly if they are treated with antibiotics and intravenous fluids without vitamin K supplementation), and in states of fat malabsorption (O'Reilly 1970). Hepatic dysfunction also potentiates the response to warfarin through impaired synthesis of coagulation factors (Bell 1978). Hypermetabolic states produced by fever or hyperthyroidism increase responsiveness to warfarin probably by increasing the catabolism of vitamin K dependent coagulation factors (O'Reilly 1970; Loeliger 1964). Drugs can influence the pharmacodynamics of warfarin by inhibiting the synthesis of vitamin K dependent coagulation factors, by increasing the metabolic clearance of vitamin K dependent coagulation factors and by interfering with other pathways of hemostasis.

Drugs such as aspirin (O'Reilly 1970), other non-steroidal anti-inflammatory drugs (Schulman 1989; O'Reilly 1970), high doses of penicillins (O'Reilly 1970; Brown 1974), and moxalactam (Weitkamp 1983) can increase the risk of warfarin-associated bleeding by inhibiting platelet function. Aspirin is the most important because of its widespread use and prolonged effect on hemostasis (Roth 1975). Aspirin can also produce gastric erosions which increase the risk of serious upper gastrointestinal bleeding (Graham 1986).

Sulfonamides and many broad spectrum antibiotics have the potential to augment the

anticoagulant effect of warfarin by eliminating bacterial flora and, thereby, producing vitamin K deficiency, but these agents only potentiate the anticoagulant effect of warfarin in patients on a vitamin K deficient diet (O'Reilly 1970; Udall 1965).

Oral anticoagulants have been shown to be effective in the primary and secondary prevention of venous thromboembolism, in the prevention of systemic embolism in patients with tissue and mechanical prosthetic heart valves or with atrial fibrillation; in the prevention of acute myocardial infarction in patients with peripheral arterial diseases, and in the prevention of stroke, recurrent infarction and death in patients with acute myocardial infarction (Hirsh 1992). Oral anticoagulants are indicated in patients with valvular heart disease to prevent systemic embolism although their effectiveness has never been demonstrated by a randomized clinical trial (Hirsh 1992). For most indications, a moderate anticoagulant effect with a targeted International Normalized Ratio (INR) of 2.0 to 3.0 (less intense regimen) is appropriate (Hirsh 1992).

The main limitations of oral anticoagulants are a narrow risk/benefit ratio, variability of dosage requirement and fluctuations produced by changes in diet or concomitant use of drugs (alcohol). This necessitates frequent laboratory monitoring and patient surveillance (Hirsh 1992).

3.4 Low Molecular Weight Heparins (lmwhs) and Heparinoids

The development of lmwhs for clinical use was stimulated by the observation that for equivalent antithrombotic effects in experimental models, lmwhs produce less bleeding than the heparin from which they were derived (Andriuoli 1985; Bergqvist 1994; Cade 1984;

Carter 1982; Esquivel 1982; Holmer 1982). These observations were followed by clinical studies which demonstrated that lmwhs are effective and safe antithrombotic agents for the prevention and treatment of venous thrombosis. Lmwhs have been approved for clinical use for the prevention and treatment of venous thrombosis in Europe and for the prevention of venous thrombosis in Canada and the United States.

Lmwhs are fragments of standard commercial grade heparin (stdh) produced by either chemical or enzymatic depolymerization (Ofosu 1990). Stdh is a heterogeneous mixture of polysaccharide chains with a wide molecular weight distribution which ranges from 3,000 to 30,000, with a mean molecular weight of 15,000 (Ofosu 1990; Andersson 1979; Harenberg 1990). Lmwhs are approximately one third the size of heparin. Like stdh they are heterogenous in size with a molecular weight range of 1,000 to 10,000 and a mean molecular weight of 4,000 to 5,000 (Ofosu 1990).

Depolymerization of stdh results in a change in its anticoagulant profile, in its bioavailability and pharmacokinetics, and in its effects on platelet function and experimental bleeding. Two other glycosaminoglycans have also been developed for clinical use. These are dermatan sulfate and the Organon heparinoid (Orgaran) which is a mixture of heparan sulfate (the major component making up 80% of the mixture) and smaller amounts of dermatan sulfate and chondroitin sulfates (Ofosu 1990).

3.4.1 Anticoagulant Effects of Lmwhs

Like stdh, lmwhs produce their major anticoagulant effect by binding to ATIII through a unique pentasaccharide sequence (Bjork 1982; Rosenberg 1979; Casu 1981; Choay

1981; Choay 1983; Hook 1976; Lindahl 1979; Lindahl 1984; Rosenberg 1975; Rosenberg 1979). This pentasaccharide sequence is present on approximately one third of the stdh chains and is present on less than one third of lmwh molecules. The binding of the pentasaccharide to ATIII produces a conformational change in the ATIII molecule (Olson 1981; Villanueva 1977) which enhances its ability to inactivate the coagulation enzymes thrombin (factor IIa) and factor Xa (Rosenberg 1975). Stdh and lmwhs catalyze the inactivation of thrombin by ATIII acting as a template which binds both the plasma co-factor through the unique pentasaccharide sequence and thrombin to form a ternary complex (Bjork 1982; Rosenberg 1979; Rosenberg 1975; Olson 1982). A minimum chain length of 18 saccharides (including the pentasaccharide sequence) is required for ternary complex formation. In contrast, to catalyze the inactivation of factor Xa by ATIII heparin needs only to bind to ATIII (Bjork 1982; Rosenberg 1979; Rosenberg 1975; Olson 1982). Thus, even low molecular weight heparin fragments can catalyze this reaction, provided that they contain the high affinity pentasaccharide. Virtually all stdh molecules contain at least 18 saccharide units while only 25% to 50% of the different lmwhs contain fragments with 18 or more saccharide units (Lindahl 1984; Holmer 1981; Holmer 1986). Therefore, compared with stdh which has a ratio of anti-factor Xa to anti-factor IIa activity of approximately 1:1, the various commercial lmwhs have anti-factor Xa to anti-IIa ratios which vary between 4:1 and 2:1 depending on their molecular size distribution.

3.4.2 Pharmacokinetics of Lmwhs

The plasma recoveries and pharmacokinetics of lmwhs differ from stdh because of

differences in the binding properties of the two sulfated polysaccharides to plasma proteins and endothelial cells (Ofosu 1990; Lane 1986; Barzu 1984; Barzu 1985).

Lmwhs bind much less avidly to heparin-binding proteins than stdh (Ofosu 1990; Lane 1986; Lijnen 1983; Preissner 1987; Dawes 1991; Sobel 1991) a property which contributes to the superior bioavailability of lmwh at low doses and their more predictable anticoagulant response (Handeland 1990). Lmwhs do not bind to endothelial cells in culture (Barzu 1984; Barzu 1985; Barzu 1987) a property which could account for their longer plasma half life (Boneu 1988; Briant 1989; Frydman 1988). Lmwhs are cleared principally by the renal route and their biological half life is increased in patients with renal failure (Boneu 1988; Palm 1987).

Lmwh preparations have a lower affinity than stdh for von Willebrand factor (vWF), (Sobel 1991) a finding which could contribute to the observation that they produce less experimental bleeding than stdh for equivalent anticoagulant effects (Andriuoli 1985; Bergqvist 1994; Esquivel 1982; Ockelford 1982).

3.4.3 Antithrombotic and Hemorrhagic Effects of Lmwhs in Experimental Animal Models

The antithrombotic and hemorrhagic effects of stdh have been compared with lmwhs, the ORG heparinoid and dermatan sulfate in a variety of experimental animal models (Andriuoli 1985; Bergqvist 1985; Esquivel 1982; Ockelford 1982; Hobbelen 1987; Van Ryn-McKenna 1989; Van Ryn-McKenna 1989). In these models of thrombosis, temporary venous stasis is produced by ligating an appropriate vein and blood coagulation is stimulated by injecting either serum, factor Xa, thrombin, or tissue factor (Van Ryn-McKenna 1989;

Van Ryn-McKenna 1989; Ockelford 1982). When compared on a gravimetric basis, lmwhs are slightly less effective than heparin as antithrombotic agents but produce much less bleeding than heparin in models measuring blood loss from a standardized injury (Bergqvist 1985; Esquivel 1982; Hobbelen 1987; Ockelford 1982). The differences in the relative antithrombotic to hemorrhagic ratios among these sulfated polysaccharides could be due in part to their different effects on platelet function (Sobel 1991; Fabris 1983; Fernandez 1986) and vascular permeability (Blajchman 1989).

3.4.4 Lmwhs in Prevention of Thrombosis: Clinical Trials

Lmwhs have a number of advantages over stdh. The observations that lmwhs have a longer plasma half life and a more predictable anticoagulant response than stdh allow lmwhs to be administered once daily and without laboratory monitoring. The observation in experimental animals that lmwhs produce less bleeding than stdh for an equivalent antithrombotic effect has allowed patients to be treated with higher anticoagulant doses of lmwhs without compromising patient safety. This latter potential advantage of lmwhs has been demonstrated in one prophylactic study in which stdh produced a significant increase in bleeding when its dose was increased to match the anticoagulant effect ex-vivo of a lmwh (Levine 1991), and in two studies comparing high doses of a lmwh with full doses of stdh for the treatment of venous thrombosis (Hull 1991; Prandoni 1991). Lmwhs have been evaluated for the prevention and treatment of venous thromboembolism and have been shown to be highly effective.

3.4.5 Lmwhs in the Prevention of Post-PTCA Re-stenosis: Experimental and Clinical Studies

Low molecular weight heparins, when used in animal models of angioplasty, have been reported to prevent the development of intimal thickening and stenosis (Wilson 1991; Dryjski 1988; Currier 1991). However, in the one clinical trial in which it was evaluated, lmwh failed to prevent re-stenosis after coronary angioplasty (Faxon 1992). The negative results of this study could have been due to the low dose used and the short duration of therapy.

3.5 Novel Antithrombotic Compound (Table II and III)

A large array of new compounds designed to inhibit specific molecular interactions which are believed to be important in thrombogenesis have been developed. These include: 1) inhibitors of vWF-dependent platelet adhesion to platelet glycoprotein receptor Ib (GPIb); 2) inhibitors of platelet glycoprotein receptor IIb/IIIa (GPIIb/IIIa)-dependent platelet aggregation; 3) platelet thrombin receptor antagonists; 4) inhibitors of thrombin generation - tissue factor pathway inhibitor (TFPI), activated protein C (APC), inhibitors of factor Xa; and 5) direct thrombin inhibitors. The development of these inhibitors has been made possible by advances in molecular biology and in knowledge of the structural chemistry of binding sites on receptors and ligands involved in thrombogenesis.

3.5.1 Platelet Function Inhibitors

Aspirin is a very selective inhibitor of platelet function since it only inhibits platelet aggregation mediated by activation of the arachadonic acid/TXA_2 pathway. This selective

activity may explain the limited effectiveness of aspirin in inhibiting coronary reocclusion clinically (de Bono 1992; Hsia 1990) and in experimental models of thrombolysis (Golino 1988; Golino 1989). Nevertheless, despite its limited selectivity, aspirin is effective in preventing death and re-infarction after coronary thrombolysis (ISIS-3 1992).

The limitations of aspirin have generated considerable interest in developing new and more potent inhibitors of platelet aggregation. These fall into four main classes: 1) the non-specific inhibitor, Ticlopidine; 2) the TXA_2 receptor antagonists and synthetase inhibitors; 3) compounds which compete with vWF for binding to platelet GPIb; and 4) compounds which compete for binding of fibrinogen and other adhesive proteins to platelet GPIIb/IIIa. Of these, the most promising group of compounds are those which compete with fibrinogen and the other adhesive proteins for binding to the platelet glycoprotein receptor GPIIb/IIIa. Ticlopidine is currently the only one of the four classes of new compounds that is approved for clinical use.

3.5.2 Ticlopidine

Ticlopidine is a relatively new antiplatelet drug with an entirely different mechanism of action than aspirin (Saltiel 1987). Ticlopidine is a thienopyridine derivative which inhibits platelet aggregation induced by a variety of agonists, including ADP, possibly by altering the platelet membrane and blocking the interaction between fibrinogen and its membrane glycoprotein receptor, GPIIb/IIIa (Di Minno 1985). The inhibitory effect of ticlopidine is delayed for 24 to 48 hours after its administration, suggesting that the antiaggregating effects are caused by metabolites (Saltiel 1987). Ticlopidine has been evaluated in patients with

stroke (Gent 1989), transient cerebral ischemia (Hass 1989), unstable angina (Balsano 1990), intermittent claudication (Arcan 1988; Balsano 1989; Janzon 1990), and in patients having aorto-coronary bypass surgery (Limet 1987). Ticlopidine was significantly more effective than aspirin in reducing stroke in patients with transient cerebral ischemia or minor stroke (Hass 1989); more effective than placebo in reducing the risk of the combined outcome of stroke, myocardial infarction or vascular death in patients with thromboembolic stroke (Gent 1989); more effective than an untreated control group in reducing vascular death and myocardial infarction in patients with unstable angina (Balsano 1990); more effective than placebo in reducing acute occlusion of coronary bypass grafts (Limet 1987), and more effective than controls in improving walking distance (Balsano 1989) and reducing vascular complications in patients with peripheral vascular disease (Balsano 1990; Arcan 1988; Janzon 1990).

Ticlopidine has a number of troublesome side effects, the most common of which are diarrhea and skin rash, and the most serious, neutropenia. Nevertheless, ticlopidine can be used in patients with aspirin allergy or gastrointestinal intolerance of aspirin.

In one clinical study (Kitazume 1988) a combination of ticlopidine, aspirin, and the calcium channel antagonist nicorandil significantly reduced post-PTCA re-stenosis whereas aspirin alone had no effect at 28 weeks.

3.5.3 Inhibitors of Platelet Adhesion

Platelet adhesion occurs when glycoprotein receptors on nonactivated platelets bind to the ligands in the subendothelial extracellular matrix exposed during vascular injury

(Sakariassen 1987; Phillips 1991; Hynes 1992). The most important ligands are collagen and vWF but platelets may also adhere to other subendothelial proteins including fibronectin, laminin, vitronectin, and thrombospondin (Sakariassen 1987; Phillips 1991; Hynes 1992; Sixma 1984; Handa 1986; Sakariassen 1986). The platelet membrane glycoprotein receptors for the various adhesive matrix molecules are GPIb/IX, GPIa/IIa, GPIc/IIa, vitronectin receptor and GPIV (GPIIIb).

Platelets exposed to elevated levels of fluid shear stress bind to vWF and then aggregate in the absence of exogenous agonists (Chow 1992). vWF binds to two receptors on the platelet membrane, GPIb and GPIIb/IIIa, both of which are involved in shear stress-induced platelet aggregation (Moake 1988). Shear stress stimulates binding of vWF multimers to GPIb on the surface of inactivated platelets. This binding interaction then activates platelets and exposes functional GPIIb/IIIa integrin receptors which bind vWF multimers and undergo aggregation. Shear stress-induced aggregation is inhibited in part by a monoclonal antibody (10E5), which blocks vWF binding to GPIIb/IIIa.

The molecular mechanism responsible for shear stress-induced vWF/GPIb binding is uncertain. Shear-stress could either alter the structure of vWF or it could alter some characteristic of platelet surface GPIb and permit ligand binding to occur (Moake 1988). Whatever the mechanism high shear-stress initiates vWF-dependent transmembranous influx of Ca^{2+} and induces platelet aggregation. This platelet response is not inhibited by ASA (Chow 1992) and requires a functional platelet GPIIb/IIIa complex.

Binding of collagen to its glycoprotein receptors also stimulates platelet activation with the exposure of the integrin GPIIb/IIIa in its functional form.

Inhibition of vWF-dependent platelet adhesion can be achieved by interfering with binding of vWF to GPIb or by inhibition of vWF multimerization (Sixma 1984; Handa 1986; Sakariassen 1986; Miller 1991; Ikeda 1991; Bellinger 1987).

Monoclonal antibodies against GPIb or GPIb/IX have been studied in experimental animal models of thrombosis (Miller 1991; Hanson 1989). Monoclonal antibodies raised against GPIb have antithrombotic effects in guinea pigs subjected to laser-induced injury to mesenteric small arteries (Miller 1991). These antibodies produce irreversible thrombocytopenia in nonhuman primates (Hanson 1989). Peptides mimicking the GPIb binding domain of vWF inhibit ristocetin-induced GPIb-dependent platelet agglutination (Handa 1986; Ikeda 1991) inhibit platelet deposition in high-shear flow models in vitro (Ikeda 1991) and reduce thrombus formation under arterial flow conditions in experimental animal models (Badimon 1990). The template bleeding time is prolonged at concentrations of these peptides which exhibit antithrombotic effects (Miller 1991; Hanson 1989; Badimon 1990).

Moderate antithrombotic effects are also observed in animal models of arterial thrombosis when anti-vWF neutralizing antibodies (Miller 1991; Bellinger 1987) are administered intravenously at doses that produce a marked prolongation of the bleeding time. Similarly moderate antithrombotic effects are produced in animal models of arterial thrombus by inhibiting vWF multimerization using aurin tricarboxylic acid (ATA) at doses that produce marked prolongation of the bleeding time (Strony 1989).

3.5.3.1 Inhibitors of Platelet GPIIb/IIIa-Dependent Recruitment

Platelet aggregation can be triggered by platelet adhesion to either collagen or vWF (under conditions of high shear) or by exposure to ADP, TXA_2 or thrombin. Platelet activation by these agonists results in the expression on the platelet surface of functional GPIIb/IIIa receptors for fibrinogen, and other adhesive glycoproteins, leading to calcium-dependent inter-platelet linkages (Phillips 1991; Hynes 1992). The functional GPIIb/IIIa receptor binds with a number of adhesive glycoprotein ligands, including fibrinogen, vWF, fibronectin, vitronectin and thrombospondin. Of these, fibrinogen is the most important because it is present in much greater concentrations in plasma than the other adhesive glycoproteins. Arg-Gly-Asp (RGD) serves as the integrin recognition sequence in the adhesive proteins interacting with this receptor (Phillips 1991; Hynes 1992). Fibrinogen contains two RGD sequences in each α chain (Phillips 1991). An additional site on the carboxy-terminal of each gamma chain of fibrinogen also binds to GPIIb/IIIa. This site consists of a dodecapeptide sequence that is not found in other adhesive proteins and contains a Lys-Ala-Gly-Asp sequence.

Platelet aggregation is inhibited by monoclonal antibodies directed against GPIIb/IIIa, by naturally occurring peptides containing RGD, or by dodecapeptide sequences, and by synthetic competitive analogs.

3.5.3.2 Monoclonal Antibodies Directed Against GPIIb/IIIa

Inhibition of the platelet GPIIb/IIIa receptor by murine monoclonal antibodies prevents thrombosis in experimental models of vascular injury (Coller 1989; Gold 1988;

Yasuda 1988) and significantly shorten the time to t-PA-induced reperfusion after thrombotic coronary occlusion (Gold 1988; Coller 1990). In dogs with experimental coronary thrombosis, 7E3, a monoclonal antibody against GPIIb/IIIa, accelerates thrombolysis and prevents rethrombosis (Gold 1988; Yasuda 1988; Coller 1990).

The dosages required to achieve antithrombotic effects with murine monoclonal antibodies against GPIIb/IIIa, essentially eliminate GPIIb/IIIa receptor-function on all circulating platelets, and produce substantial experimental bleeding at sites of tissue injury in nonhuman primates (Hanson 1989). Thrombocytopenia also develops in nonhuman primates following the administration of murine monoclonal antibodies (Hanson 1989). In patients, antithrombotic doses of these monoclonal antibodies also prolong template bleeding, although to-date no severe spontaneous abnormal bleeding has been reported in clinical trials. Preliminary studies using "humanized" anti-GPIIb/IIIa monoclonal antibodies in patients (Coller 1990; Iuliucci 1991) at risk of arterial thrombotic events are reportedly free of both bleeding events and significant thrombocytopenia at doses exhibiting antiplatelet effects in patients with unstable angina (Gold 1990) and myocardial infarction who are also treated with thrombolytic agents (Kleiman 1991).

3.5.3.3 Natural Antiplatelet Peptides

A number of naturally occurring cysteine-rich single-chain polypeptides have been isolated from snake venoms that potentially inhibit the binding of fibrinogen to GPIIb/IIIa receptors and abolish platelet aggregation. This group of RGD-containing peptides include trigramin (Cook 1989; Huang 1987), bitistatin (Mellott 1989; Shebuski 1990; Bush 1989)

echistatin (Bush 1989; Savage 1990), kistrin (Yasuda 1991) and applaggin (Chao 1989; Savage 1990). In experimental animals all of these polypeptides, referred to as disintegrins, produce dose-dependent inhibition of platelet aggregation ex vivo and thrombus formation in vivo. Accelerated tPA-induced thrombolysis with prevention of subsequent reocclusion have also been demonstrated experimentally with some of these polypeptides (Shebuski 1990; Yasuda 1991). For example, bitistatin (Shebuski 1990) augments the effect of heparin in accelerating thrombolysis and prevents reocclusion following tPA-induced thrombolysis in canine models of coronary thrombolysis.

These biologic peptides inhibit binding of all RGD-containing adhesive proteins with platelet GPIIb/IIIa receptors with affinities similar to monoclonal antibodies, although their effects are short-lived in vivo. Barbourin, a peptide isolated from the southwestern pygmy rattlesnake Sistrurus m. barbouri, differs from the other snake venom peptides in that it specifically inhibits the binding of adhesive proteins with human platelet GPIIb/IIIa without affecting the binding to GPIIa/IIIb on other cells (Scarborough 1991). This specificity is a consequence of the substitution of ARG for LYS, forming the unique recognition sequence KGD.

RGD-containing snake venom polypeptides (Gan 1988; Dennis 1990) enhance and maintain coronary arterial thrombolysis with recombinant tissue plasminogen activator (rtPA) in dogs (Yasuda 1991).

3.5.3.4 Synthetic Antiplatelet Peptides

GPIIb/IIIa-antagonist peptides have been synthesized and characterized in vitro and

in vivo as competitive inhibitors of platelet GPIIb/IIIa binding with the adhesive proteins (Charo 1991; Hanson 1991; Strony 1991; Kessler 1991). These peptides, which are potent when synthesized in a cyclic configuration, inhibit platelet aggregation in a dose-dependent manner in vitro and in vivo and produce antithrombotic effects in experimental models of thrombosis (Charo 1991; Hanson 1991; Strony 1991; Kessler 1991; Haskel 1991). In experimental animal models, the tetrapeptide analogue Arg-Gly-Asp-O-methyltyrosine amide prevents reocclusion after successful tPA-induced thrombolysis in the femoral arteries of dogs (Haskel 1989; Haskel 1991), and the cyclic hepatopeptide MK-852 is an effective antithrombotic compound in experimental models of arterial thrombosis (Haskel 1991).

Synthetic cyclic peptides containing the KGD sequence also inhibit the binding of human platelets with adhesive proteins but with greater specificity for platelet GPIIb/IIIa than the integrins on other cells (Charo 1991; Hanson 1991).

3.6 Thrombin Receptor Antagonist

The platelet thrombin receptor is a 425 amino-acid, seven-transmembrane, G-protein-coupled molecule with an amino-terminal extracellular domain that undergoes activation by thrombin-mediated cleavage at Arg 41 in the LDPR/S amino acid sequence (Vu 1991; Vu 1991; Hung 1992; Coughlin 1992). Severance of this terminal peptide creates a neo-amino-terminus that activates the receptor as a tethered ligand (Vu 1991; Vu 1991). Thrombin interacts with an acidic region on the receptor through an anion-binding exosite in a manner analogous to its interaction with hirudin (Rydel 1990). Each platelet has, on average, about a thousand copies of the thrombin receptor. The thrombin receptor is also present on

endothelium and vascular smooth muscle cells.

In vitro, platelet thrombin receptor activity is inhibited by monoclonal antibodies and synthetic peptides targeting specific receptor sites (Hung 1992). In preliminary studies, novel synthetic thrombin receptor antagonist peptides (TRAPs) comprising portions of the neo-amino-terminus, hirudin-like binding sequence, or other extramembranous domains also inhibit thrombin receptor function in vivo (Hanson 1992; Wilcox 1992). TRAPs do not inhibit the cleavage of fibrinogen by thrombin but they may block other thrombin receptor-dependent responses at sites of vascular injury including the mitogenic stimulation of vascular smooth muscle cell proliferation (Glenn 1988; Carney 1992) and leukocyte chemotaxis and cell adhesion receptor expression (Hanson 1992; Wilcox 1992).

3.7 Thromboxane A_2 Synthetase Inhibitors

Thrombin and TXA_2 are both important mediators of platelet activation (Ganguly 1974; Moncada 1979; Fitzgerald 1988; Owen 1988). TXA_2 causes platelet activation and coronary vasoconstriction. Studies in experimental animal models of thrombolysis and in man using activation markers suggest that reocclusion after thrombolytic therapy is contributed to by TXA_2 production and release at the site of the lysing coronary thrombus (Coller 1990; Willerson 1991). There is experimental evidence that inhibition of both TXA_2 and thrombin activity may be more effective than inhibition of either one alone in shortening the time to reperfusion and in the prevention of re-occlusion (Golino 1988; Golino 1989; Fitzgerald 1989). Recent studies showed that when added to hirulog, Ridogrel, a combined TXA_2 synthetase inhibitor and receptor antagonist (De Clerck 1989; De Clerck

1989) reduces the frequency of reocclusion after tPA-induced lysis of experimental coronary thrombosis in dogs more effectively than hirulog. In this model, inhibition of thrombin alone, or TXA_2 alone, did not prevent reocclusion. Thus both TXA_2 and thrombin might contribute to the process of reocclusion. Ridogrel (Ashton 1986; Bush 1984) was more effective than either a thromboxane receptor antagonist (Bush 1984) or a thromboxane synthetase inhibitor (Golino 1990) when either of these classes of thromboxane inhibitors were used alone.

3.8 ATIII-Independent Thrombin Inhibitors

Several ATIII-independent inhibitors are now available. These include hirudin, hirudin fragments, argatroban, and the peptide chloromethyl ketone inhibitor, D-Phe-Pro-ArgCH_2Cl (PPACK) and its derivatives. Although all of these inhibitors bind directly to thrombin, they have different mechanisms of action as described below. The potential advantage of the ATIII-independent inhibitors is that unlike heparin, these agents can access and inactivate thrombin that is bound to fibrin (Weitz 1990). This potential advantage appears to be real since these inhibitors have proven to be more effective than heparin in experimental animal models of venous and arterial thrombosis (Heras 1989; Agnelli 1990; Agnelli 1992) and as adjuncts to tPA-induced thrombolysis using a canine model (Yasuda 1990). These observations illustrate the importance of inhibiting fibrin-bound thrombin to achieve optimal antithrombotic effects.

3.8.1 Hirudin and Its Derivatives

Hirudin is a 65 amino acid residue protein isolated from the salivary glands of the medicinal leech. It is a potent and specific thrombin inhibitor which is now available through recombinant DNA technology. It forms an essentially irreversible, stoichiometric complex with thrombin. Analysis of the crystal structure of the thrombin-hirudin complex illustrates that hirudin makes extensive contact with thrombin as it binds to both the active centre and the substrate recognition site of the enzyme (Rydel 1990). Hirudin inhibits thrombin by forming a stoichiometric complex with a dissociation constant that has been reported to be as low as 20 fM (Stone 1986).

A novel class of synthetic C-terminal peptide fragments of hirudin have been developed (Chang 1990) as thrombin inhibitors (Maraganore 1989; Maraganore 1990). The first of these fragments, hirugen, is a synthetic dodecapeptide comprising residues 53 to 64 of the carboxy-terminal region of hirudin (Maraganore 1989). Hirugen binds only to the substrate recognition site of thrombin but does not bind to the catalytic centre of the enzyme. As a result hirugen blocks the interaction of thrombin with fibrinogen, platelets and other substrates but does not inhibit the amidolytic activity of the enzyme. By adding D-Phe-Pro-Arg-Pro-$(Gly)_4$ to the amino-terminal region, hirugen has been converted from a weak competitive inhibitor to a potent bivalent inhibitor known as hirulog (Maraganore 1990). Like hirudin, hirulog blocks both the active centre and the substrate recognition site of thrombin. However, active site inhibition is transient because once complexed, thrombin can slowly cleave the Pro-Arg bond on the amino terminal extension, thereby, converting hirulog to a weaker, hirugen-like species.

In experimental animals, hirudin inhibits the formation of venous thrombi and blocks

intravascular coagulation when administered intravenously or subcutaneously (Markwardt 1986). Hirudin is much more effective than high dose heparin (Heras 1989; Kelly 1991) or aspirin (Lam 1987) in reducing platelet deposition and thrombosis after angioplasty in pigs. Hirudin also interrupts platelet-dependent thrombus formation at sites of mechanical deep arterial injury in pigs (Heras 1989) and nonhuman primates (Kelly 1991), although the doses required to inhibit platelet deposition produce corresponding impairment in hemostatic function. Clinical trials are currently being conducted to evaluate the relative efficacy and safety for both venous and arterial thrombotic outcomes in patients.

The initial results of Phase II trials with hirudin in unstable angina and in acute myocardial infarction have been encouraging. In patients with unstable angina using a 3:1 randomization allocation to 3 days with hirudin versus heparin, bleeding complications have not been increased and paired angiography (baseline and at follow-up, prior to any coronary intervention) points to improvement in culprit vessel clot lysis (Topol 1994). Similarly, as an adjunct to thrombolysis in acute myocardial infarction in 214 patients, hirudin compared favourably to heparin with respect to sustaining infarct vessel patency and reducing morbid events (TIMI 5 Trial 1993). It has also been associated with no increase in bleeding complications compared with heparin (TIMI 5 Trial 1993). Thus, both randomized, heparin controlled, angiographic trials have provided evidence for improvement in coronary artery thrombus dissolution or prevention of new thrombus formation with hirudin as compared to heparin.

Since thrombin has also been reported to stimulate VSMC proliferation, its inactivation by hirudin may prevent post-PTCA re-stenosis. In one study, recombinant desulphatohirudin

was tested in a rabbit femoral angioplasty model (Sarembock 1991) and at twenty-eight days after angioplasty the hirudin group exhibited significantly less stenosis than animals treated with heparin. Similar inhibitory effects of hirudin on VSMC proliferation were reported in baboons (Hanson 1992). The results of these studies suggested that thrombin plays a role in the pathogenesis of re-stenosis after angioplasty (Sarembock 1991, Hanson 1992).

3.8.2 Argatroban

The synthetic arginine derivative, argipidine or Argatroban, is a competitive inhibitor of thrombin (Kikumoto 1984) that interacts with the active site of the enzyme. It has a half-life of only a few minutes. Although Argatroban is a relatively potent antithrombin in vitro (Kikumoto 1984), it fails to inhibit platelet-dependent thrombus formation in vivo.

3.8.3 PPACK and Its Derivatives

The tripeptide chloromethyl ketone D-Phe-Pro-ArgCH_2Cl (PPACK), irreversibly inhibits thrombin by alkylating the active centre histidine (Kettner 1979). This synthetic antithrombin is unique among the ATIII-independent, direct antithrombins because it potently and irreversibly inactivates both soluble and thrombus-bound thrombin (Hanson 1988; Lumsden 1993; Kelly 1989; Kotze 1990; Krupski 1990). Recent crystallographic studies confirm the tight interactions of this molecule with thrombin's catalytic pocket in addition to its covalent derivatization of His-57 in the catalytic triad (Bode 1989). Since thrombin binds to fibrin through a site distinct from its catalytic centre, PPACK readily inhibits clot-bound thrombin (Weitz 1990). Systemic infusions of PPACK into nonhuman

primates interrupts the formation of platelet-rich thrombi on Dacron vascular grafts (Hanson 1988), vascular stents (Krupski 1990), and hemodialyzers (Kotze 1990), whereas heparin and/or aspirin have no effect. Short intravenous infusions of PPACK produce lasting interruption of platelet deposition at sites of surgical carotid endarterectomy because this agent irreversibly inactivates thrombin generated by, and bound to, forming thrombus (Hanson 1988). In contrast, hirudin produces only transient inhibition presumably because there is slow dissociation of the thrombin-hirudin complex once the drug is cleared from the circulation (Kotze 1990). Despite the potential advantages of PPACK, the long-term toxicities associated with systemic or local administration have yet to be determined.

Recently, a PPACK derivative, D-Phe-Pro-Arg-borate, has been developed which is a more specific inhibitor of thrombin than the parent molecule (Kettner 1990). This competitive antithrombin peptide blocks thrombin's catalytic site (Kettner 1990) and exhibits potent antithrombotic effects in several different animal models of arterial thrombosis (Knabb 1991; Kelly 1991). Two additional synthetic antithrombin peptides, D-Phe-Pro-Arg-H and D-MePhe-Pro-Arg-H have anticoagulant and antiplatelet effects when administered either intravenously or orally in a number of animal species (Bagdy 1992). These Benzamidine-based compounds also exhibit significant antithrombotic effects in vivo (Markwardt 1986).

Catalytic site-directed antithrombins are less specific than natural or bivalent antithrombin peptides. This may be a serious limitation because specificity is likely to be critical for achieving therapeutic efficacy without inducing toxic side-effects.

The direct thrombin inhibitors have been compared with heparin, aspirin or platelet

GPIIb/IIIa receptor antagonists in a number of different animal models of tPA-induced thrombolysis. In all of these studies the direct thrombin inhibitors proved to be more effective than other antithrombotic agents at accelerating thrombolysis and preventing reocclusion. In a coronary thrombosis model in dogs, hirudin was more effective than heparin, aspirin or a peptide RGD-containing analogue in accelerating tPA-induced thrombolysis (Fitzgerald 1989). The superiority of hirudin over heparin in preventing thrombosis during and after thrombolysis and in permanently inactivating clot-bound thrombin has also been demonstrated in a study using a rabbit jugular vein model (Agnelli 1990).

Both recombinant hirudin and hirulog are undergoing clinical testing for the prevention of venous thrombosis after surgery (Ginsberg 1992). In addition, the effectiveness of these agents is also being examined during coronary reperfusion therapy with thrombolytic drugs (TIMI 5 Trial 1993; Lidon 1993).

The relative antithrombotic and antihemostatic effects of a number of the direct antithrombins have been compared (Kelly 1991; Hanson 1988; Kettner 1990; Kelly 1989). All direct antithrombins tested interrupt platelet and fibrin deposition and prevent thrombotic occlusion in a dose-dependent manner.

3.9 Direct Factor Xa Inhibitors

Two ATIII-independent factor Xa inhibitors, a tick anticoagulant peptide (TAP) and a leech anticoagulant peptide (Antistasin), have been developed. TAP is a 60-amino acid-polypeptide which was originally isolated from the soft tick Ornithodoros moubata (Waxman

1990) and subsequently made recombinantly in yeast (rTAP) (Vlasuk 1991). It is a potent and selective inhibitor of factor Xa, which unlike heparin can access and inhibit factor Xa within the prothrombinase complex. rTAP has been shown to effectively prevent venous thrombus formation in rabbits (Vlasuk 1991) to suppress systemic elevations of FPA induced by intravenous administration of thromboplastin in conscious Rhesus monkeys (Neeper 1990), and to inhibit thrombosis in a silastic femoral arteriovenous shunt in baboons, a model which has been used extensively (Hanson 1988; Hanson 1988) to simulate arterial thrombosis produced under conditions of high shear.

The relative effects of rTAP, rHirudin, and heparin have been compared in a canine model of rtPA-mediated coronary thrombolysis (Shebuski 1990; Haskel 1991). Both rTAP and rHIR, but not heparin, significantly accelerated rtPA-mediated thrombolysis and prevented acute reocclusion. Heparin had a modest effect on enhancing thrombolytic reperfusion but failed to prevent or significantly delay reocclusion even in doses which elevated the APTT approximately 8-fold over baseline values.

Like TAP, recombinant antistasin (rATS) is a potent and selective inhibitor of factor Xa. Antistasin was originally isolated from the Mexican leech, Haementeria officinalis (Dunwiddie 1989; Nutt 1988; Nutt 1991). rATS has a molecular weight of 13,341 and its anticoagulant activity persists for over 30 hours after a single subcutaneous injection. This long duration of action reflects a prolonged period of absorption coupled with a rather long plasma half-life (Dunwiddie 1992). rATS exhibits no detectable inhibition of thrombin at molar ratios as high as 500 to 1 (Dunwiddie 1989). The in vivo antithrombotic effects of rATS following continuous intravenous infusion have been demonstrated in a rabbit model

of venous thrombosis (Vlasuk 1991) and a rhesus monkey model of mild disseminated intravascular coagulation (Nutt 1991).

The superiority of rTAP over heparin in experimental models of thrombolysis probably reflect the ability of the direct factor Xa inhibitor to access factor Xa with in the prothrombinase complex (Beguin 1988; Hemker 1987).

The specific Xa inhibitors, rATS and rTAP, have been reported also to reduce injury-induced stenosis in the femoral arteries of rabbits (Sarembock 1992). The antiproliferative effects of these agents were attributed to inhibition of thrombin generation.

3.10 Activated Protein C

Thrombin activates the natural antithrombotic zymogen protein C by cleaving the amino-terminal dodecapeptide when bound to thrombomodulin on the vascular endothelial membrane surface (Esmon 1989). APC inhibits coagulation and prolongs the APTT by inactivating activated factors V and VIII (factor Va and factor VIIIa) on endothelial and platelet surfaces. By so doing, APC inhibits thrombin generation induced by thrombin and factor Xa.

The effectiveness of APC as an anticoagulant has been shown by Hanson and colleagues (Hanson 1993) who induced the activation of endogenous protein C by the injection of low doses of thrombin into baboons with arteriovenous shunts. The activated protein C had antithrombotic effects which could be blocked with an antibody that prevents protein C activation.

Natural and recombinant forms of APC have been developed and studied in vitro and

in experimental models of thrombosis and hemostasis (Gruber 1989; Gruber 1990). APC inhibits platelet deposition in baboon models of acute arterial thrombosis (Gruber 1991), prevents experimental venous thrombosis, and interrupts re-thrombosis after experimental thrombolysis (Grinnell 1987). When combined with urokinase in baboons (Gruber 1991), APC had additive effects in preventing the accumulation of fibrin and platelets onto Dacron vascular grafts.

In contrast to the findings with antiplatelet agents and the direct antithrombins, the administration of APC in effective antithrombotic doses is not associated with any detectable prolongation of bleeding times (Gruber 1989; Gruber 1990). A soluble thrombomodulin has also been developed as potential means for generating APC endogenously (Hanson 1990). In addition, the possibility of engineering thrombin to promote selective endogenous activation of protein C is being evaluated (Wu 1991).

3.11 Tissue Factor Pathway Inhibitor

Exposure of blood to tissue factor contained in the depths of the lipid-rich atherosclerotic plaque is thought to be an important mechanism for reocclusion following successful thrombolysis (Haskel 1991; Weiss 1989; Wilcox 1989). Tissue Factor Pathway Inhibitor (TFPI) (formerly known as lipoprotein-associated coagulation inhibitor or LACI) (Broze 1988) forms a complex with activated factor X and this factor Xa-TFPI complex then binds to and inhibits activated factor VII within the tissue factor-factor VIIa complex. TFPI has been cloned and limited studies with recombinant TFPI have been performed in a canine femoral artery model. Thrombosis was induced by two methods and recanalization

was produced by tPA infusion. TFPI infusion prevented reocclusion following tPA in the arteries subjected to intimal injury (Haskel 1991). These findings suggest that tissue factor plays a role in reocclusion after successful thrombolysis and suggest a new approach for adjuvant antithrombotic therapy.

3.12 Other Agents for Prevention of Post-PTCA Re-Stenosis

Over the years a number of agents have been tested for prevention of post-PTCA re-stenosis after angioplasty. Each will be discussed in turn.

3.12.1 Omega-3 Fatty Acids

The classical epidemiological studies in Greenland Eskimos (Kronman 1980) have stimulated a great deal of interest in the possibility that fish oil could be beneficial in the prevention of cardiovascular diseases. Cod-liver oil or its products has been shown to inhibit atherosclerosis in a hyperlipidemic swine model (Weiner 1986) and intimal hyperplasia in autologous vein grafts in a normal dog (Landymore 1985). This may be due to a number of effects of omega-3 fatty acids. Thus, these agents have an antiaggregatory effect on platelets (Goodnight 1981), induce a favourable change in lipid profile (Harris 1983; Phillipson 1985; Slack 1987), and inhibit PDGF-like factors that may cause VSMC proliferation and migration (Fox 1988). To date, results of five clinical trials testing the effect of omega-3 fatty acids on post-PTCA re-stenosis have been inconclusive (Dehmer 1988; Grigg 1989; Milner 1989; Reis 1989; Slack 1987). In two of these studies (Dehmer 1988; Milner 1989) inhibition of re-stenosis was achieved, but in the other three trials (Grigg 1989; Reis 1989; Slack 1987)

no reduction in the rate of re-stenosis was observed.

3.12.2 Corticosteroids and Other Agents

Inflammatory cells including macrophages, lymphocytes, and neutrophils have been identified in the atherosclerotic plaque (Munro 1988). These cells release cytokines and growth factors (PDGF, bFGF, Interleukin 1, 6) which may contribute to the formation of the atherosclerotic plaque (Munro 1988). Since an inflammatory response is stimulated by vessel damage such as occurs with angioplasty, corticosteroids have been used in an attempt to reduce inflammation. Indeed, the corticosteroid agent dexamathasone and other anti-inflammatory agents have been reported to inhibit VSMC proliferation secondary to balloon-induced arterial injury in one experimental study (Gordon 1987) and one clinical trial (Hirayama 1992) . However, another clinical trial that evaluated pretreatment with methylprednisolone did not report a beneficial effect (Pepine 1990).

Intracellular calcium has been shown to regulate VSMC proliferation and vasospasm. Thus, inhibition of calcium transport (influx) by calcium channel blockers has the potential to inhibit these processes. The contribution of calcium to vasospasm (Willerson 1991) and to re-stenosis after angioplasty has been addressed in two clinical trials which evaluated the effect of calcium channel blockers. Neither diltiazem (Corcos 1985) or nifedipine (Whitworth 1986) had an effect on the rate of restenosis after angioplasty.

3.12.3 Agents Modulating Cholesterol and Lipid Profile

Elevated levels of LDL cholesterol are a well established risk factor for atherosclerosis.

Accordingly, the effect of lowering blood cholesterol using lovastatin and/or colestipol has been studied in patients undergoing angioplasty who have elevated cholesterol levels. The results of these studies are contradictory. Two clinical trials (Sahni 1989; Zhao 1992) have reported that lovastatin or the combination of lovastatin and colestipol therapy reduced the rate of re-stenosis after angioplasty, but in another study, lovastatin in combination with colestipol (Hollman 1989) failed to produce a beneficial effect.

3.12.4 Antagonist of PDGF: Triazolopyrimidine (Trapidil)

A role for PDGF in the pathogenesis of VSMC proliferation has been suggested. Accordingly, triazolopyrimidine, a PDGF antagonist, administered one week before and six months after PTCA was evaluated and found to have a small, but non significant beneficial effect on re-stenosis rates (Okamoto 1991).

3.12.5 Angiotensin Converting Enzymes (ACE) Inhibitors

Angiotensin II (ANGII) infused into rats with denuded carotid arteries doubles the normal rate of intimal thickening (Deamen 1991) and also enhances DNA synthesis and VSMC proliferation at the site of injury (Deamen 1991). Inhibition of angiotensin II (ANGII) receptors by losartan, an ACE inhibitor, has been reported to reduce balloon injury-induced myointimal proliferation in a rat carotid model (Kauffman 1991).

A number of experimental studies have documented that ACE inhibitors (cilazapril or perindopril) inhibit arterial intimal thickening induced by balloon injury in rat and guinea pig models (Powell 1991; Osterrieder 1991; Clozel 1991; Powell 1989) reduce intimal

hyperplasia in rat and rabbit carotid artery vein grafts, and in vascular allografts in rats (O'Donohoe 1991; Roux 1991; Plissonnier 1991). In other studies, ACE inhibitors did not inhibit VSMC proliferation in injured arteries and vascular grafts in baboons (Hanson 1991). A study by Clowes et al. (Clowes 1991) reported that a combination of heparin and cilazapril significantly reduced myointimal hyperplasia after balloon denudation in a rat carotid artery model. However, in one clinical trial, cilazapril administered for six months did not prevent re-stenosis or favourably influence the overall clinical outcome following PTCA (Serruys 1992).

The mechanism by which ACE antagonists inhibit VSMC proliferation is not clear. One theory proposes that bradykinin accumulates during ACE inhibitor therapy and stimulates EDRF and PGI_2 release from nearby uninjured endothelium; these vasodilators and platelet antagonists then suppress VSMC proliferation at the site of injury (Dzau 1991). Another theory proposes that ANGII regulates expression of VSMCs beta-transforming growth factor (beta-TGF) (Gibbons 1990), as well as autocrine PDGF-AA (Dzau 1991). Beta-TGF inhibits the proliferation of VSMCs, while PDGF-AA stimulates proliferation. Studies comparing the effect of basic-FGF and ANGII with basic-FGF alone on VSMC in culture have demonstrated that the combination potentiates DNA synthesis (Gibbons 1989). Presumably injury to the vessel results in the release of beta-TGF from the basement membrane, but circulating ANGII, platelet-derived PDGF-AB, and basic fibroblast growth factor, override the antiproliferative effects of beta-TGF. The net result is VSMC proliferation (Dzau 1991; Gibbons 1989; Lindner 1991; Lindner 1991).

3.12.6 Inhibitors of Insulin-Like Growth Factor-1 (Angiopeptin)

Exposure of cultured VSMC to PDGF results in release of insulin-like growth factor-1 peptide (ILGF1P) into the medium. The ILGF1P acts synergistically with PDGF to promote cell proliferation (Clemmons 1985). Inactivation of these growth factors would therefore have the potential to inhibit myointimal hyperplasia. Angiopeptin and other analogues of somatostatin have potent inhibitory effects on growth hormone release and ILGF1P production. In rats, rabbits and pigs, angiopeptin inhibits (Lundergan 1991; Calcagno 1991; Santoian 1992), intimal thickening that occurs in the carotid artery, a vein graft or a coronary artery after balloon injury. It has been postulated that angiopeptin may inhibit autocrine or paracrine regulation of cell growth. Since native somatostatin inhibits both lymphocyte proliferation and leukocyte migration, its synthetic analog angiopeptin may also exhibit antiproliferative effect in vivo (Lundergan 1991). However, this possibility remains to be tested.

3.12.7 Alpha-Tocopherol (Vitamin E) and Free Radical Scavengers

Vitamin E (Boscoboinik 1991) and superoxide dismutase (Rao 1992), free radical scavenging enzymes, have been reported to suppress VSMC growth in vitro (Boscoboinik 1991) and the antioxidant agent probucol has been shown to reduce intimal thickening in a swine model of re-stenosis (Schneider 1992). These observations support a role for active oxygen species (e.g. $O_2^{\cdot}$,H_2O_2, $^{\cdot}OH$) in the pathogenesis of the VSMC mitogenic response (Boscoboinik 1991; Rao 1992).

3.12.8 Cyclosporin A (CyA)

T-Lymphocytes and monocytes are found in atherosclerotic plaques (Munro 1988; Dartsch 1989) and in injury-induced myointimal proliferative lesions in the rat carotid artery (Hanson 1993). To explore the potential role of these cells in intimal thickening, CyA was administered to rats before balloon-injury. CyA inhibited intimal thickening two weeks after balloon injury (Hanson 1993). Further studies are needed to confirm these findings.

3.12.9 Antineoplastic Therapy (Colchicine, Vincristine and Actinomycin D)

The process of VSMC proliferation has some similarities to benign neoplasia. Accordingly, the possibility that antineoplastic therapy would prevent VSMC proliferation has been tested. Colchicine therapy (Currier 1989) was administered to rabbits undergoing angioplasty and was shown to inhibit post-PTCA re-stenosis. A combination of intravenous vincristine and actinomycin D therapy also suppressed VSMC proliferation in rabbits 3 days after endothelial injury to the aorta (Burath 1989). In addition to its inhibitory effect on post-PTCA re-stenosis in experimental animals, colchicine has also been demonstrated to have a beneficial effect on post-PTCA re-stenosis rates at 6 months follow up in patients (O'Keefe 1991).

3.12.10 Modification of Genes at the Angioplasty Site

Recent advances in DNA technology have made it possible to modify genes. Genetic material can be introduced into cells using lysosomes and/or viral vectors (Nabel 1991). Both endothelial and smooth muscle cells could be targeted and functionally modified to

increase their synthesis of heparan sulphate or other antiproliferative factors and so lead to the inhibition of atherosclerosis and post-PTCA stenosis (Nabel 1991; Lynch 1992). These possibilities are under active investigation.

3.12.11 Inhibitors of Signal Transduction Pathway of Cell Division

In response to vascular injury, a variety of mitogenic stimuli (such as PDGF and bFGF) induce activation of proto-oncogenes such as c-fos, c-myc, and c-myb. These observations have raised the possibility that nuclear proto-oncogene activation is a final common pathway through which many diverse mitogenic signals converge and lead to DNA synthesis and VSMC mitosis. Inhibition of this common pathway of cell proliferation by antisense oligodeoxynucleotides results in marked inhibition of VSMC proliferation both in cultured cells and in experimental animals (Shi 1993; Speir 1992). These findings provide an opportunity for a new strategy for prevention of post-PTCA re-stenosis. To date, most therapies used for the prevention of re-stenosis after angioplasty have attempted to inhibit the actions of a specific mitogen that acts on the VSMC surface.

3.12.12 Local Versus Systemic Administration of Antiproliferative Agents

To block restenosis, agents such as heparin, corticosteroids, hirudin, ACE inhibitors, and colchicine must be given in high doses. This results in adverse side effects which limit their clinical utility. Local administration of these agents at the angioplasty site allows lower doses to be used and may improve their applicability. The recent introduction of new

technology such as coronary angioscopy and echocardiography (Mancini 1991) provides a means for precise local administration of these agents (Bonan 1992; Hong 1992; Liu 1992; Villa 1992) using a new perforated angioplasty catheter (Wolinsky 1990; Wolinsky 1991).

3.13 New Devices and Techniques for Lumen Stenosis Correction

With the aid of advances in medical technology, new atherectomy devices (mechanical drills, laser or ultrasound catheters) have been developed (Kaltenbach 1989; Kensey 1987; Foschi 1990; Siegel 1988; Kaufman 1989; Sanborn 1991). Their major disadvantages are high rates of vessel wall perforation, associated with drill and laser techniques, and failure of ultrasound catheters to recanalize the non-calcified fibrotic lesion. A variety of intraluminal devices such as vascular stents have been developed and are currently under clinical evaluation (Schatz 1991; Seurruys 1991). The stent is introduced into the coronary artery on an angioplastic catheter. At the point of stenosis it expands and keeps the stenotic lumen open. However, to date the re-stenosis rates associated with stents has not been well documented. In an attempt to prevent re-stenosis, a slow release vehicle containing antiproliferative agents has been incorporated into stents (Levy 1992), but further evaluation is needed to assess the potential benefits of these approaches.

CHAPTER 4. MATERIALS AND METHODS

To explore the role of anticoagulant therapy in preventing rethrombosis following fibrinolysis and coronary post-PTCA re-stenosis, we developed animal model systems a) to compare heparin and ATIII-independent thrombin inhibitors in terms of their ability to

accelerate clot lysis and to prevent reocclusion after tPA-induced thrombolysis: and b) to evaluate various thrombin inhibitors in terms of their ability to prevent injury-induced stenosis.

4.1 Rat Model of tPA-Induced Thrombolysis

4.1.1 Reagents: Tissue-type plasminogen activator (Lot number L9124AX) was provided by Genentech, Inc., San Francisco, CA, while recombinant desulphatohirudin (rHH5-7033) was a gift from Dr. R.B. Wallis, Ciba-Geigy Pharmaceuticals, West Sussex, U.K. and hirulog (BG8967), was supplied by Biogen, Inc., Cambridge, MA. Unfractionated sodium heparin derived from porcine intestinal mucosa was purchased from Organon Teknika, Inc., and PPACK was obtained from Calbiochem Corp., San Diego, CA.

4.1.2 Surgical Procedures: Fifty-eight male Sprague Dawley rats (500-750g) purchased from Charles River of Canada were included in the study. The rats were anaesthetized with a mixture of 50% oxygen, 50% nitrous oxide, and 1 to 3% Isoflurane (Forane, Anaquets, A Division of Canadian Oxygen Limited, Mississauga, Ontario, Canada) introduced into an anaesthesia chamber of a rodent anaesthetic machine (Med-Vet Anaesthetic System Inc.,# 466 Briar Hill Avenue, Toronto, Ontario, Canada). Once induced, anaesthesia was maintained with the same mixture given by mask. Both external jugular veins and the right common carotid artery were then exposed through midline cervical incision. The right and left jugular veins were cannulated using polyethylene catheters (PE# 60, Intramedic Non-Radiopaque Polyethylene Tubing, Clay Adams, Division of Becton Dickinson and Company,

Parsippany, New Jersey, USA) attached to a 21 gauge needle (B-D needle 20 gauge 1 inch long, Becton Division and Company, Franklin Lakes, NJ, USA) for drug administration. The right common carotid artery was also cannulated for simultaneous recording of arterial blood pressure on a Dynograph R-711 physiologic recorder (Beckman Instruments, Inc., Fullerton, CA, USA). The right external iliac artery and the abdominal aorta were isolated through a midline laparotomy incision. A No. 2 Fr Fogarty catheter (Arterial Embolectomy Catheter # 2 Fr., Baxter Healthcare Corporation, Edwards LIS Division, Santa Ana, CA, USA) was then introduced into the right external iliac artery and a cuff-type ultrasonic blood flow meter (Model T101, Transonic Systems, Inc., Ithaca, NY, USA) was placed around the aorta immediately proximal to the bifurcation. After 10 minutes stabilization, the baseline blood flow was determined.

4.1.3 Induction of Arterial Thrombus: To produce endothelial injury, the Fogarty catheter was advanced into the distal abdominal aorta, the balloon was inflated with 0.25 to 0.3 ml of air, and the catheter was then passed up and down 13 times over a 30 mm length of aorta. After deflating the balloon, the Fogarty catheter was removed, and a catheter composed of PE# 50 tubing was placed in the right external iliac artery to monitor the post-stenotic arterial pressure. To produce stenosis, the segment of aorta immediately distal to the right renal artery was externally constricted by 3 ligatures tied 1 mm apart around a 21 gauge needle. The needle was then carefully withdrawn leaving the ligatures in place. This technique produces stable, high-grade stenosis. We demonstrated this stenosis in early pilot studies using contrast media, and defined the extent of stenosis by angiography, (2-3 ml of

Omnipaque 350 Winthrop Laboratories, Aurora, Ontario, Canada).

Two tourniquets were then placed 5 mm apart above and below the stenotic portion of the aorta, and the lumen was totally occluded for 30 minutes. The combination of endothelial injury and vascular stasis resulted in spontaneous thrombus formation. The tourniquets were then removed. Blood flow, and pre- and post-stenotic arterial pressures were continuously monitored. Animals were excluded from further study if there was spontaneous clot lysis (n=3), if t-PA failed to produce thrombolysis (n=7), or if the animal died prior to completion of the study (n=4). Thus, a total of 44 rats completed the study.

4.1.4 Experimental Protocol: All animals were given a 1 mg/kg bolus of t-PA, followed by an infusion of 1 mg/kg over 30 minutes. In addition, the animals were randomly assigned to one of five treatment groups; Group I: bolus of 1 ml/kg saline followed by a maintenance infusion of 2 ml/kg/hr over 80 minutes; Group II: bolus of 40 U/kg heparin followed by a maintenance infusion of 40 U/kg/hr over 80 minutes; Group III: bolus of 1 mg/kg hirudin followed by a maintenance infusion of 2 mg/kg/hr over 80 minutes; Group IV: bolus of 0.6 mg/kg hirulog followed by a maintenance infusion of 2 mg/kg/hr over 80 minutes; and Group V: bolus of 0.5 mg/kg PPACK followed by a maintenance infusion of 1.4 mg/kg/hr over 80 minutes. In each group, the volume of infusion was identical.

The doses of antithrombotic agents were chosen based on pilot studies which demonstrated that these amounts prolonged the activated partial thromboplastin (APTT) time 4-fold over baseline values. All drugs were initiated 10 minutes after the release of occlusive tourniquets. The t-PA infusion was given over 30 minutes, whereas the saline cr

antithrombotic agents were administered for a total of 80 minutes. Thus, the antithrombotic agents or saline were administered for an additional 50 minutes after the t-PA infusion was terminated. In this way, the effect of these drugs on reocclusion could be examined.

4.1.5 End Points: The systemic, and pre- and post-stenotic arterial pressures and blood flow were recorded continuously. Based on these measurements, the following end points were determined: (a) the time to lysis; the time (in minutes) required to restore blood flow, (b) the number of reocclusions (a reocclusion was defined as a fall in blood flow and post-stenotic arterial pressure to values recorded prior to t-PA infusion); (c) percentage of time the vessel remained patent (the total amount of time that the vessel was patent was expressed as a percentage of the total 80 minutes study period); and (d) the number of reocclusions per hour.

4.1.6 APTT Analysis: The APTT was measured before, and 15, 30, 45 and 80 minutes after initiating drug treatment. Instantaneous APTT measurements were obtained by placing three drops of whole blood drawn directly from an arterial line into a Biotrack Coagulation Monitor fitted with an APTT test cartridge (Ciba-Corning Diagnostic Corp., Medfield, MA, USA).

4.1.7 Statistical Analysis: Analysis of variance was used to compare the data in the various treatment groups. The Duncan test was used for multiple comparisons.

4.2 Rat Model of Injury-Induced Stenosis

4.2.1 Pilot Study: Assessment of Anticoagulant Effects of Stdh, Lmwh, Hirudin in Rats

The effect of stdh, lmwh, hirudin, and saline on blood coagulation assays was established in 20 male, retired breeder Sprague-Dowley rats (purchased from Charles River of Canada) with average weight of 450 g. Each agent was tested in 5 rats.

Anaesthesia: Anesthesia was induced using a mixture of 50% oxygen, 50% nitrous oxide and 1 to 3 % of isoflurane (Forane, Anaquest, Mississauga, Ontario, Canada, A Division of Canadian Oxygen Limited) delivered with a rodent anaesthetic machine (Med-Vet Anaesthetic System Inc., Toronto, Canada) into an induction chamber. Anaesthesia was maintained with the same gas mixture delivered by a face mask.

Surgery: The ventral area of the neck was shaved and surgically prepared. The left common carotid artery was cannulated and used for blood collection. The free end of the cannula was occluded with a stainless steel pin, tunnelled subcutaneously to the nape of the neck and exteriorized. The cannula was secured in place with 2 sutures and the skin was closed in a routine manner. Following the surgery, the rats were randomly allocated into one of five treatment groups.

Anticoagulant Treatments: All rats received single sc. injection of the following agents: group I (n=5) received stdh in a dose of 2 mg/kg, group II (n=5) received low molecular weight heparin (Enoxaparin$^{R)}$ in a dose of 10 mg/kg, group III (n=5) was given hirudin, 6 mg/kg, whereas group IV (n=5) served as a control and was given 1 ml/kg of saline.

Reagents: Stdh and hirudin were of the same origin as those ones used in the

fibrinolytic study. Lmwh (Enoxaparine^{R}, 100 anti-Xa units/mg) was provided by Rhone-Poulenc, 8580 Esplanade, Montreal, Quebec, Canada.

Blood Sample Collection: At 0.5, 1, 3, 6 hour intervals after sc. injection of hirudin, and also at 12 hours following the sc. injections of stdh, lmwh and saline, 700 μl blood was collected into microcentrifuge tubes (#500, Quality Scientific Plastic) prefilled with 70 μl of 3.8% trisodium citrate. Blood volume lost as a result of blood collection was replaced with an equivalent volume of Lactated Ringers' solution. After mixing the blood with the anticoagulant, the samples were centrifuged for 15 minutes at 10,000 rpm using a Fisher microcentrifuge model 235A (Fisher Scientific Company, USA). The platelet poor plasma was then removed and stored in aliquots at -70°C for thrombin clotting time determination.

Assays: Thrombin clotting times (TCT) were measured using 2 U/ml of thrombin (Park Davis, Canada) in a calcium buffer system. Fifty microliters of rat plasma was added to 150 μl of buffer containing calcium and 50 μl of thrombin. The end point, fibrin formation, was determined using a semi-automated instrument, ST4 (Diagnostica Stago , Wellmark, Guelph, Ontario, Canada).

At each time interval, the activated partial thromboplastin time (APTT) was instantaneously measured by collecting blood from the arterial cannula directly into an APTT test cartridge fitted into a Biotrac Coagulation Monitor (Ciba-Corning Diagnostic Corp., Medfield, MA, USA).

Statistical Analysis: Analysis of variance (ANOVA) was used to evaluate the differences between means, and unpaired student t-tests were used to compare the results of each time interval within the different treatment groups.

4.2.2 Pilot Study: Evaluation of Degree of Balloon-Induced Injury in Rat Common Carotid Artery

Sixteen Spraque-Dawley male rats weighing between 500 to 650 g (retired breeders), were purchased from Charles River of Canada. After anesthesia was induced, as previously described, the ventral cervical area was shaved and the external carotid artery was exposed through a midline cervical skin incision by splitting the cervical muscles at the level of the mandibular angle. A 5 mm segment was ligated distally with a 4-0 silk suture and a small tourniquet (consisting of 4-0 silk suture covered with PE# 60 tubing) was then placed proximally (just proximal to the external carotid ramification of the common carotid artery) to occlude blood flow. A Fogarty catheter (# 2 Fr.) was inserted through a small arteriotomy and was advanced through the common carotid artery into the aortic arch. The balloon was then inflated with 0.3 ml of air and the vessel wall was damaged by ten passes over the entire length of the artery. The catheter was removed, and after closure of the arteriotomy with a 4-0 silk suture, the skin incision was closed using 4-0 Dexon interrupted sutures. The rats were then randomly allocated into four groups (4 rats/group) and the extent of arterial injury was evaluated using four different methods, (a) quantitative angiography (n = 4), (b) Evan's blue staining (n = 4), (c) light microscopy (n = 4), and (d) scanning electron microscopy (n = 4). Each of these methods is briefly described below.

(a) Quantitative Angiography: A catheter (PE3 180 tubing connected to B-D # 16 gauge. 1.5 inch needle) was introduced into the left ventricle through a small sternotomy incision. A ventro-dorsal radiograph was taken after injecting 3 ml of Omnipaque # 350 (Winthrop Laboratories, Aurora, Ontario, Canada) using a Phillips 6 inch Image Intensifier

(XG-3000-SPX-5.67), and Super 100 Three-Phase X-ray Generator (Holland) set to 60 kV and 1/70 seconds. The X-ray image was projected onto an 8 x 10 inch cassette (Hasley X-Ray Products Inc., Brooklyn, New York, USA) that was positioned under the rat and filled with Kodak TML film (Kodak Canada Inc., 3500 Eglinton Avenue West, Toronto, Ontario, Canada). Exposed film was processed using a Kodak RP X-Omat Processor (Model # MGAM, Kodak Canada Inc.). The luminal diameters of the injured and control carotid arteries were measured directly from the radiograph positioned on a X-ray film viewer using a Mitutoyo digital calliper (Model CD-6" P, ITM Instruments Inc., 5715 Kennedy Road, Mississauga, Ontario, Canada).

(b) Evans Blue staining: Rats were given 50 mg/kg Evans Blue (Sigma Chemical Company, St. Louis, MO, USA) which was diluted in 1 ml of saline and given as a bolus into the penile vein immediately after balloon injury. The animals were euthanized 30 minutes later with a barbiturate overdose. Just prior to euthanasia, each rat was given 1,000 IU of stdh (Leo Laboratories, Ajax, Ontario, Canada) intravenously through the penile vein. Both common carotid arteries were then resected, opened longitudinally, and photographed.

(c) Light Microscopy: After draining the blood by transecting the external jugular veins and the cranial vena cava, the rats were infused with Kreb's solution containing 1 U/ml heparin followed by formaldehyde under 100 mm Hg pressure. After pressure fixation, the carotid arteries were resected, and 3-mm cross sectional segments from the middle part of both the control and denuded carotid arteries were submitted for light microscopic evaluation. The sections were embedded in paraffin and 5-μm sections were stained with haematoxylin-eosin and viewed at 10, 20, 100, and 400 X magnification.

(d) Scanning Electron Microscopy (SEM): After balloon injury, the abdominal aorta was cannulated under partial aortic occlusion using PE# 180 tubing fitted to a 16 gauge needle. Heparin (1,000 IU/kg) was given, and 60 minutes later the rats were euthanized with an intravenous injection of 250 mg of Pentobarbital. Both external jugular veins and the cranial vena cava were transected for venous drainage, and the arterial tree was then perfused with Kreb's solution for 10 minutes and then pressure-fixed by infusion of 2.5% glutaraldehyde for five minutes under 100 mm of Hg pressure. Both carotid arteries were resected and prepared for SEM as follows. The carotid arteries were opened longitudinally and the adventitial surface was glued onto a coverglass with cyanoacrylic glue (Eastman Kodak 910, Toronto, Canada). The flattened and glued arteries were then immersed for two hours in 1% OsO4 buffered with 0.1 M sodium cacodylate, after which they were dehydrated in a Bomar Spec 90 critical point drier, sputter coated with gold for 1.5 minutes using a Polaron sputter-coater, and then examined with a Philips 501 B scanning electron microscope at 15 kV accelerating voltage.

4.2.3 Effect of Thrombin Inhibitors on Injury-Induced Carotid Artery Stenosis:

Experimental Procedures

A total of 75 Sprague-Dawley male rats (retired breeders from Charles River of Canada) weighing 500 to 550 grams was used for these studies. The rats were randomly allocated into one of five treatment groups. The first dose of the selected treatment (labelled I to V) was intravenously administered through the penile vein immediately prior to balloon injury. Subsequent treatment was given by sc. injections at appropriate time

intervals. Both the surgeon and the technician were blinded as to treatment allocation, with the exception of the sham group. Treatment groups consisted of:

Group I (n=15), saline 0.5ml/kg sc., b.i.d. for 7 days (1 ml/kg/day).

Group II (n=15), stdh in a dose of 2 mg/kg sc., b.i.d. for 7 days (4 mg/kg/day in 1 ml).

Group III(n=15), lmwh 10mg/kg sc., b.i.d. for 7 days (20 mg/kg/day in 1ml).

Group IV (n=15), hirudin 6mg/kg sc.,q.i.d. for 7 days (24 mg/kg/day in 1 ml).

Group V (n=10), sham, skin incision only.

Once the first dose of treatment was given to the rats, Groups I-IV then underwent balloon injury to either the left or right common carotid artery. In contrast, the 10 rats in Group V served as a control group and underwent skin incision but no carotid injury was performed (sham).

Reagents: Stdh, hirudin, and lmwh were obtained from the same sources as those used in the pilot study.

End Point Assessment

Two weeks after balloon injury, all 75 rats were euthanized and the extent of stenosis was evaluated using three different methods, a) quantitative angiography, b) resin casting, and c) planimetry. In each case, the damaged vessel was compared with the noninjured vessel. The techniques are described below.

(a) Quantitative Angiography Procedure: Quantitative angiography was performed as described in Section 4.2.2a and in each animal the diameter of the left and right common carotid artery was compared.

(b) Resin Casting Procedure: After pressure fixation, five millilitres of resin cast was

prepared according to the factory recommendations using Batson's #17 resin cast kit (Analychem Corporation Ltd., Victoria Pk. Avenue, Markham, Ontario, Canada) and was infused under 100 mm of Hg pressure via the thoracic cannula for approximately 45 minutes. Once hardened, the aortic and carotid artery resin casts were resected together with the surrounding arterial wall sheath and were stored in 10% buffered formaldehyde solution. The tissue sheath was slipped over the cast and used for histological assessment. Resin casts were flushed in tap water and then stored.

(c) Planimetry: The most stenotic segment of the injured carotid artery was identified by examining the angiographic films and the resin cast. A 3 mm section containing the most stenotic area was resected together with the corresponding segment of the contralateral noninjured vessel and was submitted for histology. Each carotid artery segment was embedded in paraffin, cut into 5 μm cross-sections, stained with haematoxylin-eosin, and then assessed by planimetry.

Lumen Assessment by (a) Angiography, (b) Resin Cast, and (c) Planimetry

a) Angiographic assessment: The diameters of the most stenotic portion of the injured artery and the corresponding uninjured artery were measured using a Mitutoyo digital calliper directly from X-ray films. The cross-sectional areas of the most stenotic segment of the injured and the corresponding uninjured artery were calculated and the luminal area (i.e. stenosis) of the injured vessel was expressed as a difference in % of the cross-sectional area of uninjured artery - injured artery as follows:

$$\% \text{ Stenosis} = 100 - (\pi r^2_{INJ} / \pi r^2_{UN}) \times 100$$

b) Resin cast assessment: A 10 mm segment that contained the most stenotic lesion

was cut from the cast of the injured artery and a corresponding segment was cut from the uninjured vessel using a specially designed cutter. The cast segments were weighed on an electronic balance (Mettler basic balances # BB 244, Mettler Instrument AG, CH-8606 Greifensee, Switzerland). As an index of stenosis, the difference between the weight of the cast of the control artery and the injured artery were expressed as a percentage using the following formula:

$$\% \text{ Stenosis} = 100 - [(wt_{INJ}/wt_{UN}) \times 100]$$

where wt_{INJ} = weight of injured carotid cast

and wt_{UN} = weight of uninjured carotid cast

(c) Morphometric/Planimetric Analysis: Using magnified prints of histologic slides of carotid cross-sections the lumen-neointima boundary (B), internal elastic lamina (I.E.L.) and the external elastic lamina (E.E.L.) circumference were measured using a computerized planimetric system (Kontron Mop-Videoplan, Carl Zeiss Canada Ltd., 45 Valleybrook Dr., Don Mills, Ontario, Canada, M3B 2S6). The luminal intima and medial areas were calculated by first using the formula Perimeter (P) = 2π r. From the equation for perimeter, the radius (r) of each of the boundary, I.E.L. and E.E.L. was calculated for both the injured and uninjured arteries. The Luminal Area (Ao) of both the injured ($A0_{inj}$) and uninjured arteries ($A0_{un}$) was then calculated using the formula $Ao = \pi r^2$. The cross-sectional area of lumen of injured artery was expressed as a percentage of the uninjured artery using the formula $\% = [(A0_{INJ}/A0_{UN}) \times 100]$.

Intimal Thickening (A_1): The cross-sectional area of intima (neointima) was calculated from the injured internal elastic lamina ($I.E.L._{INJ}$) and boundary (B) as follows:

$A_1 = \pi \times (I.E.L._{INJ}/ 2\pi)^2 - \pi \times (B/ 2\pi)^2$

The neointimal area of injured artery was expressed as a percentage (%) of luminal cross -sectional area of control artery using the following formula:

% of Intima = $[(A_{1\ INJ}/ A_{1\ UN}) \times 100]$

Medial area (A_2)

The cross-sectional area of media was calculated as a difference between area calculated from E.E.L. and I.E.L. for both injured and uninjured vessels as follows:

$A_2 = \pi \times (E.E.L./2\pi)^2 - \pi \times (I.E.L./ 2\pi)^2$

Difference in Area = A_2 un. - A_2 inj. (A_2 un. = 100%)

A positive number indicates a decrease in the cross-sectional area of media of the injured artery while a negative number indicates an increase in the area of the injured artery. In the final analysis, the calculated medial cross-sectional area of the injured artery was expressed in a percentage (%) of the area of contralateral uninjured artery as follows:

% of vasoconstriction = 100 - $[(A_3$ un. / A_3 inj.) x 100]

$A_3 = \pi \times (I.E.L./ 2\pi)^2$

CHAPTER 5. RESULTS

5.1 Effect of Adjunctive Therapy Consisting of Heparin or ATIII-Independent Thrombin Inhibitors on tPA Induced Fibrinolysis and Rethrombosis in Rat Aortic Model of Thrombosis

Arterial Thrombus Formation and Time to tPA-Induced Lysis

A total of 44 rats was investigated. The application of the external constrictor produced > 95% stenosis of the distal aorta as demonstrated by angiography (figure 2). The combination of stasis and endothelial damage resulted in formation of a thrombus rich in red blood cells and fibrin (figure 3), which occluded the vessel and eliminated blood flow. The time to restoration of blood flow after tPA administration represents the time to clot lysis. The mean times to clot lysis and their associated SE were as follows: saline (n=12) 10.5 ± 1.6, heparin (n=7) 6.7 ± 2.2 , hirudin (n=7) 7.4 ± 2.2, hirulog (n=9) 4.4 ± 1.9 and PPACK (n=9) 4.2 ± 1.9 minutes . Hirulog and PPACK administration significantly (p=0.02) shortened the time to t-PA-induced lysis as compared to the saline control, whereas heparin and hirudin did not (figure 4).

Restoration of Blood Flow and Reocclusion

t-PA restored blood flow in all animals included in the study. However, with concomitant saline treatment, there was cyclical lysis and reocclusion (figure 5). Although heparin had virtually no effect on this pattern of blood flow, all of the other agents significantly ($p < 0.002$) reduced the number of reocclusions and significantly ($p < 0.002$) increased the portion of time that the vessel remained patent in the 80-minute period of observation (figure 6). Comparing the number of reocclusions during the time the vessel remained patent, hirudin, hirulog and PPACK significantly ($p < 0.05$) reduced the number of reocclusions whereas heparin did not (figure 7).

APTT Results: There was no prolongation of the APTT in the control animals given saline and t-PA (figure 8). In contrast, heparin, hirudin, hirulog and PPACK produced an approximately 4-fold prolongation of the APTT significantly. Except at the 80 minute time

point, heparin and hirudin produced a greater prolongation of the APTT than did PPACK or hirulog.

5.2. Rat Model of Injury-Induced Carotid Artery Stenosis:

5.2.1 Pilot Study: Assessment of Anticoagulant Effect of Stdh, Lmwh, and Hirudin in Rat Model:

Stdh (2mg/kg) prolonged the APTT more than 7-fold and the TCT more than 6-fold over the baseline values throughout the 12 hour observational period.

Lmwh (10mg/kg) prolonged the APTT 2.5-fold over the baseline value during the first three hours; however, by 6 hours, the APTT had returned to near baseline levels.

Hirudin (6mg/kg) prolonged the APTT 2.5-fold and the TCT more than 4.5-fold over the baseline for the entire observational period of 6 hours.

Saline (1ml/kg) had no effect on the APTT or TCT.

The effects of stdh, lmwh, hirudin and saline treatment on coagulation assays (APTT and TCT) at 0, 0.5, 1, 3, 6 and 12 hour time intervals after sc. injection are summarized in figure 9.

5.2.2 Pilot Study: Evaluation of Degree of Balloon-Induced Injury in Rat Common Carotid Artery:

Evans Blue: All denuded arterial surfaces stained blue while control flow surfaces remained unstained (see figure 10) since the Evans blue only binds to subendothelial structures and does not stain intact endothelium.

Light Microscopy: Occasional tears in the intima and/or media were seen. No intact endothelium remained after balloon injury and occasional platelet aggregates, white cells, and fibrin were seen on the subendothelial surface (see figure 12).

Scanning Electron Microscopy: No intact endothelium remained in the balloon-injured arteries. Clumps of platelets and fibrin were occasionally seen (see figure 11).

Angiographic Evaluation of Lumen Following Balloon Injury

There was no significant difference in lumen diameter between injured and uninjured common carotid artery immediately after the balloon injury. A representative example of an angiogram of the common carotid arteries immediately after balloon injury to the left common carotid artery is shown in figure 13.

5.2.3 Effect of Thrombin Inhibitors on Injury-Induced Carotid Artery Stenosis :

Lumen Assessment by Angiography

An example of an angiogram two weeks after balloon injury to the left common carotid artery is shown in figure 14. In the control (Saline group) balloon injury produced significant ($p<0.0002$) stenosis when compared with the sham group (22.9 ± 3.0 and -0.04 ± 1.6%, respectively). Standard heparin and hirudin did not significantly reduce the amount of stenosis. In contrast, Lmwh significantly ($p < 0.008$) reduced injury-induced stenosis from 22.9 ± 3.0% to 8.8 ± 7.8%.

These results are summarized in figure 15 and table I.

Stenosis Assessment as determined by Resin Casting

An example of a resin cast of the common carotid arteries and the aortic arch made

two weeks after balloon injury is shown in figure 16. A 10 mm segment of resin cast that contained the most stenotic segment was cut from the damaged carotid artery and a corresponding segment was also cut from the noninjured vessel. The weight of these segments were then compared, with the difference reflecting the degree of injury-induced stenosis. Using this surrogate index of stenosis, the saline treated control animals developed significant ($p < 0.0002$) stenosis 2 weeks after balloon injury when compared with the sham treated group (46.7 ± 4.8% and 1.9 ± 3.5%, respectively). Treatment with stdh or hirudin had no significant effect on the extent of stenosis (34.9 ± 5.9% and 33.4 ± 7.3%, respectively). In contrast, treatment with lmwh significantly ($p < 0.006$) decreased the stenosis from 46.7 ± 4.8% to 22.5 ± 8.3%). Figure 15 and table I summarize these results.

Intimal Thickening Assessment by Planimetry at the Point of Maximal Stenosis

Histologic cross sections of injured and noninjured common carotid arteries are shown in figure 18.

Balloon injury caused considerable neointima formation. Thus, compared to the sham group in which there was no measurable intima, there was a 21.8 ± 3.4% increase in the percent of the lumen occupied by neointima ($p < 0.001$). Stdh and hirudin had no effect on neointima formation (22.0 ± 4.1 and 21.9 ± 2.2%, respectively). In contrast, lmwh significantly ($p < 0.005$) reduced the neointimal cross-sectional area from 21.8 ± 3.4% to 11.3 ± 2.1%. These results are summarized in figure 17 and table I.

Effects of Balloon Injury on the Cross-Sectional Area of Media as Assessed by Planimetry: Balloon injury produced a 28.6 ± 7.3% increase in the cross-sectional area of media. The cross-sectional areas of media were 15.0 ± 8.9%, 19.9 ± 6.9%, and 13.2 ± 5.3%

in animals given stdh, hirudin, and lmwh, respectively; values were not significantly different from that in the saline control group. Thus, none of the treatments significantly affected the increase in the cross-sectional area of media that occurred after injury. The results are summarized in figure 19.

Balloon Injury-Induced Vasoconstriction as Determined by Planimetry: There was a trend for more vasoconstriction in the animals given stdh, lmwh, and hirudin than was seen in the saline-treated control group (figure 20), but these differences were not significant.

CHAPTER 6. GENERAL DISCUSSION

6.1 Rat Model of tPA Induced Thrombolysis:

Our rat model of tPA-induced thrombolysis was developed in order to determine whether thrombin inhibitors administered in doses which produced equivalent antithrombin effects ex-vivo are equal in terms of their ability to accelerate lysis and to prevent reocclusion. In this model, an external tourniquet was placed around the distal aorta with extensively damaged intima. The combination of vascular injury and severe stenosis (figure 2) resulted in formation of a thrombus rich in red cells and fibrin (figure 3). Because of the marked stenosis (figure 2), cyclical lysis and reocclusion followed successful tPA-induced thrombolysis and restoration of blood flow. This process is similar to events seen in other animal models (Hugh 1977; Gold 1988; Shebuski 1990), and in patients (Pompa 1991).

Using this arterial thrombolysis model, we evaluated the effectiveness of a number of thrombin inhibitors when used as adjuncts in tPA-induced thrombolysis. In doses producing equivalent anticoagulation (as determined by APTT, see figure 8), ATIII-

independent inhibitors are more effective than heparin at preventing reocclusion (figures 5 and 6) and increasing the percentage of the time that the vessel remains patent (figure 6). Furthermore, both hirulog and PPACK accelerate the time to clot lysis (figures 4 and 5).

The aim of adjuvant therapy is to limit fibrin accretion during the thrombolytic process and to prevent reocclusion after thrombolysis. There is evidence that thrombolytic therapy induces a procoagulant state which retards clot lysis. The procoagulant state is only partially inhibited by heparin (Rapold 1990; Weitz 1991). The activation of the coagulation system during thrombolysis is believed to occur through a number of mechanisms. Thrombin bound to fibrin (Weitz 1990), to the subendothelial matrix (Bar-Shavit 1989), or to soluble fibrin degradation products (Weitz 1991) is protected from fluid-phase inhibitors and hence can amplify the coagulation pathways. Plasmin can contribute to thrombin generation by directly activating prothrombin (Eisenberg 1988), factor V (Lee 1989), or contact factors (Ewald 1993).

While heparin is effective at inhibiting free thrombin and thrombin generated by the action of plasmin (Eisenberg 1988), it is limited in its ability to inactivate thrombin bound to fibrin (Weitz 1990), fibrin degradation products (Weitz 1991), or to the subendothelial matrix (Bar-Shavit 1989). In contrast to heparin, the ATIII-independent inhibitors are able to inhibit thrombin bound to these sites. As a result, the direct thrombin inhibitors are better than heparin at preventing rethrombosis, and PPACK and hirulog also accelerate thrombolysis.

Using various experimental animal systems and humans, other investigators have also demonstrated that ATIII-independent thrombin inhibitors shorten the time to lysis and

prevent reocclusion equally or more effectively than heparin (Gold 1988; Shebuski 1990; Neuhaus 1993; Lidon 1993). Recent studies in humans support these concepts. Thus, Lidon et al. (1993) performed a phase II study in patients with acute myocardial infarction that compared hirulog with heparin in terms of their effect on early angiographic patency rates when used in conjunction with streptokinase. A total of 45 patients entered the study, and patients were randomized in a two-to-one fashion to receive either hirulog (30 patients) or heparin (15 patients) concomitant with streptokinase (1.5 million U over 90 minutes) and aspirin (325 mg). Heparin was given at a dose of 1000 U/hr, adjusted according to the activate partial thromboplastin time, whereas hirulog was started at a dose of 0.5 mg/kg/hr, and after 12 hours, the dose was increased to 1.0 mg/kg/hr. Coronary patency (TIMI grade 2 to 3) was higher in the hirulog group than in the heparin group at 90 minutes (82% and 29%, respectively; $p < 0.01$) and 120 minutes (86% and 43% respectively; $p < 0.005$). In addition, a trend was found for less bleeding in the hirulog group. These findings indicate that hirulog is better than heparin at accelerating streptokinase-induced thrombolysis presumably because it is a more effective inhibitor of clot-bound thrombin.

The superiority of the direct thrombin inhibitors on heparin was also shown in a second study (TIMI 5 Trial Group 1993). This study included 214 patients with acute myocardial infarction who received t-PA and aspirin and were randomized to receive either concomitant hirudin or heparin. Patency at 18 to 36 hours was higher in patients given hirudin than in those given heparin, and there were fewer reocclusions. These beneficial effects were seen in the absence of increased bleeding events. Thus, the studies in humans support our findings in experimental animals that the direct thrombin inhibitors are better

than heparin in the setting of thrombolytic therapy.

6.2 The Effect of Thrombin Inhibitors on Injury-Induced Carotid Artery Stenosis:

Smooth muscle cell hyperplasia, vasoconstriction and thrombosis are involved in the pathogenesis of stenosis seen in atherosclerosis (Fuster 1992; Fuster 1992) and in the re-stenosis which occurs in about 30% to 40% of cases following PTCA (Leimgruber 1986; Mata 1985; Chesebro 1987). A role for thrombin in the mechanism of stenosis has been suggested, since this enzyme has the potential to regulate all of these processes (Moon 1989; Inoue 1989; Yanagisawa 1988; Fenton 1988; Vu 1991; Vu 1991; Hung 1992; Coughlin 1992; Glenn 1988; Carney 1992). Recently thrombin has also been shown to stimulate VSMC proliferation (Bar-Shavit 1990; Wilcox 1992; Vu 1991; Vu 1991).

To test the possibility that thrombin modulates injury-induced stenosis, we used stdh, lmwh and hirudin at concentrations that produced similar prolongation of the TCT (figure 9). All of the agents were administered for one week.

In this model, balloon injury was used to produce endothelial denudation (figure 10 and 11) and medial damage (figure 12). Within two weeks, balloon injury caused significant stenosis of the carotid artery as assessed by angiography (figure 15a), resin casting (figure 15b), and morphometry. The morphometric studies demonstrate that this stenosis is due to neointimal hyperplasia (figure 17).

In our model system, there was little vasoconstriction 2 weeks after balloon injury. In contrast, other investigators (Clowes 1983) have suggested that vasoconstriction is an important contributor to the stenosis that occurs after vascular injury. Although the reasons

for these differences are unclear, we took great efforts to limit the amount of vasoconstriction. For example, in pilot we ensured that the resin casting material and the Omnipaque did not induce vessel constriction.

In the present study, only lmwh inhibited injury-induced stenosis. Standard heparin and hirudin failed to block the stenotic process when used at concentrations with equivalent anti-thrombin activity. The failure of hirudin to prevent stenosis suggests that the effectiveness of lmwh is not the result of its ability to block thrombin. This concept is supported by the work of other investigators who demonstrated that non-anticoagulantly active fractions of heparin have antiproliferative effects both in cultures of smooth muscle cells (Castellot 1984; Castellot 1987; Wright 1989) and in vivo (Guyton 1980). In fact, heparin fragments as small as hexasaccharides have antiproliferative effects in tissue culture systems (Castellot 1984; Castellot 1987; Wright 1989).

Unlike lmwh, standard heparin did not block stenosis in our model system. However, we used these agents in concentrations that produced equivalent prolongation of the TCT. Thus, the total daily dose of lmwh was 20 mg/kg while that of standard heparin was only 4 mg/kg. Since lmwh has a mean molecular weight about one-third that of standard heparin (5,000 and 15,000, respectively), we estimate that we delivered about 15-times more molecules of lmwh than standard heparin. Based on these considerations, the lower dose of standard heparin is the most likely explanation for its failure to exert an inhibitory effect on stenosis. This concept is supported by a review of the literature that demonstrates a beneficial effect of standard heparin on blocking stenosis (Clowes 1985; Wilson 1991; Dryjski 1988; Guyton 1980; Currier 1991; Edelman 1994) when it is used in daily doses of

7.2 to 24 mg/kg. In contrast, we used only 4 mg/kg/day.

The mechanism by which non-anticoagulantly active heparin prevents smooth muscle cell proliferation is unknown. Our studies are consistent with the concept that this effect is not due to thrombin inhibition. It has been shown that heparan sulfate, which is synthesized by vascular smooth muscle cells and endothelial cells (Kjellen 1980; Kanwar 1979; Hedman 1982; Fedarko 1986; Fritz 1985), has antiproliferative properties (Castellot 1981; Fritz 1985; Wright 1989; Castellot 1987; Castellot 1982). Vascular injury causes the release of heparitinase from alpha-granules of platelets (Wright 1989) which can degrade surface-bound heparan sulfate thereby converting the smooth muscle cells into a synthetic phenotype. By competing with heparan sulfate for heparitinase, large concentrations of heparin or low molecular weight heparin prevent degradation of surface-bound heparan-sulfate (Mutoh 1993). Since these agents act as competitive inhibitors, their effects are dose-dependent (Castellot 1984; Reilly 1986; Wright 1989). Only high concentrations (Clowes 1985; Wilson 1991; Dryjski 1988; Guyton 1980; Currier 1991) will have this protective effect thereby explaining why low molecular weight heparin, but not standard heparin, was effective in our study.

We conclude that (a) thrombin does not play a significant role in the pathogenesis of stenosis and intimal thickening following angioplasty and (b) the inhibitory effect of lmwh molecule on injury-induced stenosis reflects its nonanticoagulant fractions as suggested by others (Castellot 1984; Wright 1989).

6.3 Future Directions

Thrombolysis:

Our studies have demonstrated that the direct thrombin inhibitors are better than heparin at accelerating pharmacologic thrombolysis with t-PA. Further experiments could be done to determine whether these agents also accelerate endogenous fibrinolysis. To examine this possibility, we have developed a rabbit model of jugular vein thrombosis. In a pilot study, rabbits with jugular vein thrombi were randomized into three treatment groups. Group 1 received 12 hours of treatment with hirudin, Group 2 received 12 hours of treatment with heparin while Group 3 served as controls and were given 12 hours of saline. Approximately 60 h later the animals were killed and the clot size was assessed. Whereas the jugular thrombi had doubled in size over this time interval in the animals given saline or heparin, in those given hirudin, the clots were actually half of their initial weight. Thus, these preliminary findings suggest that unlike heparin, hirudin accelerates endogenous fibrinolysis. This occurs because hirudin can block clot-bound thrombin thereby preventing clot accretion. Further studies are underway to confirm these findings.

Injury-induced Stenosis:

To confirm our findings with low molecular weight heparin, a dose response study should be done. Rats should be treated with various doses of low molecular weight heparin after balloon angioplasty and their effects on injury-induced stenosis determined. In addition to confirming the present findings, this type of study would also help us to identify the optimal dose of low molecular weight heparin.

One limitation of our studies, and those of other investigators, is that we are monitoring the proliferative response that occurs after normal vessels are damaged.

However, in patients, angioplasty is done on atherosclerotic arteries and the mechanisms responsible for injury-induced proliferation may be different in diseased vessels than in normal arteries. This may explain why heparin and low molecular weight heparin have not been shown to be effective in preventing restenosis in patients. To test these concepts, we need to develop an animal model that more closely resembles the situation that exists in humans. One possibility is to use corpulent rats that are prone to develop spontaneous atherosclerosis. The effects of low molecular weight heparin on injury-induced restenosis could be tested in these animals since here we would be damaging an already diseased vessel.

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TABLE I. Effect of one week therapy with saline, stdh, lmwh, and hirudin on stenosis and intimal thickening in the injured common carotid artery at two week follow-up.

Treatment Group Group (n=5)	Stenosis % Angio Mean ± SE	Stenosis % Res.Casting Mean ± SE	Intima % Planimetry Mean ± SE
Saline	22.9 ± 3.0	46.7 ± 4.8	21.8 ± 3.4
Stdh	28.4 ± 2.2	34.9 ± 5.9	22.0 ± 4.1
Lmwh	8.8 ± 7.8*	22.5 ± 8.3*	11.3 ± 2.1*
Hirudin	28.2 ± 1.6	33.4 ± 7.3	21.9 ± 2.2
Sham	0.04 ± 1.6**	1.8 ± 3.5**	0.0 ± 0.0**

p vs. Saline; * p≤0.05; ** p≤0.01

TABLE II. Inhibitors of Coagulation.

	Inhibitor	Inactivates
1)	Heparin	Non-bound thrombin Non-bound factor Xa (Factors IXa + XIa)
2)	Low Molecular Weight Heparins	Non-bound factor Xa Non-bound thrombin (Factor IXa + XIa)
3)	Hirudin Hirudin Fragments Chlormethylketones and Related Small Molecules	Fibrin-bound thrombin Free-thrombin
4)	Tick Anticoagulant Protein Antistasin	Factor Xa bound in prothrombinase complex Free factor Xa
5)	Tissue Pathway Inhibitor	Tissue factor/factor VIIa complex
6)	Activated Protein C	Factors V and VIIIa

TABLE III. Inhibitors of Platelet Function.

	Inhibitor	Effect
1)	Aspirin	Thromboxane A_2 synthesis Prostaglandin G_2 + H_2 synthesis Prostaglandin I_2 synthesis
2)	Thromboxane A_2 Receptor Antagonists	Thromboxane A_2-mediated platelet activation
3)	Thromboxane A_2 Synthetase Inhibitors	Thromboxane A_2 synthesis
4)	Ticlopidine Clopidogrel	Inhibition of platelet aggregation by ADP and other agonists (mechanism unknown)
5)	Glycoprotein IIb/IIIa Inhibitors	Inhibit platelet aggregation induced by ADP, thrombin, thromboxane A_2 and collagen by inhibiting binding of fibrinogen to GPIIb/IIIa.
6)	Thrombin receptor-binding inhibitors.	Inhibit platelet activation by thrombin.
7)	von Willebrand factor-binding inhibitor.	Inhibit binding reaction between von Willebrand factor and platelet glycoprotein Ib.

Figure 1: Regulatory mechanisms limiting the extension of thrombus formation. Intravascular extension of thrombus is limited by multiple protective mechanisms. The most important of these mechanisms are related to the presence of thrombin and/or intact endothelium and include (1) inactivation by complex formation with plasma antithrombin III (AT III), (2) facilitation by endothelial heparin-like molecules of the inactive thrombin-antithrombin III complex formation, (3) down-regulation of thrombin formation through destruction of surface-bound factor VIIIa and factor Va by activated protein C (Act PC) formed by thrombomodulin-dependent thrombin cleavage, and thrombin-mediated release from endothelium of (4) tissue plasminogen activator (t-PA), (5) prostacyclin (PGI_2), and (6) nitric acid (NO). Additionally, intact endothelium adjacent to forming thrombus inactivates adenosine diphosphate (ADP) and vasoactive amines released from activated platelets (Reproduced with permission from Hematology, fourth edition, edited by Williams WJ, Beutler E, Erslev AJ, Lichtman, 1990, McGraw-Hill Ryerson, Inc., New York).

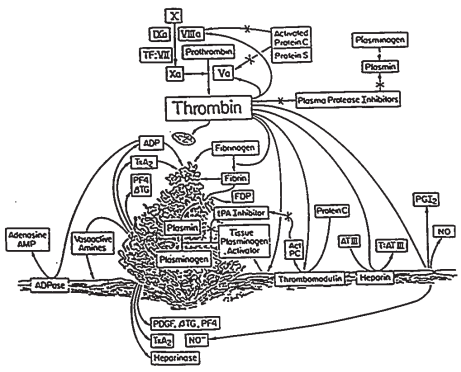


Figure 2. Angiogram of the rat aorta illustrating the high-grade stenosis produced by application of an external constrictor.

Figure 3. Histologic cross section of the occlusive thrombus which is rich in red cells and fibrin.

Figure 4. Effect of thrombin inhibitors on the time to t-PA-induced thrombolysis. The bars represent the means, whereas, the lines above the bars represent the standard deviation of each mean. Hirulog and PPACK significantly ($p=0.02$) accelerate the time to t-PA-induced clot lysis.

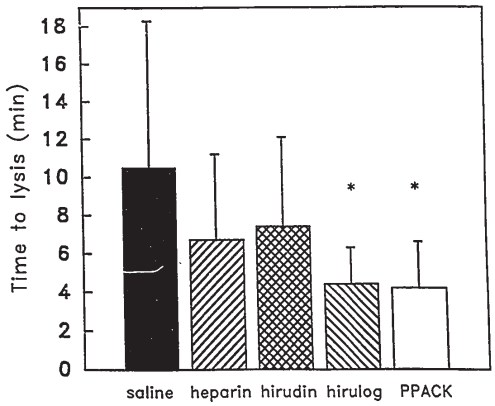


Figure 5. Schematic representation of vessel patency throughout the 80 minute observation period. Closed bars represent absence of blood flow, whereas open bars reflect a patent vessel.

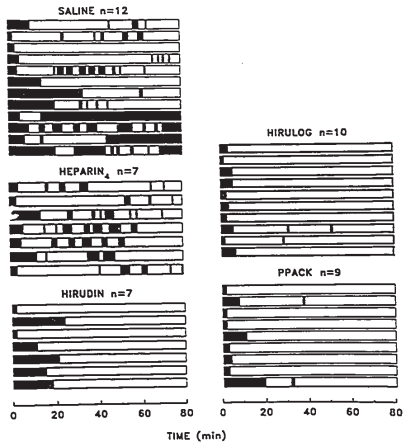


Figure 6. Effect of thrombin inhibitors on the percentage of time during the 80 minute observation period that the vessel remains patent. The bars represent the means, whereas, the lines above the bars represent the standard deviation for each mean. Hirudin, hirulog, and PPACK significantly ($p<0.002$) increased vessel patency, whereas heparin did not.

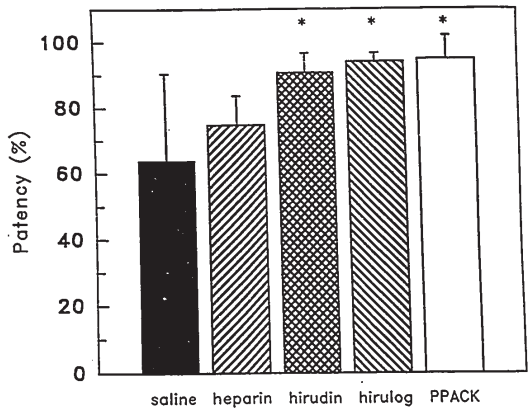


Figure 7. Effect of thrombin inhibitors on the number of reocclusions per hour that the vessel remains patent. The bars represent the means, whereas, the lines above the bars represent the standard deviation of each mean. Hirudin, hirulog and PPACK significantly ($p<0.05$) reduced the number of reocclusions, whereas heparin didn't.

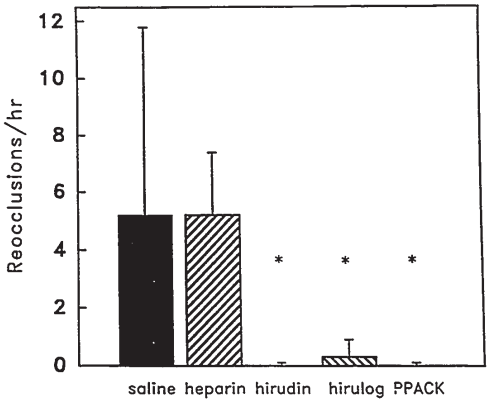


Figure 8. Effect of saline (o), heparin (•), hirudin (▾), hirulog (▫), and PPACK (▪) on the APTT. The individual symbols at each time interval represent the means, whereas, the bars represent standard errors of each mean.

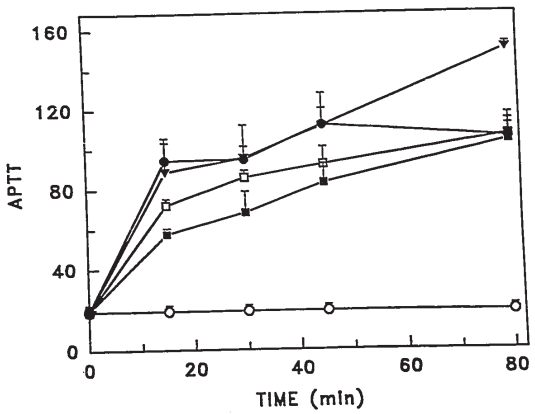


Figure 9. Effect of a single sc. injection of saline, heparin, low molecular weight heparin, and hirudin on blood coagulation assays (APTT, TCT). Stdh, lmwh, and hirudin significantly ($p<0.05$) elevated both assays, whereas saline did not.

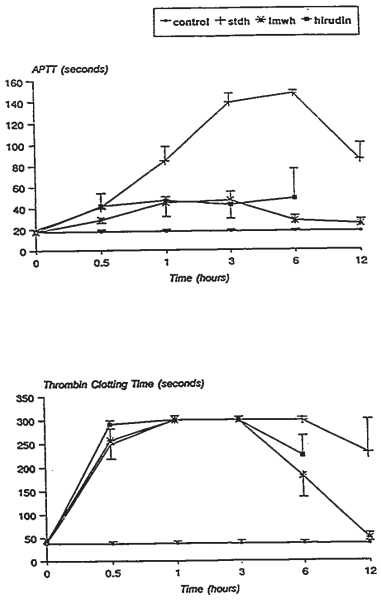


Figure 10. Evan's blue staining of the injured and noninjured common carotid arteries. The flow surface of the control nondenuded artery (R) is not stained, however, the artery with denuded endothelium (L) is stained blue.

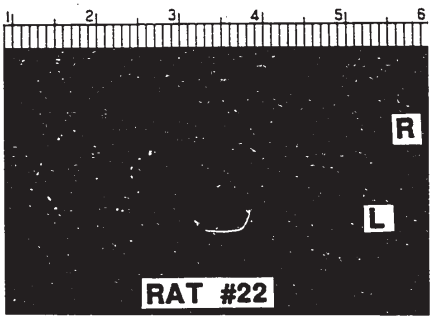
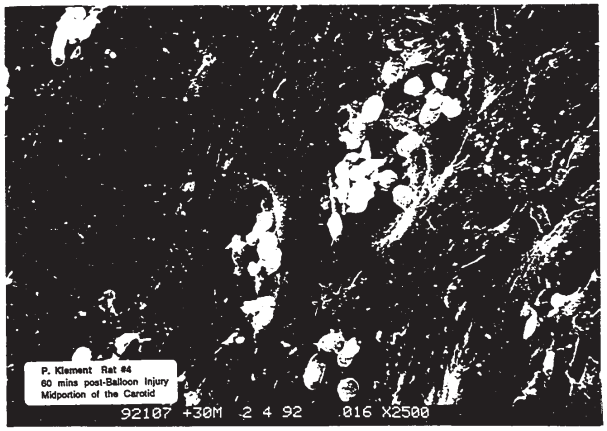


Figure 11. Scanning electron microscopic view of the flow surface of the common carotid artery (A), 60 minutes after endothelial denudation (magnification 2500x), and (B) uninjured flow surface with intact endothelial cells for comparison (magnification 1250x). Denuded flow surface (A) is covered with clumps of platelets, fibrin, and leukocytes.

A



B

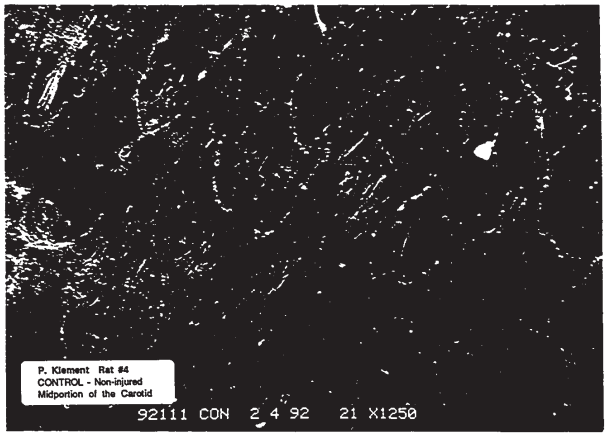


Figure 12. Histologic cross-section of the left common carotid artery, 60 minutes after balloon injury shows partial rupture of the media (arrows) and presence of a mural thrombus [Osmium staining, (A) magnification 40x and (B) magnification 200x].

A

B

Figure 13. Angiogram of the common carotid arteries of the rat immediately after balloon injury. The lumen of the injured carotid artery did not change diameter immediately after denudation.

Figure 14. Angiogram of the common carotid arteries two weeks after balloon injury. There is 50% stenosis of the injured carotid artery (A) when compared to the uninjured contralateral artery.

Figure 15. Effect of stdh, lmwh, hirudin, and saline treatment on injury-induced stenosis as determined by A) angiography and B) resin casting. Only lmwh significantly inhibited stenosis ($p<0.05$ by angiography and $p<0.05$ by resin cast) when compared to saline treatment.

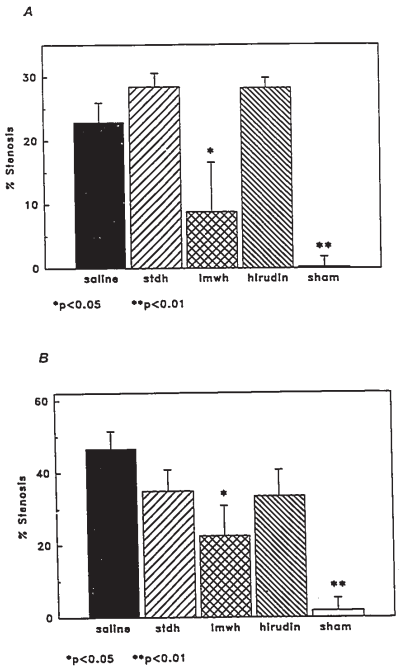


Figure 16. Picture of a resin cast of the common carotid arteries and aortic arch two weeks after injury. The injured carotid artery (L) is stenotic when compared to the uninjured contralateral artery (R).

Figure 17. Effect of stdh, lmwh, hirudin, and saline treatment on injury-induced intimal thickening as assessed by measurements of cross-sectional area. Only lmwh therapy significantly ($p<0.005$) inhibited intimal thickening when compared to saline treatment.

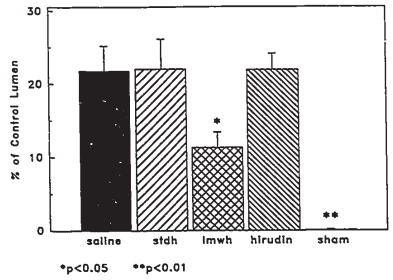
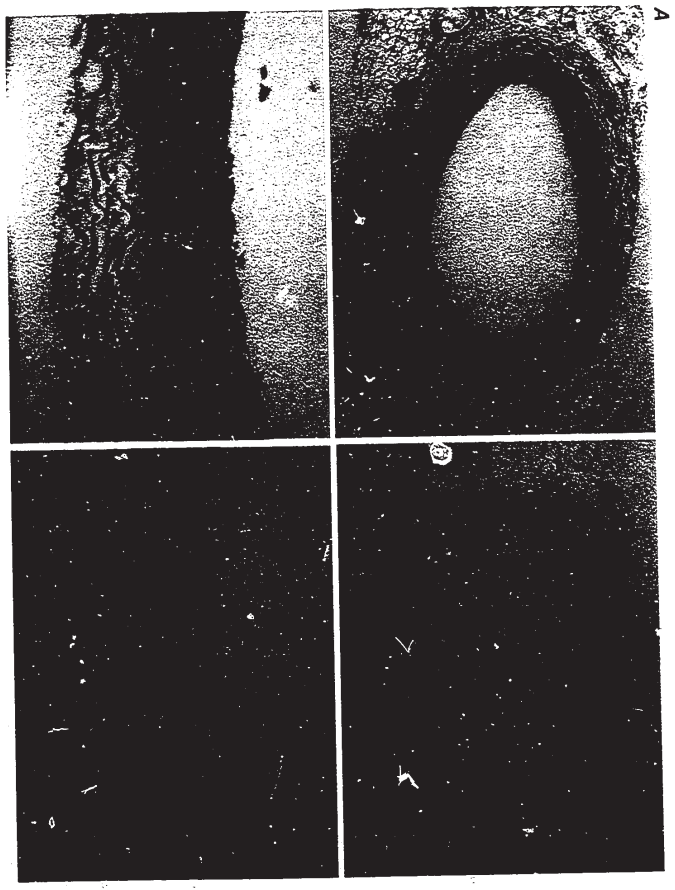


Figure 18. Representative histologic cross-section of the injured (A) and uninjured (B) common carotid artery from the rat treated with saline for one week. Cross-section was taken at the point of maximal stenosis. (Haematoxylin & eosin and Movat's pentachrome staining, magnification 40x and 200x).



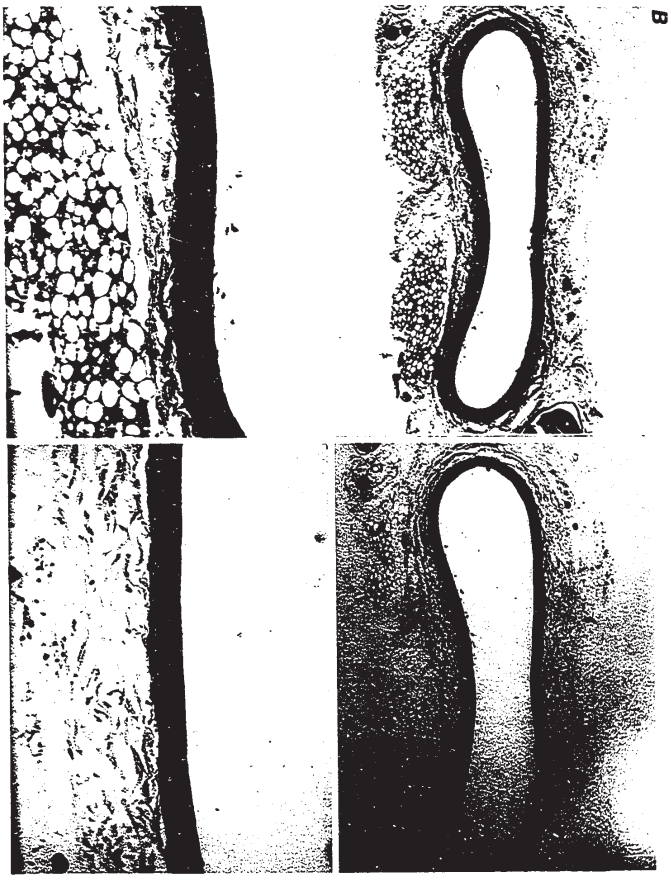


Figure 19. Effect of stdh, lmwh, hirudin, and saline treatment on the cross-sectional area of media as determined by planimetry. None of the treatments had an effect on the cross-sectional area of the media.

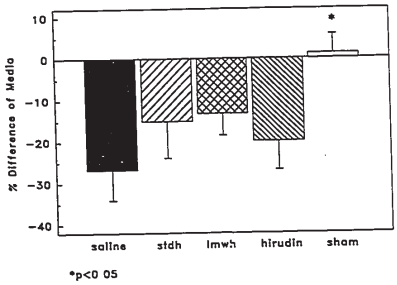


Figure 20. Effect of stdh, lmwh, hirudin, and saline treatment on vasoconstriction as assessed by planimetry. None of the agents had a significant effect on vasoconstriction.