ENDOTHELIAL PERMEABILITY IN TUMOURS

CHARACTERISTICS OF ENDOTHELIAL PERMEABILITY IN TUMOURS

AND

THE ROLE OF NEUTROPHILS

By

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ABSTRACT

Vascular hyperpermeability is a common characteristic among many tumour types, especially those that grow in ascites form. With these, the exudate that flows out of the circulation collects as ascites fluid in the cavities within which these tumours are growing. In the past, this hyperpermeability has been attributed to the production of vascular permeability factor (VPF) by tumours. VPF has been found to bind to endothelial cells and lead to an increased vascular permeability. In the present study, the role of polymorphonuclear leukocytes (neutrophils) in tumour vascular hyperpermeability was investigated. Hey-3 tumour cells were grown into masses on the chick embryo chorioallantoic membrane (CAM). Interstitial neutrophilia was found to be a common feature at the tumour-host interface. Horseradish peroxidase was injected into the circulation and allowed to perfuse for five minutes. The density of labelled vesicles within the endothelial cell cytoplasm was calculated to be 0.99 +/- 0.28 vesicles/μm^2. This vesicular density was comparable to that of N-formyl-methionine-leucine-phenylalanine (a chemotactic peptide for neutrophils)-treated CAM (1.04 +/- 0.09 vesicles/μm^2), but very different from control CAM (0.51 +/-

0.09 vesicles/μm^2). In order to rule out any immune response to foreign cells, murine hepatocyte masses were grown on the CAM and vesicular density was calculated to be 0.54 +/- 0.03 vesicles/μm^2. Through chemotaxis assays with the Boyden chamber, it was observed that Hey-3 tumour cells in culture were producing a chemotactic factor that is an attractant for human neutrophils. Once in the area, neutrophils do possess the potential to increase vascular permeability. Thus, neutrophils play a role in vascular endothelial hyperpermeability in tumours.

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1.0 INTRODUCTION

1.1 VASCULAR ENDOTHELIUM

The importance of the vascular endothelium has only been realized in the last decade, and it is now known to be a dynamic tissue involved in many aspects of vascular physiology and homeostasis (Davies & Hagen 1993). The endothelium performs many important functions; for instance, it metabolizes a variety of substances, as well as acting as an antithrombogenic surface and a selective permeability barrier. The endothelial cell is a pluripotent cell responsible for producing growth factors, coagulation-related factors, and factors controlling vascular tone (reviewed by Davies & Hagen 1993, Shah 1992). The focus of this thesis is the role of the endothelium in vascular permeability.

1.1.1 Structure

The vascular endothelium is a typical simple squamous epithelium characterized as a confluent monolayer of thin, flattened cells (Junqueira et al.

1992). These endothelial cells line the intimal layer of all arteries and veins, and are the sole constituents of capillaries. Between these endothelial cells are intercellular junctions which serve as sites of adhesion as well as seals to prevent the flow of material through the intercellular spaces (Junquiera et al. 1992, Majno 1965). Tight junctions, or zonula occludens, surround individual endothelial cells and bind them to adjacent cells, closing off the intercellular spaces. They are located on the luminal side of intercellular spaces. The primary function of tight junctions is to prevent the flow of materials between endothelial cells by forming a tight seal. Other intercellular junctions include zonula adherens and gap junctions. While gap junctions are responsible for communication between cells, zonula adherens function to attach one cell to the next.

The endothelium, like other epithelial layers, possesses an underlying basement membrane, or basal lamina, which is composed mainly of type IV collagen, laminin, and proteoglycan (Junqueira et al. 1992, Majno 1965). The basal lamina serves as a selective barrier which controls the exchange of macromolecules between the cells and the surrounding connective tissue. Subjacent to the basement membrane in capillaries and venules are pericytes, which are believed to aid in the production of the basement membrane.

Endothelia can be grouped into two types depending on the structure of the endothelial cells (Junquiera et al. 1992). The continuous endothelium is made up

of a sheet of interconnected squamous endothelial cells and characterized by the lack of fenestrae in its wall. This type is found in most tissues, including muscle, connective, exocrine, and nervous. Fenestrated endothelia are characterized by large fenestrae in the walls of endothelial cells. This type of endothelium is found in tissues where the free exchange of substances occurs, such as in the kidney, intestine and endocrine glands. Macromolecules have been found to cross the endothelium by way of these fenestrae and thus is an important means of transendothelial transport of macromolecules (Junqueira et al. 1992). In continuous endothelia, on the other hand, numerous pinocytotic vesicles are present. These vesicles are responsible for the exchange of macromolecules between the circulation and the extravascular tissue.

1.1.2 Vascular Permeability

The vascular endothelium is very different from other simple squamous epithelia; it is highly permeable to water and water soluble molecules as well as water soluble macromolecules and macromolecular tracers (Landis & Pappenheimer 1965, Renkin 1964, Renkin 1977). Thus, the endothelium forms a relatively impermeable surface that regulates the passage of ions, molecules, and fluid as well as cells between the circulation and the extravascular space. In the normal state, the net flow across the endothelium is less than 0.05 % of the cardiac

output (Davies & Hagen 1993). Flow across the endothelium occurs by both ligand specific and non-specific binding, as well as by passage between cells.

Various capillary beds have been used for in vivo studies of vascular permeability (Landis & Pappenheimer 1965, Pappenheimer et al. 1951). Through early studies in the 1950s, Pappenheimer (1951) introduced the concept of a two pore system being responsible for the permeability seen in these blood vessels. According to the diameters of the particles that were able to pass through, these were referred to as small and large pore systems. The intercellular junction is now believed to be the small pore system equivalent, while the fenestrae and pinocytotic vesicles are referred to as the large pore counterparts (Junqueira et al. 1992).

Cellular and intercellular transport occurs across the exchange vessels in the body, specifically the capillaries and non-muscular venules (Renkin 1992). The post-capillary venules are believed to be the sites where most of the cellular transcytosis occurs (Curry & Joyner 1988). Water and small lipid soluble substances are able to penetrate the cell surface and diffuse through the endothelium (Renkin 1992). Lipophilic molecules are also believed to pass through intercellular junctional pores. Large molecules such as plasma proteins are able to penetrate the endothelial cell layer, but the mechanism of transport is still a matter of controversy.

Most blood vessels are lined with a continuous endothelium and the maintenance of normal permeability depends on the presence of plasma proteins, especially albumin and orosomucoid (Renkin 1992). Albumin plays a central role in microvascular exchange (Michel 1992). When bound, it reduces the permeability of endothelial cells to macromolecules (Hurley & Curry 1985, Michel & Phillips 1985) maintaining vascular exchange in a normal range. Due to the importance of albumin in normal vascular permeability, many studies in transendothelial transport have been performed by tracking the movement of labelled albumin (Michel 1992, Milici et al. 1987).

1.1.2.1 Mechanisms of Transcytosis

Small hydrophobic and hydrophilic molecules can passively diffuse across the endothelium or through intercellular spaces (Junqueira et al. 1992). Once these have crossed the luminal plasma membrane, they diffuse through the cytoplasm to the opposite cell surface where they are discharged. On the other hand, two structural elements are believed to be responsible for the active vascular transcytosis of larger molecules (Palade et al. 1979): macromolecular transport by a transcytotic vesicular system (Simionescu et al. 1987) and movement through interendothelial junctions (Rippe & Haroldsen 1987).

1.1.2.1.1 *Vesicular Transport*

Vesicular transcytosis occurs through coated and uncoated vesicles (Alberts et al. 1994, Scheenberger & Hamlin 1984). Coated vesicles are those that possess receptors on their membranes to which molecules bind. Thus, this form of endocytosis is receptor-mediated and specific. On the other hand, uncoated vesicles are those that do not depend on ligand-receptor recognition.

One of the key structural features of the vascular endothelial cell layer is that many vesicles are visible in the cytoplasm (Bennett et al. 1959, Bruns & Palade 1968, Majno 1965, Palade 1961, Simionescu et al. 1974). These 70 nm spheres have most often been observed as single vesicles, fused chains, or as vesicles opening onto the luminal or abluminal surface of endothelial cells (Bruns & Palade 1968, Simionescu et al. 1974).

Much of the evidence for the movement of plasma proteins through the endothelium lends support to the vesicular system of transendothelial transport, but controversy exists in this area of study as well. Four hypotheses have been proposed to explain the mechanism of transendothelial vesicular transport (reviewed by Michel 1992, Shasby 1988). The first, and least accepted, hypothesis states that vesicles do not exist free within the cytoplasm, but rather that these uncoated membrane-bound vesicles are arranged in static, fused clusters. The vesicles within a cluster communicate with each other and with the

extracellular space at the luminal and abluminal surfaces (Bundgaard et al. 1979, Bundgaard et al. 1983, Frokjaer-Jensen 1980). These investigators also believe that the transfer of macromolecules is more likely to occur between cells, as opposed to through them. Contrary to this, the endocytotic uptake of macromolecules (fluid-phase endocytosis) has been observed by many groups (Davies et al 1984, Kakaoka & Tavassoli 1984, Milici et al. 1987, Tavassoli et al. 1986, Williams et al 1984). These investigators have provided evidence for the endocytosis and exocytosis of macromolecules by the endothelium. Milici and his colleagues (1987) observed the binding of labelled bovine serum albumin (BSA) to vesicles open on the luminal surface of the endothelium as well as to depressions that could represent the early stages of vesicle formation. They were also able to observe BSA in cytoplasmic vesicles and in vesicles releasing their contents at the abluminal surface.

The three remaining hypotheses for transendothelial transport deal with the transient formation of vesicular structures (reviewed by Michel 1992). The first is referred to as the "shuttle" or "ferryboat" system. According to this mechanism, single vesicles pinch off at the luminal surface to form free vesicles that move through the cytoplasm and release their contents at the opposite surface of the endothelial cell (Palade 1960). The second is referred to as the fusion-fission hypothesis (Clough & Michel 1981, Loudon et al. 1979). This mechanism

suggests that once a vesicle is formed at the luminal surface, it travels part way through the endothelial cell where it fuses with another vesicle in order to transfer its contents. After a few of these fusions, the macromolecules will have moved to the abluminal surface of the cell, with which the vesicle fuses and expels its contents. The third hypothesis is that vesicles are fused, forming a channel that extends from the luminal to the abluminal surface of the endothelial cell (Milici et al 1987, Simionescu et al. 1975). These chains of vesicles were first observed many years ago (Bruns & Palade 1968, Palade & Bruns 1968) and were believed to be the structural equivalents of the small pore system (Pappenheimer et al. 1951). The small pores would allow the movement of macromolecules through the endothelium.

Many more investigators report and accept vesicular or transendothelial channel-mediated transcytosis of macromolecules across the endothelium (Garlick & Renkin 1970, Palade 1953, Renkin 1964, Shasby 1988). Palade and Simionescu were the first to present data to support vesicular and channel transcytosis (Clementi & Palade 1969, Simionescu et al. 1972). Both of these groups have described receptor-mediated and receptor independent endocytosis as well as having observed the transendothelial transfer of a wide range of tracers, including horseradish peroxidase, ferritin, and labelled dextran (reviewed by Shasby 1988). It has also been reported that uncoated vesicles (receptor independent) are more

important that coated ones (Scheenberger & Hamlin 1984); they are also much less common in the endothelium than uncoated vesicles. These investigators have observed that the larger tracers are not seen moving through the intercellular spaces, suggesting that their transport of these tracers occurs transcellularly.

1.1.2.1.2. *Interendothelial Transport*

Studies with large tracers such as horseradish peroxidase (~ 5 nm) and ferritin (~ 11 nm) found these macromolecules within the cytoplasmic vesicles (reviewed by Palade et al. 1979), while also sometimes being seen within the intercellular spaces (Karnovsky 1967). If tracers were passing through the interendothelial spaces, then the vesicular transport system may not be as important as had been believed. When more controlled experiments were conducted, these large tracers were not seen within the intercellular spaces (Simionescu et al. 1973, Simionescu et al. 1975). In fact, when Williams and Wissig (1975) repeated Karnovsky's experiments (1967), they did not find any horseradish peroxidase or cytochrome C (~ 3 nm) between the endothelial cells. Investigators repeatedly found that the interendothelial junctions were impermeable to tracers such as ferritin, myoglobin (~ 3 nm), and hemepeptides (~ 2 nm) (Bruns & Palade 1968, Simionescu et al. 1973, Simionescu et al. 1975). Simionescu found that tracers with a diameter

greater than 2 nm were not able to pass through the intercellular spaces at all (Simionescu et al. 1978).

Soon afterwards, Simionescu and his colleagues realized that there were detectable differences in the organization of the microvascular endothelium (Simionescu et al. 1978). They observed variations in vesicle density, frequency of transendothelial channels, and in the appearance of interendothelial spaces. In the capillaries, microperoxidase tracers with diameters of less than 2 nm passed through the endothelial cells by way of vesicles and transendothelial channels, but not through intercellular spaces. More importantly, though, they observed that in approximately 25 % of post-capillary venules, these microperoxidase tracers were visible in the intercellular spaces. This suggested that, although it may occur rarely, macromolecular tracers are able to pass through the intercellular spaces.

In summary, by utilizing vesicles and intercellular spaces, the endothelium is specialized in the bulk transport of solutes and macromolecules from the circulation into the extravascular space.

1.2 SOLID TUMOUR GROWTH

Tumour growth can be divided into two distinct, but oftentimes indiscernible, phases. The early phase is an avascular one, with the masses being too small for detection. The tumours are able to maintain this size for many years and will not grow any larger without a nutrient supply, consequent on the development of a vascular supply. In this prevascular phase of solid tumour growth, the tumours are usually thin and their cell population is limited (Folkman 1990). Once tumours are penetrated by blood vessels, they enter the vascular phase and begin to grow rapidly (Klagsbrun et al. 1977).

These "vascular phase" solid tumours are composed of two discrete, but dynamically interacting compartments: the tumour cells themselves and the stroma throughout which they are scattered (Dvorak 1986). This organization occurs in tumours as well as in normal tissues where an avascular area borders on a vascular one. An example of this is the skin, where the avascular epidermis ("tumour cell compartment") is adjacent to the vascularized connective tissue dermis ("tumour stroma"). All solid tumours, regardless of their site of origin, require a stroma if they are to grow beyond a minimum diameter of 1 to 2 mm (Folkman 1985). Beyond this size, diffusion of nutrients through the matrix is not

sufficient to supply the tumours demands, so in order to nourish the entire tumour as well as remove wastes, a vascular supply is required.

The tumour cell compartment is organized into sheets or clumps of malignant tumour cells (Dvorak et al. 1991). Coursing between these tumour cell masses are bands of connective tissue stroma. Tumour stroma is composed of three distinct elements: a vascular supply, inflammatory cells, and connective tissue elements (Cotran et al. 1989). The new blood vessels as well as fibroblasts arise due to the proliferation and inward migration of nearby cells. Interstitial fluid and inflammatory cells move out of the local vasculature through transport and diapedesis, respectively; the collagen fibrils and proteoglycans that make up the connective tissue are synthesized and secreted by the connective tissue cells, predominantly fibroblasts (Dvorak et al 1991).

1.2.1. Tumour Stroma

1.2.1.1. Interstitial Connective Tissue

Tumour stroma contains matrix components such as fibrin, fibrinogen, fibronectin, interstitial collagen, elastin, and glycosaminoglycans (Dvorak et al. 1983, reviewed in Dvorak 1986, Yeo & Dvorak 1993). In experimental animal models as well as in humans, tumour stroma is composed of interstitial connective

tissue elements and, in carcinomas, a basal lamina, which is found to exist between the tumour cells and the connective tissue (Yeo & Dvorak 1993).

Interstitial connective tissue accounts for a large percentage of the tumour stromal composition and, interestingly, it is formed from the circulating blood elements as well as from the surrounding connective tissue (Yeo & Dvorak 1993). From the blood, the tumour acquires water, plasma proteins, and many types of inflammatory cells. Tumour connective tissue contains all of the same components as normal connective tissue: collagen, fibrin, fibronectin, tenascin, elastin, sulfated proteoglycans, glycosaminoglycan hyaluronan, interstitial fluid, blood vessels, fixed tissue cells (i.e. fibroblasts) as well as inflammatory cells (Yeo & Dvorak 1993). On the other hand, tumour stroma is also very different from normal connective tissue (Dvorak et al 1991). It is structurally very disorganized and lacking functionally. In fact, tumour stroma strongly resembles the scar tissue of healing wounds; changing over time in a process that is very similar to that of wound healing (Dvorak 1986, Dvorak et al 1992, Dvorak et al 1991, Nagy et al 1988).

Various tumours differ in their stromal content either qualitatively or quantitatively. Regardless of tumour type, though, fibrin is a consistent component of connective tissue, varying only in distribution (Dvorak 1986, Dvorak et al. 1991, Nagy et al. 1988). In carcinomas, tumours of epithelial cell

origin (Cotran et al. 1989), fibrin is found between tumour cells and is very abundant at the tumour-host interface (Yeo & Dvorak 1993). This fibrin deposition is an early event in tumour stroma formation as it is in wound healing. Blood vessels use the fibrin matrix as a scaffolding to move toward the tumour mass in response to angiogenic stimulatory factors (Yeo & Dvorak 1993).

Tumour stromal fibrin is formed when fibrinogen leaks out of the vasculature and comes into contact with tissue factor in the extravascular tissue (Yeo & Dvorak 1993). This activates the extrinsic pathway of the clotting cascade which leads to the formation of a fibrin gel matrix (reviewed in Nagy et al. 1988). Experimentally, it was shown that fibrin turnover is more rapid in tumours than in wound healing tissue (Brown et al. 1989). Fibrinogen was found to accumulate similarly in tumours and in wounds, but the initial extravasation and clotting of fibrinogen in tumours exceeded that of wounds. In these, the fibrinogen extravasation and fibrin accumulation returned to normal after a few days, but remained persistently elevated in the tumours. Based on these findings, the tumour vasculature was said to be exceedingly more permeable than that of healing wounds and normal tissue. Also, most microvasculature is lined by a continuous endothelium, with a few exceptions. Therefore, in order for the fibrinogen with its large size (340 kD) to be extravasated, the vessels must be hyperpermeable (Brown et al. 1989, Nagy et. al 1988).

1.2.1.2. Tumour Vasculature

Tumours acquire a vascular supply through the process of angiogenesis. It has been observed for over 100 years that many types of solid tumours possess an increased vascularity compared to normal tissues, but it was not until the 1940s that is was revealed that new blood vessels in the area around a tumour arose from the host vasculature, and not from the tumour itself (Algire et al. 1945). In the 1960s, Folkman showed that this increased tumour vasculature was necessary for tumour growth (Folkman et al. 1963, Folkman 1985). Folkman and his colleagues also demonstrated that tumours can grow in the absence of a vascular supply until they reach a size at which passive diffusion can no longer adequately supply nutrients and remove wastes from its cells. Growth beyond this stage is dependent on angiogenesis or neovascularization, which is defined as the proliferation of blood vessels beyond the requirements of body maintenance (Folkman et al. 1981, Folkman 1985). Specifically, tumour angiogenesis refers to the sprouting of new vessels directly toward a solid tumour in response to a tumour-secreted angiogenic factor (Blood & Zetter 1990). The stromally provided vascular supply is needed by the tumour for nourishment, gas exchange, and waste removal.

Unlike organs, tumours are not supplied by only one main vessel, but, rather, by many invading branches, and as the tumour expands, even more capillary sprouts are induced (Folkman & Shing 1992). Once the tumour is embedded in

the host tissue, every increase in tumour cell population must be preceded by an increase in new capillaries invading the mass; otherwise, further tumour growth will cease (Folkman & Shing 1992, Warren 1978). Tumour-induced angiogenesis is unique because once it begins, the process will continue indefinitely until the tumour is destroyed, or the host dies (Warren 1978).

Most solid tumours are highly vascularized, but it has been shown that these vessels are very different from those of normal tissues. Vessels in tumours are mainly composed of endothelial cells, while those of normal tissue are also surrounded by pericytes (reviewed in Blood & Zetter 1990). The basement membrane that underlies the endothelium is visibly reduced in tumours as compared to normal blood vessels. Also, tumours have been found to alter their vasculature continuously throughout growth compared to normal organs, which do not do this (Folkman 1985). As the tumour increases in size the vessels in the center of the mass become compressed until, beyond 1-2 cm^3, the central area becomes necrotic due to a prolonged lack of blood flow. Jain and his colleagues have shown that the interstitial pressure is very high and uniform within the tumour, except in the outer margin, at the tumour-host interface, where it drops dramatically (Jain 1987, Jain 1994, Jain & Baxter 1988).

It has been demonstrated repeatedly that tumour vasculature is extremely hyperpermeable to plasma proteins (Brown et al. 1988a, Brown et al. 1988b,

Dewey 1959, Dvorak et al. 1984, Dvorak et al. 1986, Dvorak et al. 1988, Dvorak et al. 1991, Dvorak et al. 1992, Heuser & Miller 1986, Nagy et al. 1988, O'Connor & Bale 1984, Song & Levitt 1971, Underwood & Carr 1972, Yuan et al. 1995). Experimentally, it was observed that a variety of tracers leak out of the blood vessels of tumours much more rapidly than from those found in normal tissue. This vascular hyperpermeability was also shown to persist indefinitely (Dvorak et al. 1991). Heuser and Miller (1986) demonstrated that different types of tumours show different degrees of hyperpermeability Using Sprague-Dawley rats, they showed that the vasculature of a rapidly growing Walker 256 carcinosarcoma was more permeable to fluorescent tracers than the slow growing chondrosarcoma. They suggested that the mechanisms responsible for protein transport from the Walker 256 carcinosarcoma may also be involved in fast growth of the tumour as opposed to slow growing ones like the chondrosarcoma.

Dvorak and his colleagues (1988) proposed two hypotheses to account for the vascular hyperpermeability associated with solid tumour growth. The first was that this increased permeability was due to inherent structural defects within the tumour blood vessels. This could mean anything from improper junctions being formed between endothelial cells to the vessels being lined with a different endothelium (i.e. a fenestrated endothelium that would be more permeable to plasma proteins). Also, this defect in the tumour vasculature could be secondary

to tissue injury, since tumours have been known to exhibit ischemic damage. The second hypothesis postulated that the vessels would become permeable due to a cytokine that was released by tumour cells or the host inflammatory cells (as in inflammation).

Using fluorescence, light, and electron microscopy in animals bearing solid transplantable tumours, Dvorak and his colleagues (1988, 1991) were able to show that the transport of macromolecular tracers was an exclusive property of vessels in two locations in tumours: at the tumour-host interface (Brown et al. 1989, Kohn et al. 1992, Senger et al. 1993) and in the connective tissue both surrounding and separating individual tumour nodules. These hyperpermeable vessels were determined to be ultrastructurally intact mature venules and small veins (Brown et al. 1989, Kohn et al. 1992, Senger et al. 1993) which were lined by a continuous endothelium with normal amounts of underlying basement membrane and pericytes. This latter finding is different from that which was described by Blood and Zetter (1990); they found that vessels in tumours are rarely surrounded by pericytes. Open interendothelial junctions were also determined to be very uncommon (Brown et al. 1989, Dvorak et al. 1988, Senger et al. 1993). Also, damaged endothelial cells were not encountered (Senger et al. 1993). Hyperpermeability did not affect all blood vessels equally and was also found to

be patchy along the length of a single vessel (Dvorak et al. 1988, Dvorak et al. 1991, Kohn et al. 1992).

1.2.1.2.1. *Vesiculo-Vacuolar Organelles*

Dvorak and his colleagues concluded that the mechanism by which tracers move out of the vasculature of tumours is the same as that in normal tissues (Yeo & Dvorak 1993). Through their studies, they were able to conclude that the pathway of macromolecular tracer transport across venules and small veins involved structures that they named vesiculo-vacuolar organelles (VVOs) (Kohn et al. 1992, Dvorak et al. 1996).

Vesiculo-vacuolar organelles (VVOs) are described as "grape-like clusters of interconnecting uncoated vesicles and vacuoles bound by trilaminar unit membranes" (Dvorak et al. 1996). These are said to transverse the entire vascular endothelium, connecting the vascular lumen with the extravascular space. Through specimen tilting on the electron microscope, it was shown that individual VVO vesicles and vacuoles were able to communicate with each other and the endothelial cell plasma membrane through stomata (Dvorak et al 1996). These stomata are believed to resemble the caveolae of capillary endothelium.

Using C3HeB/HeJ mice, Dvorak and her colleagues (1996) were able to show that VVOs are also found in the endothelium of normal subcutaneous tissue

vessels. Kohn and her colleagues (1992), had previously determined that VVOs were the primary mechanism of transcytosis in the vessels of normal tissues of mice and guinea pigs. In addition, these VVOs were more complex than vesicles and vesicular chains that were described by Palade and his colleagues (Palade et al. 1988). In fact, these VVOs were found within the endothelial cells of normal blood vessels with about the same frequency, but with smaller amounts of tracer within them (Senger et al. 1993). This suggests that the differences seen in permeability between normal blood vessels and those within tumours is not due to differences in the number or structure of VVOs, but rather to the upregulation function of the VVOs within the tumour blood vessels. From these findings, Senger and his colleagues concluded that tumour vascular hyperpermeability was due to a change in endothelial cell function attributable to a cytokine.

1.2.1.2.2. *Vascular Permeability Factor/Vascular Endothelial Growth Factor*

Fluid collects in the interstitial space of solid tumours due to an increased vascular permeability and those tumour that are able to grow within body cavities (ascites form) subsequently produce ascites fluid. It was hypothesized that these tumours produce a cytokine-like factor that induces vascular permeability. The first evidence to support this hypothesis was presented by Senger and his colleagues (1983) who found that serum-free tumour cell culture media, as well as

ascites fluid, caused blood vessels of normal guinea pig skin to become hyperpermeable to tracers. They isolated a protein that caused this hyperpermeability effect, exhibited heparin binding potential, and had an electrophoretic motility M_r 34-42 000. Antibodies to this vascular permeability factor (VPF) were found to decrease ascites fluid accumulation with guinea pig line 10 tumour cells (Senger et al. 1983).

In vitro, it was discovered that human tumour cell lines also synthesized and secreted this heparin-binding protein (Senger et al. 1986). Using ^{125}I-labelled albumin in the Miles assay (Miles & Miles 1952), VPF protein expression was found to be higher in tumourigenic cells compared to non-tumourigenic ones. Therefore, there were several lines of evidence that a tumour-secreted factor was acting on blood vessels to cause the increased extravasation of plasma proteins into the tumour-associated extravascular space leading to fluid accumulation. Identical or similar proteins were also identified in a diverse number of tumour cell lines (Dvorak et al. 1992). This fluid accumulation was also found to contribute to the clotting of fibrinogen into fibrin, which has been seen to occur in a number of animal and human tumours.

Not only has VPF been implicated in vascular permeability, but Connolly and his colleagues (1989) found that tumour-secreted VPF stimulated endothelial cell growth. Therefore, VPF is also commonly referred to as vascular endothelial

growth factor (VEGF). There is evidence that suggests that tumour VPF and pituitary VEGF are encoded by the same gene (Gospodarowicz et al. 1989, Senger et al. 1993). Thus, VPF/VEGF is a cytokine with many functions; it modifies endothelial cells to cause increased vascular permeability, and it directly stimulates the growth of vascular endothelial cells. It has also been implicated in playing a role in stromal formation (Dvorak et al. 1992) and angiogenesis (Senger et al. 1993).

Through in vitro experiments, it was discovered that VPF/VEGF induces a signal transduction cascade in endothelial cells (Brock et al. 1991). Two minutes following exposure to serum-free media, VPF caused a three to four-fold increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in endothelial cells from human umbilical vein (HUVEC) and bovine aorta and pulmonary artery. This effect was not seen in aortic smooth muscle cells, fibroblasts, or neutrophils. Exposure to thrombin or histamine had the same results, but factors that inhibit these had no effect on actions of VPF/VEGF. From this evidence it was deduced that VPF/VEGF acted on endothelial cells through receptors that were independent from those for thrombin and histamine. In addition to the above, VPF/VEGF was found to stimulate inositol 1,4,5-triphosphate (IP_3) accumulation in HUVECs. This led Brock and his colleagues (1991) to conclude that VPF/VEGF was acting through the signal transduction pathway that involves phospholipase C (PLC)

activation. Not too long after this, two receptors for VPF/VEGF were identified; Flt, a *fms*-like tyrosine kinase, which binds VPF/VEGF with a high affinity (de Vries et al. 1992) and KDR, which binds with an equally high affinity (Terman et al. 1992).

Thus, VPF/VEGF has been seen to cause a rapid increase in vascular permeability (within five minutes after exposure) and to have a temporary (less than 30 minutes) effect (Senger et al. 1983). These same investigators also found that VPF/VEGF does not cause endothelial cell damage, mast cell degranulation, nor does it respond to standard antihistamines. At concentrations less than a low of 1 nM, VPF/VEGF was seen to effectively induce increased vascular permeability (Senger et al. 1993).

VPF/VEGF has also been found to be a potent angiogenic factor and mitogen (Claffey et al. 1996, Connolly et al. 1989, Detmar et al. 1995, Dvorak et al. 1995, Guidi et al. 1995, Keck et al. 1989, Millauer et al. 1993) which is related to platelet-derived growth factor (PDGF) (Leung et al 1989). Dvorak and his colleagues (1995) have actually suggested that the angiogenesis stimulated by tumours, and within certain non-neoplastic states, shares a common pathogenesis that is initiated by the synthesis and secretion of VPF/VEGF. To illustrate this experimentally, a normally slow-growing tumour line with a low vascular density (SK-MEL-2) was transfected with sense VPF/VEGF cDNA and grown in mice.

The tumours that resulted were found to grow rapidly and develop a dense blood supply in comparison with the normal SK-MEL-2 tumours (Claffey et al. 1996). These experiments suggest that VPF/VEGF promotes and enhances tumour growth by stimulating angiogenesis. VPF from keratinocytes was found to be biologically active, and to have the ability to stimulate dermal endothelial cell proliferation by a paracrine mechanism (Detmar et al. 1995). Thus, it can be concluded that VPF plays two roles in angiogenesis. Directly, it stimulates endothelial cells to proliferate, migrate, and alter their pattern of gene expression (Dvorak et al. 1995). Indirectly, VPF renders these same endothelial cells hyperpermeable so that they leak plasma proteins into the extravascular space leading to the clotting of fibrinogen into a fibrin gel matrix across which the cells can migrate.

The next step was to determine where within the tumour the protein could be found. Immunostaining guinea pig tumours and a human brain lymphoma for VPF lead to the observation that the factor was localized in new blood vessels which the tumour had stimulated (Dvorak et al 1991), specifically within the endothelial cells and basement membrane, as well as in the blood vessels within the peritoneal walls of line 1 and line 10 tumours grown in ascites form. A rapid decrease in VPF staining was also observed with tumour destruction. Blood vessels in the immediately adjacent normal tissue surrounding the tumour also immunostained for VPF. These were venules and small veins that were less than 0.5 mm away

form the periphery of the tumour mass. With the injection of a tracer (colloidal carbon) it was observed that VPF staining co-localized with the carbon, which suggested to the investigators that VPF is responsible for the vascular hyperpermeability (Dvorak et al. 1991, Senger et al. 1993). VPF/VEGF accumulation within endothelial cells is believed to be a mechanism for retaining and concentrating the protein (Senger et al. 1993). It is not known, however, what structures the protein binds to. Binding to the two receptors (de Vries et al. 1992, Terman et al. 1992) and its binding to heparin (Senger et al. 1983) accounts for some of the immunostaining. Using mouse ovarian tumour (MOT) ascites tumour-bearing mice, Hong and his colleagues (1995) observed that VPF/VEGF was primarily found on the abluminal plasma membrane of endothelial cells and within cytoplasmic vesicles and vacuoles that make up the VVOs, especially those that were close to the abluminal surface (Hong et al. 1995). Thus, if this cytokine was responsible for the upregulation of VVO function in order to increase vascular permeability, the location of VPF/VEGF deposits corresponded closely to the distribution expected.

Although VPF/VEGF was originally isolated as a tumour-secreted protein, it has since been found to be synthesized and secreted by a number of normal and inflamed (non-neoplastic) human and animal tissues (Berse et al. 1992, Brown et al. 1995a, Brown et al. 1995b, Dvorak et al. 1995, Fava et al. 1994, Kamat et al.

1995, Olson et al. 1994, Yeo et al. 1993). The highest levels of VPF mRNA have been found in normal lung, kidney, heart, and adrenal gland (Berse et al. 1992). Detectable levels were seen in liver, spleen, gastric mucosa, and breast. The protein has also been found in ovaries (Olson et al. 1994) and corpus luteal cells (Kamat et al. 1995). Normal and rheumatoid arthritic synovial fluids and tissues have also been shown to contain VPF (Fava et al. 1994). Brown et al. (1995b) found VPF within the male genital tract and semen. These findings suggest that VPF is probably produced by normal tissues to maintain a baseline permeability. Inflammatory effusions (Yeo et al. 1993) as well as skin showing delayed hypersensitivity reactions (Brown et al. 1995a, Dvorak et al. 1995) have also been observed to contain VPF.

1.3 INFLAMMATION

Inflammation is defined as "the reaction of vascularized living tissue to local injury" and the inflammatory response is the first step in the cascade that leads to the healing and repair of the damaged tissue (Cotran et al. 1989). Acute inflammation lasts for a short period of time (a few minutes to a couple of days) and involves the exudation of fluid and plasma proteins and the migration of leukocytes, primarily neutrophils, to the injured area. On the other hand, chronic inflammation is longer lasting, and involves the migration of lymphocytes and macrophages to the area as well as the proliferation of blood vessels and connective tissue.

Acute inflammation can be divided into three main events: (i) changes in the vasculature (vasodilatation) that result in increased blood flow to the area, (ii) changes in the vascular endothelium (increased permeability), predominantly in post-capillary venules, that leads to the movement of plasma proteins and leukocytes into the extravascular space, and (iii) migration and accumulation of leukocytes, fibrinogen, and other proteins in the area (Cotran et al. 1989, Guyton 1991).

1.3.1. Cellular Events: Leukocyte-Endothelial Cell Interaction

A critical step in inflammation is the migration of leukocytes (primarily neutrophils and monocytes) to the area of injury (Cotran et al. 1989). Through phagocytosis, leukocytes are able to ingest foreign antigens as well as necrotic tissue. Leukocytes can also induce tissue damage by releasing enzymes, cytokines, and oxygen radicals. The migration of leukocytes from the vascular lumen to the extravascular space can be divided into a series of localized events: margination, rolling, and adhesion in the lumen; diapedesis or transmigration across the endothelium; and migration through the extravascular space toward a chemoattractant (reviewed by Cotran et al. 1989).

Leukocytes, along with erythrocytes, are found in the center of blood vessels in normally flowing blood, leaving a plasma layer in contact with the endothelium (Cotran et al. 1989, Guyton 1991). As blood flow slows, leukocytes come into contact with the endothelial cells, along which they roll and transiently adhere. Eventually, the endothelium can become lined with leukocytes (pavementing). Leukocyte adhesion is dependent on interactions between adhesion molecules on the endothelial cell and leukocyte surfaces.

Adhesion molecules are divided into three families (reviewed by Bevilacqua & Nelson 1993, Cotran et al. 1989, Elliott & Finn 1993, Smith 1993a, Smith 1993b, Vadas & Gamble 1990): selectins, immunoglobulins, and integrins. E-selectin

and P-selectin are found on the endothelial cell surface while L-selectin is found on leukocytes. The immunoglobulin family includes three endothelial cell adhesion receptors: ICAM-1 (intercellular adhesion molecule 1), ICAM-2, and VCAM-1 (vascular cell adhesion molecule 1). These immunoglobulin receptors are known to bind to integrins that are found on the leukocyte cell surface. The β_2 integrins CD11a/CD18 and CD11b/CD18 interact with ICAM-1 and ICAM-2 while $\alpha_4\beta_1$ integrin (VLA-4) binds to VCAM-1.

The loose adhesion that is associated with rolling involves the binding of the selectins (P-, L-, and E-selectin). While rolling, the leukocytes may become activated by factors released by the endothelium or by other cells in the area. Once activated, the leukocyte adhesion increases and the cells form more stable bonds with the endothelium, largely due to ICAM-1 and ICAM-2 binding to their integrins. Once bound, the leukocytes move along the endothelium until they encounter an intercellular space into which they insert pseudopodia. The leukocytes transmigrate through the endothelium until they are located between the endothelial cells and basement membrane. They eventually move through the basement membrane probably by releasing collagenases. As with increased vascular permeability, leukocyte diapedesis occurs predominantly in post-capillary venules (Cotran et al. 1989, Dvorak et al. 1986, Smith 1993).

1.3.2 Polymorphonuclear Leukocytes (Neutrophils)

Polymorphonuclear leukocytes (neutrophils) are a major cellular component of inflammation. Transendothelial migration of neutrophils is both a local and transient event; limiting the extent and duration of inflammation (Vadas & Gamble 1990). During the inflammatory reaction, neutrophils only migrate across the endothelium in the area of injury and, at sites of inflammation, this migration occurs through post-capillary venules (Cotran et al. 1989, Dvorak et al. 1986, Vadas & Gamble 1990).

The adhesion of neutrophils to the endothelium during rolling primarily involves the selectin family of adhesion molecules (Smith 1993a). L-selectin is constitutively expressed on the surface of unstimulated neutrophils and the activation of the vascular endothelium leads to the expression of P- and E-selectin (Bevilacqua & Nelson 1993). Once cells encounter one of the CD18 integrins, they stop rolling and bind firmly to the endothelium (Smith 1993a), but for this to occur CD18 needs to be upregulated. When neutrophils bind to selectins, CD18 integrin expression may be upregulated or chemotactic factors could also upregulate CD18 (Smith 1993a).

Chemotactic gradients across endothelial cell monolayers in vitro have been shown to promote the migration of neutrophils (Furie et al 1984, Taylor et al. 1981), but unless the endothelial cells are stimulated no more than 40 % of the

neutrophils will pass through (Smith et al. 1988). Smith and his colleagues found that stimulating endothelial cell cultures for three hours with interleukin-1β (IL-1β), tumour necrosis factor α (TNFα), or lipopolysaccharide (LPS) resulted in an increase in neutrophil transmigration. Although, IL-1 and TNF play an important role in neutrophil transmigration (Smith 1993b), neither are capable of directly affecting neutrophils after they come in contact with the endothelium.

1.4 CHEMOTAXIS

Chemotaxis is defined as the locomotion of cells along a chemical gradient in response to an attractant (Cotran et al. 1989). The term was first used in 1884 by Wilhelm Pfeffer in reference to the directional movement of plant sperm during migration (reviewed by Wilkinson 1974, Wilkinson 1982). It is important to differentiate chemotaxis from chemokinesis, which is defined as the accelerated random movement of cells in response to a chemical stimulus (Cotran et al. 1989, Ribaudo & Kreutzer 1985). It is also necessary to keep in mind that cells may also exhibit random movement, independent from any chemical gradient that may exist in the area.

The studies in chemotaxis increased greatly with the development of Boyden's chamber assay (Boyden 1962), especially those dealing with the chemotactic response of leukocytes. In Boyden's technique, the cells are separated from the chemotactic substance by a porous filter. If the substance is a chemoattractant, the cells move through the pores of the filter, along the chemical gradient.

1.4.1 Leukocyte Chemotaxis

In leukocyte chemotaxis, the cells move toward the area of highest chemoattactant concentration, where they accumulate (Wilkinson 1974, Wilkinson

1982). De Bruyn (1946) considered that the motion of leukocytes was similar to that of amoebae in shape and, like amoebae, leukocytes put out pseudopodia in the direction of movement. He believed that their was cytoplasmic streaming with a distinct "sol" and "gel" phase as in the amoebae. On the other hand, Ramsey (1972a, 1972b) did not consider leukocyte movement to be amoeboid, since he did not observe pseudopod formation and found that cytoplasmic flow was continuous. He instead described lamellipodia, which were flattened extensions of a cell used in locomotion. These lamellipodia are found on all sides of the cell, but the cell contents only flow into one of them; this is the direction of movement. If a chemoattractant were present, the cell contents would flow into the lamellipodium which faced its direction.

The presence of chemoattractant substances was found to only influence the direction of movement of neutrophils (Dixon & McCutcheon 1936, Ramsey 1972a), without affecting the speed of the movement. Thus, the function of a chemotactic gradient is to determine into which lamellipodium the cytoplasmic contents will flow. Ramsey (1972a) found that neutrophils migrated toward a chemoattractant at a constant speed of 10 μm/minute. If a chemoattractant is present, but no gradient exists, there is only an increase in the random movement of a leukocyte, or chemokinesis (Wilkinson 1974).

1.4.2 Chemoattractants

Both exogenous and endogenous substances can act as chemoattractants for neutrophils. The most common are bacteria and bacterial products; some of these peptides possessing the N-formyl-methionine terminal amino acid (Cotran et al. 1989, Murphy 1976). Other common chemotactic agents are components of the complement system (especially C5a), products of the lipoxygenase pathway of arachadonic acid metabolism, and prostaglandins (Cotran et al. 1989, Murphy 1976). When a chemoattractant is added to leukocytes in a non-gradient fashion, an increased number of cells are seen with a motile morphology (Malech et al. 1977). When added as a gradient, almost all of the cells are oriented toward the area of highest concentration. Some chemoattractants may stimulate chemotaxis at low concentrations but inhibit it at high concentrations (Becker 1972, Zigmond & Hirsch 1973). It has been seen that at certain concentrations, some of the agents that stimulate chemotaxis also stimulate locomotion in the cells. Rates of locomotion are not affected by the chemotactic stimulus (Harris 1954, McCutcheon 1946); rather their presence only affects direction of movement of leukocytes (Dixon & McCutcheon 1936, Ramsey 1972).

When chemotactic agents come into contact with leukocytes, they bind to specific receptors on the surface of the cells (Cotran et al. 1989). This results in the activation of the phospholipase C pathway, which leads to the formation of

inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Calcium is released into the cytoplasm, stimulating the assembly of the contractile elements responsible for cell movement. The leukocyte extends lamellipodia and pulls the rest of the cell in the direction of the chemical gradient (Ramsey 1972a, Ramsey 1972b). Locomotion involves the contractile proteins actin and myosin (Cotran et al. 1989, Murphy 1976), which are seen as a layer of microfilaments lying underneath the cell membrane. A gradient of chemoattractant induces a polarization of the neutrophil structure, leading to an increase in microfilaments at the leading end of lamellipodia (Malech et al. 1977), and the orientation of the centriole on the side of the nucleus facing the chemoattractant. Microtubules are the primary organizers of internal neutrophil contents, mobilizing the lamellipodia formation by providing a cytoskeleton (Malech et al. 1977). The microtubules also enhance unidirectional migration of the leukocytes.

In addition to stimulating locomotion, chemotactic factors are also able to induce other responses in leukocytes. It is believed that chemoattractants are able to regulate the function of adhesion molecules as well as the passage of leukocytes through the vascular endothelium (Elliott & Finn 1993). Certain chemoattractants stimulate an increase in the surface expression of β_2 integrins (CD18), allowing neutrophils to bind more firmly with ICAM-1. Chemotactic factors are able to increase the production of arachadonic acid metabolites, due to increased

intracellular DAG and calcium (Cotran et al. 1989). Leukocyte (neutrophil) degranulation and secretion of lysosomal enzymes, as well as the activation of oxidative burst (oxygen radical production) are also stimulated by chemotactic factors.

1.5 CHICK EMBRYO

1.5.1 Chorioallantoic Membrane (CAM)

In the chick embryo, the chorioallantoic membrane (CAM) serves as the primary gas exchange organ (Burton & Palmer 1989). It is also the site where nitrogenous waste is collected and stored until the egg hatches and the chick can eliminate its own waste into the environment.

The earliest evidence of the chick allantois is as a projection of the presumptive gut into the extra-embryonic coelom near the end of the second day of incubation (Leeson & Leeson 1963). Around the beginning of the fifth day of incubation, the allantois fuses with the chorion to form the chorioallantois, which is composed of the three germ layers (ectoderm, mesoderm, and endoderm). The chorioallantois lies immediately below the shell, and by the twelfth day of incubation the CAM has extended over the whole inner membrane of the shell (Sethi & Brooks 1971).

The CAM contains a dense array of capillaries which are supplied by the allantoic membrane, but become very closely associated with the endothelial cells of the overlying chorion (Romanoff 1960, Burton & Palmer 1989). By day ten, the capillary surface density has just about reached its maximal level, and beyond day eleven, there is no further increase in the area covered by the vessels (Burton

& Palmer 1989). Also by the tenth day, the capillaries are seen to have migrated from the mesoderm of the CAM up into the ectoderm (Danchakoff 1917, Romanoff 1960). The mesoderm of the CAM is composed of embryonic connective tissue, with an array of larger blood vessels coursing throughout. Its thickness varies, and is seen to be the greatest around the larger vessels (Romanoff 1960).

1.5.2 Tissue Grafting onto the CAM

The ability of the chick's CAM to support tissue grafts was shown early on by Murphy (1912) and Danchakoff (1918). Their technique proved to be invaluable in embryology, but since, has been used much more extensively, especially in the study of angiogenesis and tumour growth.

When a graft is placed onto the CAM between the seventh and ninth days of incubation, before the blood vessels have invaded the ectoderm, the cells of the ectoderm begin to proliferate rapidly, surrounding the graft (Romanoff 1960). Grafts are able to survive on the chorioallantoic membrane due to its extremely vascular nature and the chick embryo's immunologic immaturity (Glick 1976). Capillaries grow into the tissue very rapidly. The optimum time for engrafting tissue onto the CAM is between the ninth and twelfth days of incubation, since it

is during this time that the blood vessels are moving into the ectodermal germ layer (Danchakoff 1918).

The chick's CAM supports the growth of tissues from foreign species up until the 18th day of incubation. Beyond this point, the connective tissue elements increase greatly and eventually invade and replace the engrafted tissue (Murphy 1914).

1.5.3 Chick Hematology

Erythrocytes and leukocytes are both present in avian blood. As in reptiles, the red blood cells are nucleated. The leukocytes can be divided into nongranular and granular (granulocytes) populations. The granulocytes can be further subdivided into heterophils, eosinophils, and basophils (Romanoff 1960) which are functionally equivalent to the mammalian neutrophils, eosinophils, and basophils, respectively.

Heterophils, the most numerous granulocytes, contain eosinophilic rod-shaped granular bodies, instead of neutrophilic granules as in mammalian blood. The eosinophils contain granules that are much less eosinophilic than those within the heterophils, and can thus be considered pseudoeosinophils (Romanoff 1960). Three types of membrane-bound granules have been identified within the *Gallus* heterophils: small, dense granules; large, spindle-shaped, dense granules (specific

granules); and round, pale granules (Hodges 1974, Hodges 1979, Nair 1973). The heterophils are active motile cells outside of the blood stream, as are mammalian neutrophils. Their great motility is seen during inflammatory reactions where they are the first cells to the site (Nair 1973).

In adult birds, hematopoiesis takes place predominantly in the bone marrow. During embryogenesis, this process begins with the formation of the blood islands at the one-somite stage (approximately 24 hours incubation). The yolk sac becomes involved in the process between the third and fourth days of incubation (Romanoff 1960). The heterophils are not visible in the blood stream until the fifth to seventh days of incubation (reviewed in Romanoff 1960), and are in fact the only white cells present until the end of the second week of incubation. Eosinophils and basophils appear in the blood by day 18 and day 14, respectively (Sandreuter 1951).

1.6 HYPOTHESIS

The hypothesis for the present investigation is as follows.

Neutrophils play a role in tumour vascular endothelial hyperpermeability in the chick embryo chorioallantoic membrane. The mechanism of vesicular transport is not based on the vesiculo-vacuolar organelles (VVOs) described by Dvorak, but is rather due to the upregulation of free vesicular transcytosis.

1.7 OBJECTIVES

The objectives for the present investigation were as follows.

1. Establish the chick embryo chorioallantoic membrane system as an assay for the growth of human tumour cell lines.
2. Successfully grow murine hepatocytes into masses on this same chick embryo chorioallantoic membrane assay
3 Determine the labelled vesicular density in the endothelium at the tumour-host interface (correlates to vascular permeability).

4. Through comparison with vesicular density in the hepatic mass, N-fmlp-treated, and normal 16 day old CAM, establish that there is an increased vascular permeability associated with human tumours grown on the CAM.

5. Determine whether tumour vascular hyperpermeability results from the upregulation of vesiculo-vacuolar organelles (VVOs) in the chick embryo CAM.

6. Determine whether there is a distinct interstitial neutrophilia associated with tumour growth on the CAM.

7. Determine whether Hey-3 ovarian adenocarcinoma cells in culture produce a chemoattractant for neutrophils, using the Boyden chamber assay.

8. Establish whether the response of human neutrophils to Hey-3 tumour cell conditioned media is similar to their response to N-formyl-methionine-leucine-phenylalanine (a chemotactic peptide).

2.0 MATERIALS AND METHODS

2.1 PROTOCOLS

Experiments were performed with Animal Utilization Protocol approval from the McMaster University Animal Care Committee. Neoplastic cells were handled according to McMaster University Safety Committee Level 2 handling standards. All procedures were performed under aseptic contitions, with sterile equipment and media.

2.2 DESHELLING

Fertilized Delta Breeder chicken eggs were obtained from a local hatchery and incubated at 37°C for 4 days at which point they were deshelled as per Dugan et al. (1991) and Jakobson et al. (1989). Briefly, shell was chipped away from the blunt end of the egg, revealing the air pocket that is found beneath. The membrane

was punctured and a small hole was made in the shell at the opposite (pointed) end of the egg. The egg was deposited into a round-bottom bowl, covered with a lid, and placed in an incubator (37°C, 5 % CO_2, 95 % humidity).

2.3 GROWTH AND TREATMENT ON THE CAM

2.3.1 Tumour Cell Culture

The Hey-3 ovarian adenocarcinoma cell line was maintained in EB_3, a serum-free Dulbecco's modified Eagle's media (DMEM/F12). This EB_3 media was supplemented with epithelial growth factor (5 ng/ml), insulin (5 μg/ml), transferrin (10 μg/ml), phosphoethanolamine ($5x10^{-5}$ M), ethanolamine ($5x10^{-5}$ M), penicillin (100 U/ml), streptomyosin (100 μg/ml), amphotericin B (2 μg/ml), and bovine serum albumin (3 mg/ml). Cells were grown in Dulbecco's growth media supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin/streptomyosin (P/S). Following trypsinization with 1x trypsin diluted in PBS (pH 7.2), the cells were replated in Dulbecco's media supplemented with 5 % FCS and 1 % P/S. Before use, the Hey-3 cells were passaged up to 4 times in Dulbecco's media (5 % FCS).

2.3.1.1 CAM Inoculation with Hey-3 Cells

Once the Hey-3 cells had reached 50-80 % confluence in Dulbecco's media with 5 % FCS (2-5 passages), they were trypsinized and resuspended in the same supplemented Dulbecco's media. Viable cells were counted with a hemocytometer using trypan blue (0.2 %) exclusion. A cell suspension was prepared at a concentration of $2.0x10^6$ viable cells/ml.

Using a needle (27 gauge), the surface of 8 day old chick embryo chorioallantoic membranes (CAMs) were gently abraded. 50 µl aliquots (100 000 cells) of the Hey-3 cell suspension were dropped onto the abraded areas. The chick embryos were returned to the incubator and allowed to continue growing for another 8 days.

2.3.2 Hepatocyte Isolation

Collagenase (0.5 mg/ml) was dissolved in a calcium-free perfusion media, which contained sodium chloride (4.15 g), potassium chloride (0.25 g), HEPES (1.2 g), and sodium hydroxide (0.11 g) in 500 ml distilled water (pH 7.4).

C3H/HeJ mice (7-8 weeks old) were anaesthetized intraperitoneally with a Ketamine (100 mg/ml - 150 mg/kg)/Xylazine (20 mg/ml - 10 mg/kg) combination. The inferior vena cava was cannulated with PE60 tubing, in the retrograde fashion through the right atria. The liver was perfused with calcium-free perfusion media

at a rate of 5 ml/minute. As the liver was seen to clear, the hepatic portal vein was cut to allow the perfusion fluid to drain. After 2 minutes, this perfusion was switched to the collagenase solution (0.5 mg/ml) and continued for another 5 minutes (5 ml/minute).

Once perfusion was completed, the cannula was withdrawn, the liver removed and then transferred to a sterile 100 mm petri dish filled with perfusion media. The liver was gently broken apart and shaken in order to release the cells. The hepatocyte suspension was centrifuged for 5 minutes at 1000 rpm and the cells were resuspended in modified Eagle's media (α-MEM) supplemented with 10 % FCS and 1 % P/S.

2.3.2.1 CAM Inoculation with Isolated Hepatocytes

Hepatocyte cell number was calculated using a hemocytometer and viability determined by trypan blue (0.2 %) exclusion. The cells were diluted in α-MEM with 10 % FCS and 1 % P/S at a concentration of $2.0x10^6$ viable cells/ml.

The CAM surface of 8 day old chick embryos was abraded with a 27 gauge needle and 50 μl aliquots of cell suspension was dropped onto the areas as was done with the Hey-3 tumour cells. The embryos were allowed to grow for a another 8 days in a 37°C incubator (5 % CO_2).

2.3.3 N-fmlp Treatment of the Chorioallantoic Membrane

N-formyl-methionine-leucine-phenylalanine (N-fmlp) is a known chemoattractant for polymorphonuclear leukocytes (neutrophils) (Davis et al. 1982, Ribaudo & Kreutzer 1985). In order to determine whether chemotactically activated neutrophils are able to increase vascular permeability within the chick embryo CAM assay, N-fmlp was applied to the CAM.

N-fmlp was diluted in distilled water to a concentration of 10^{-4} M. 5 μl aliquots of this 10^{-4} M solution were placed onto the CAMs three times a day from day 14 to day 16 of chick embryo gestation.

2.4 MACROMOLECULAR TRACER INJECTION

2.4.1 Microinjection of Horseradish Peroxidase

Aluminosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL) were pulled with an Alexander-Nastuk micropipette puller (Industrial Science Associates, Inc., Ridgewood, NY) and beveled with a KT Brown type micropipette beveler (Sutter Instrument Co., Novato, CA). The tip of the microneedle was beveled until the outer diameter was 20-40 μm. A tuberculin syringe (1 ml) with a 23 gauge needle was attached to the microneedle through a

length of PE90 tubing. This apparatus was placed onto an injection stand in order to control the rate of flow through the needle.

Horseradish peroxidase (1 mg/ml) was diluted in sterile physiological (0.9 %) saline. 50 µl of this solution was injected into the arteriolar end of the CAM's vasculature at a rate of 20-25 µl/minute. The chick embryo was returned to the 37°C incubator and the tracer was allowed to circulate for 5 minutes, after which time the area of interest was excised. This injection procedure was used for all four experimental systems: 8 day old tumour on 16 day old CAM, 8 day old hepatic mass on 16 day old CAM, N-fmlp-treated 16 day old CAM, and normal 16 day old CAM.

2.4.2 Tissue Processing

The excised tissue from the CAMs was fixed with 2 % glutaraldehyde (EM grade) buffered in 0.1 M sodium cacodylate (pH 7.4) for 3 hours. It was then allowed to wash overnight in 0.2 M sodium cacodylate. The tissue was cut into 1 mm flat squares and these were washed with 0.05 M TRIS buffer in isotonic saline (pH 7.6). The sections were then pre-incubated in 0.05 % 3,3′- diaminobenzidine tetrahydrochloride (DAB) in TRIS buffered saline (pH 7.6) for 2 hours, in the dark, at room temperature. Following pre-incubation, the sections were again incubated in 0.05 % DAB in TRIS buffer with 0.01 % hydrogen peroxide (H_2O_2)

and 0.35 % imidazole (1,3-diaza-2,4-cyclopentadiene) for 20 minutes, in the dark, at room temperature. Imidazole was used in order to enhance the HRP stain as well as cell membranes since very little specimen staining was done otherwise.

Before conventional processing, the sections were washed in TRIS buffered saline. Tissue processing involved post-fixation in 1 % osmium tetroxide (OsO_4) buffered in 0.1 M sodium cacodylate for 1 hour at 4°C, dehydration in a graded series of alcohols (50 %, 75 %, 95 %, 100 %) and 100 % propylene oxide, followed by firm Spurr resin substitution of propylene oxide. The sections were infiltrated with Spurr resin and then flat-embedded overnight in the resin at 65°C.

80-100 nm sections were cut with a Reichert Ultracut E ultramicrotome (Reichert, Vienna, Austria) and mounted on 50 mesh formvar and carbon coated copper grids. The specimens were examined with a JEOL 1200EX Scanning Transmission Electron Microscope (JEOL USA, Peabody, MA). Images were taken, at 10 000x magnification, of the endothelium of 3 venules in the tumor-host interface of the specimen. Venules were defined as those non-muscular blood vessels with diameters of greater than 10 mm and irregular-shaped lumens.

2.5 ENDOCYTOTIC VESICLE COUNT

Using the Kontron MOP-Videoplan image analysis system (Eching, Germany) and contact sheets of the EM negatives, a vesicle count was determined in relation to endothelial cell cytoplasmic area for each test group. All endothelial cells were used in the vesicle count, regardless of whether a nucleus was present in the section, and the entire venule was evaluated. The n was the number of CAMs used from each experimental group. The number of 16 day old CAMs examined was as follows: five with tumours, four with hepatic masses, three N-fmlp-treated, and five normal (control). The compiled data was analyzed using Microsoft Excel and statistical significance (95 %) was determined with analysis of variance (ANOVA).

2.6 CHEMOTAXIS EVALUATION

2.6.1 Isolation of Neutrophils

Human peripheral polymorphonuclear cells (PMNs) were isolated from whole blood by a standard Ficoll-Hypaque protocol (Boyum, 1968).

For PMN separation, a Ficoll-Hypaque solution was prepared at room temperature by adding 3.1 ml 50 % Hypaque (sodium diatrizoate) to 7.1 ml 10.5 % Ficoll. In each centrifuge tube, 7 ml of whole blood was layered onto 3 ml of the Ficoll-Hypaque solution. This was spun at 400 g (1300 rpm) for 30 minutes. The PMN layer, lying on top of the red blood cell (RBC) fraction, was drawn out. In order to lyse any RBCs, 24 ml of ice cold distilled water was added to each 2 ml aliquot of the PMN fraction and agitated for 30 seconds. To this was added 8 ml 3.6 % sodium chloride (NaCl). This mixture was spun down at 400 g for another 15 minutes.

The pellet of PMNs was washed 3 times with physiological saline (0.9 % NaCl) and then resuspended in Hanks balanced salt solution (HBSS) with 0.5 % FCS at a concentration of $2.5x10^6$ viable cells/ml.

2.6.2 Chemotaxis Assay

Boyden chambers (Boyden 1962) were assembled using filters made of mixed cellulose esters (Millipore Filter Corp., Bedford, MA).

A standard chemotactic factor, N-formyl-methionine-leucine-phenylalanine, was used as a positive control in concentrations ranging from 10^{-4} M - 10^{-6} M, prepared by dilution in Hanks balanced salt solution (HBSS). Negative controls were HBSS, HBSS with 0.5 % FBS (HFBS), distilled water (dH_2O), EB_3 media,

and DMEM/F12 media. The test substance was conditioned media from Hey-3 cells grown to confluence in serum-free DMEM/F12 (EB_3) media (prepared as described above).

The PMNs, suspended in HBSS with 0.5 % FBS, were placed on the filter in the top compartment of the assembled Boyden chambers. The substances listed above were injected into the bottom compartment using a tuberculin syringe with a 25 gauge needle

The assays were allowed to run for 2 hours in a 37°C incubator, with 5 % CO_2. After incubation, the filters were removed, fixed and stained. This involved fixing the filter in 100 % ethanol for 3 minutes followed by staining with hematoxylin for 6 minutes, rinsing in distilled water for 30 seconds and a graded series of ethanols (70 %, 95 %, 100 %) for 2 minutes each. These were then allowed to air-dry overnight.

Two filters from each test group were cut into strips, immersed in liquid paraffin for 2-3 hours, and then embedded in paraffin for sectioning. The paraffin blocks were cut into 5 μm sections with a Riechert-Jung 2035 Biocut microtome (Cambridge Instruments, Heidelberg, Germany) and areas throughout the filter were sampled. These sections were mounted on Aptex coated slides, coverslipped with Permount, and examined.

An in-filter count was done on a 1.5 mm area of the filter section and the mean distance travelled by the cells in this area was determined. As well, the cell front within this 1.5 mm area for each filter section was determined. The cell front was defined by the distance travelled by 2 and 5 cells.

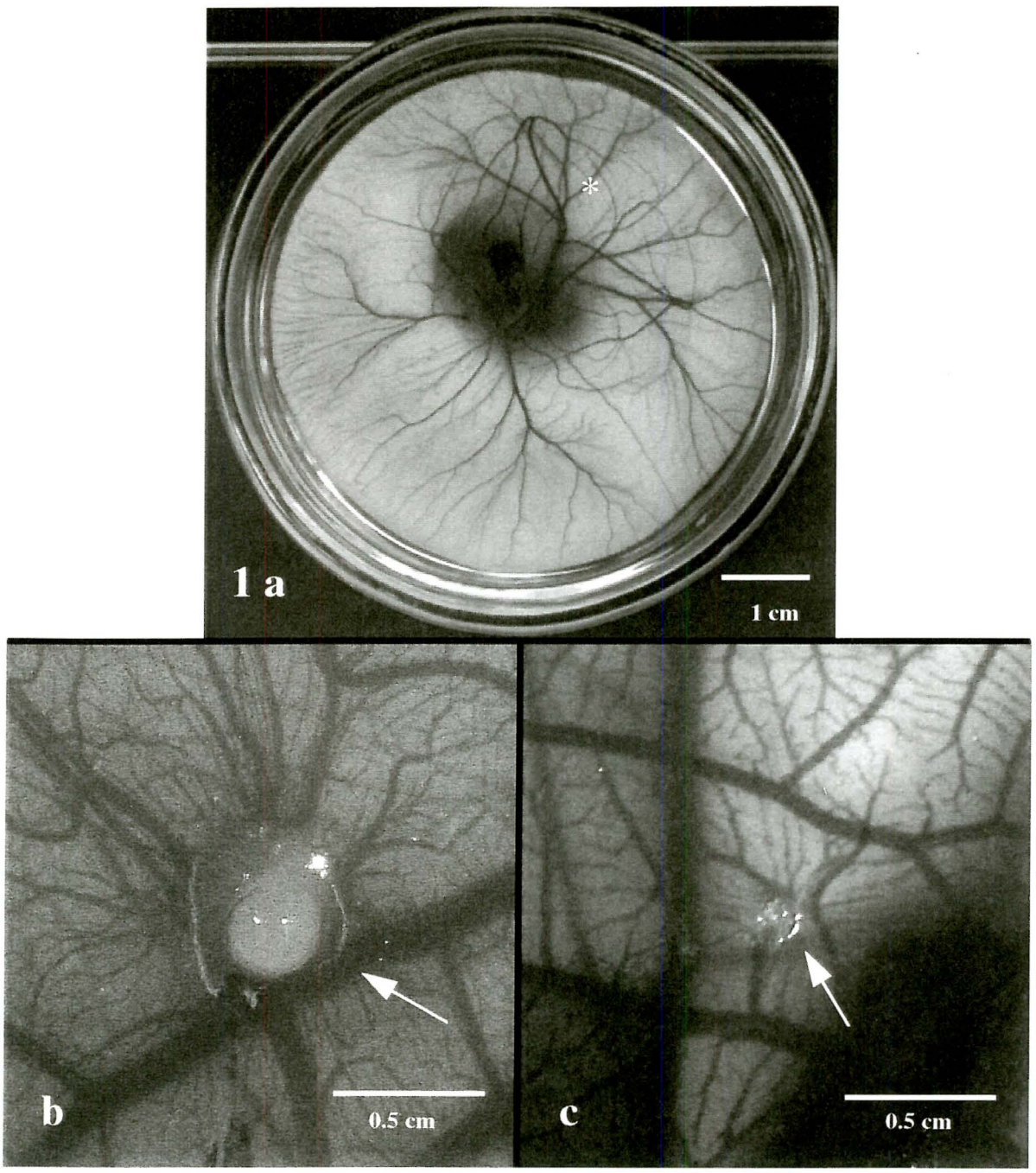
The compiled data was analyzed with Microsoft Excel and statistical significance (95 %) was determined with analysis of variance (ANOVA).

3.0 RESULTS

3.1 GROWTH ON THE CHORIOALLANTOIC MEMBRANE

Hey-3 cells (100 000/50 μl aliquot) were inoculated onto the chorioallantoic membrane (CAM) on the eighth day of chick embryo gestation. In Figure 1a, the CAM of an 8 day old chick embryo is shown and the area onto which tumour cells are placed is indicated. Tumour cell viability was determined to be greater than 98 % by trypan blue exclusion. Using a stereomicroscope, it was possible to observe white ovoid tumour masses within 24 hours. At this time, the smaller blood vessels can be seen to deviate from their normal branching pattern and curve toward the tumour. The larger vessels around the mass remain unchanged, maintaining their branching pattern. The tumour margins were richly vascularized throughout the experiment. As well, blood vessel branching deviation continues throughout tumour growth and by day 8, the area has a pin-wheel appearance. The blood vessels at the tumor-host interface consisted of pre-existing CAM vessels as well as newly induced microvessels. Figure 1b shows an 8 day Hey-3 cell tumour

Figure 1: 8 day old chick embryo and growth on the chorioallantoic membrane (CAM). **a.** 8 day old chick embryo with the CAM visible. The area of cell placement during inoculation is indicated (*). **b.** 8 day old Hey-3 tumour (arrow) on a 16 day old CAM. The pin-wheel appearance of the blood vessels radiating toward the mass is visible. **c.** 8 day old hepatic mass (arrow) on a 16 day old CAM. The mass is not as large as the tumour and few vessels are seen in the area.


1 a
1 cm
b
0.5 cm
c
0.5 cm

on the CAM of a 16 day old chick embryo. The tumours were 3-5 mm in diameter by day 8 of growth.

Hepatocytes were inoculated onto the CAM of 8 day old chick embryos using the same protocol as for Hey-3 tumour cells. The viability of the hepatocytes before inoculation was approximately 50 %, but 100 000 viable cells were placed on each CAM. Hepatocytes did not take as quickly on the CAM as did the Hey-3 tumour cells. Using a stereomicroscope, hepatocyte masses were not usually visible until 3-4 days post-inoculation. Few blood vessels were seen to deviate from their branching pattern and curve toward the mass. Although, these cells were initially delayed, they grew quickly and a few were similar in size to the tumours by day 8 post-inoculation. In Figure 1c, an 8 day old hepatocyte mass is shown.

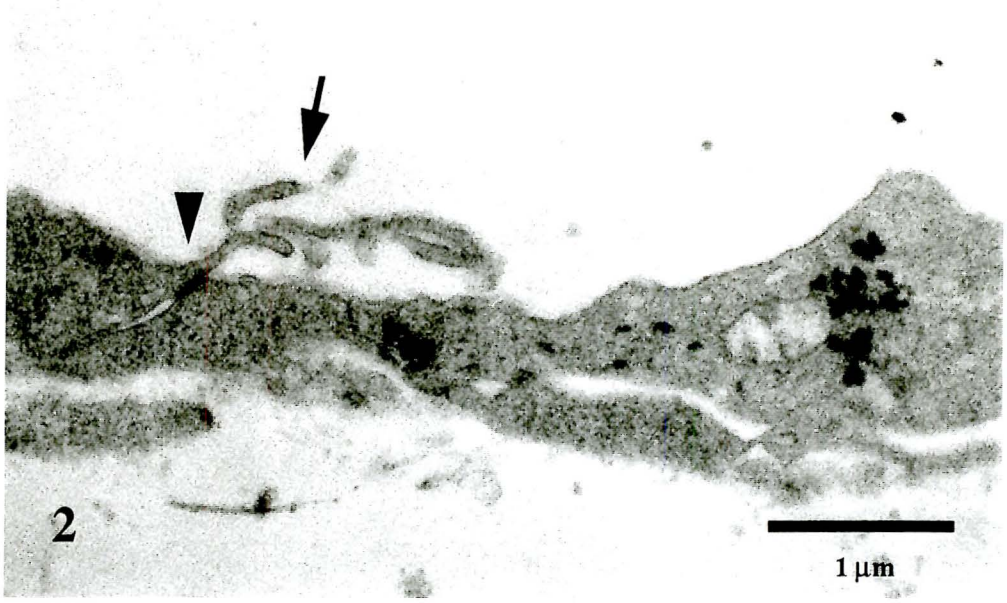
3.2 MORPHOLOGIC CHARACTERISTICS OF VASCULATURE (VENULES/SMALL VEINS)

3.2.1 Tumours

The blood vessels examined were the venules and small veins, since it has been reported that it is these vessels at the tumour-host interface that exhibit the greatest increase in tracer extravasation (Dvorak et al. 1988). At the electron microscopic level, it was possible to see that some areas of the endothelial cells were greatly folded with distinct projections extending into the lumen of the blood vessels (Figure 2). In the Hey-3 cell tumours, only continuous endothelium was found to line the vasculature.

As has been seen in many types of endothelium (Dvorak et al. 1988, Palade 1988), the endothelium of the tumour-associated blood vessels contained many cytoplasmic and plasma membrane-associated vesicles. These vesicles had a diameter that ranged from 50 nm to 500 nm. There were many more smaller, 50-100 nm vesicles than larger ones. These vesicles were seen to exist in patches along the length of venules and small veins. Essentially, vesicles were not seen within the cytoplasm all along the length and circumference of the vessels examined. This patchy leakiness has been observed in many instances (Dvorak et al. 1988). In Figure 3a, it is seen that these vesicles were found as pinocytotic

Figure 2: Micrograph of endothelial cells at the tumours-host interface. Plasma membrane projections (arrow) are extended into the lumen of the blood vessels. These extensions are most likely due to the folds in the luminal cell surface. Visible as well are interendothelial spaces with distinct cell junctions (arrowhead).

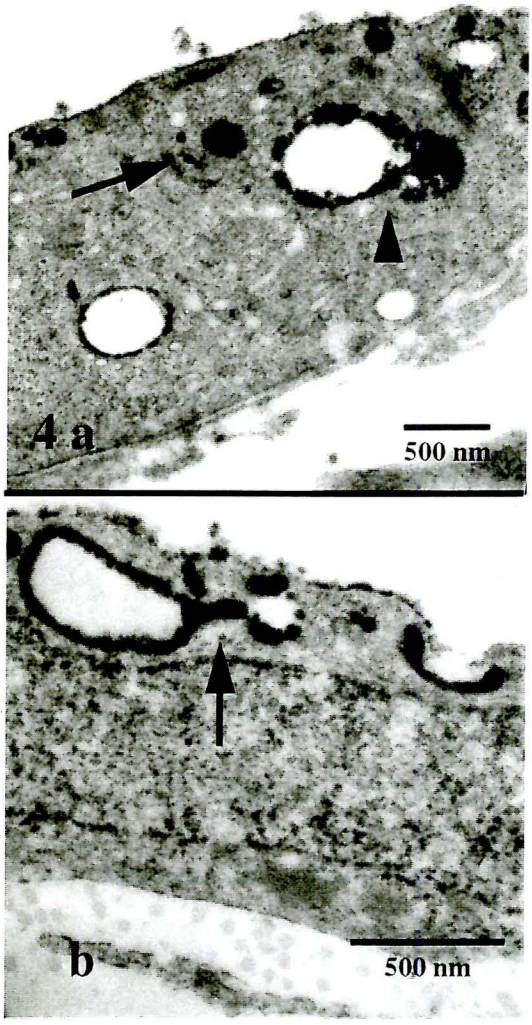

2
1 µm

omega (Ω) profiles at the luminal plasma membrane as well as free within the cytoplasm. Quite often these pinocytotic vesicles were found in the cytoplasm between the luminal plasma membrane and the nucleus (Figure 3b), causing what appears to be a deformation in shape of the nucleus in order to accommodate the larger vesicles. These larger vesicles were not seen as pinocytotic omega profiles at the luminal plasma membrane, but have been observed fused to the abluminal plasma membrane. Interendothelial junctions between adjoining endothelial cells were, in some cases, short and oriented perpendicular to the blood vessel surface. More often though, the junctions were long and oriented diagonal to the luminal plasma membrane (Figure 3b). Regardless, the membranes of the junctions were quite apposed and no tracer was visible in the area.

Pairs of fused vesicles were seen on occasion, but most vesicles were seen to exist free within the cytoplasm. Figure 4 shows two examples of fused vesicles. When these fused vesicles were found, there was always a smaller vesicle fused to a larger ones in the manner depicted in Figure 4a. In Figure 4b, a small vesicle is joined to a large one through a tube-like structure. The membranes around these structures are quite often not visible, but are rather obscured from view by the presence of the electron dense tracer (HRP/DAB). The vesiculo-vacuolar structures (VVOs) described by Dvorak's group (Dvorak et al. 1996, Kohn et al. 1992) were not seen at any time.

Figure 3: Micrograph of pinocytotic vesicles within endothelial cells at the tumour-host interface. **a.** Pinocytotic omega (Ω) profiles are seen at the luminal surface of the cells (arrow). As well, vesicles are free within the cytoplasm (arrowhead). These pinocytotic vesicles contain horseradish peroxidase (dark deposits). **b.** Vesicles are also localized between the plasma membrane and the nucleus. A diagonal intercellular space is shown as well (open arrow). In both **a** and **b**, collagen fibrils are seen to be located subjacent to the basement membrane (arrow).

Figure 4: Micrograph of pairs of fused vesicles within endothelial cells at the tumour-host interface. **a.** A small vesicle is seen fused to a large vesicle in a "∞" configuration (arrowhead). Free vesicles are also visible in the area. The arrow indicates one that is approximately 150 nm in diameter. **b.** A small vesicle is attached to a large vesicle by a tube-like structure (arrow) which is also filled with tracer.


4 a
500 nm
b
500 nm

Underlying the endothelial cells was a basement membrane and, rarely, pericytes were present. Below the basement membrane, collagen fibrils are consistently found, both in longitudinal and cross-section, associated with these leaky blood vessels.

3.2.2 Hepatocyte Masses

Venules and small veins were examined in the hepatocyte mass-host interface. It has been reported that in normal tissue and in inflammatory reactions, it is the post capillary venules that exhibit the greatest amount of tracer extravasation (Curry & Joyner 1988) and, as well, these vessels exhibit the same patchy leakiness along their length as has been seen in tumour vessels. These two characteristics were seen within the vessels associated with the hepatocyte masses. The vessels in the hepatocyte mass-host interface exhibited a prominent folding of their endothelial cells into the vascular lumen as was seen within the tumour-induced blood vessels. Only continuous endothelium was seen to line the venules that were examined. Small and large vesicles were seen within the endothelial cell cytoplasm of these blood vessels. The vesicles ranged in size, but were not found to be as large as the ones in the tumour vascular endothelium. The small vesicles were approximately 50 nm, while the large ones had a diameter of 350-400 nm, which is slightly smaller than those in the vasculature at the tumour-host interface.

In Figure 5, the vesicles can be seen as pinocytotic omega (Ω) profiles at the luminal plasma membrane as well as free in the cytoplasm; they are located away from the nucleus (Figure 5a), but also in the cytoplasm between the plasma membrane and the nucleus (Figure 5b). Fused vesicles were observed, but channels and VVOs were not seen at any instance in this system.

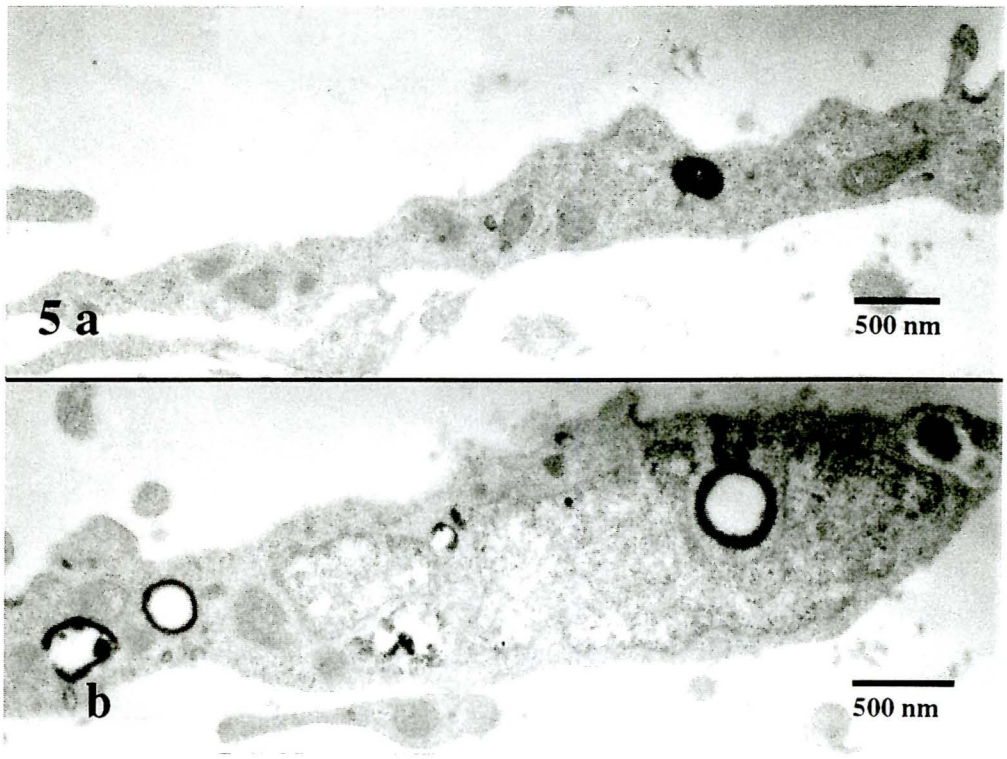
As seen in the tumour vasculature, interendothelial junctions were both short and long with no tracer present in the area. Underlying the endothelium was a basement membrane as well as some scattered pericytes. Collagen fibrils, again, were a prominent and consistent element underlying the basement membrane.

3.2.3 N-fmlp-treated CAM

N-formyl-methionine-leucine-phenylalanine (N-fmlp) was placed on the CAM. No changes were seen in the branching pattern at day 16 of CAM development. There was no discernible difference between the N-fmlp-treated and the control 16 day old CAM blood vessels.

In Figure 6, there were labelled vesicles visible in the cytoplasm of the endothelial cells. Small and large vesicles were seen, ranging in size from 50 nm

Figure 5: Micrograph of pinocytotic vesicles within endothelial cells at the hepatic mass-host interface. Tracer labelled vesicles are located free within the cytoplasm (**a**). **b.** As in the tumour endothelium, these vesicles can also be seen between the plasma membrane and the nucleus.


5 a
500 nm
b
500 nm

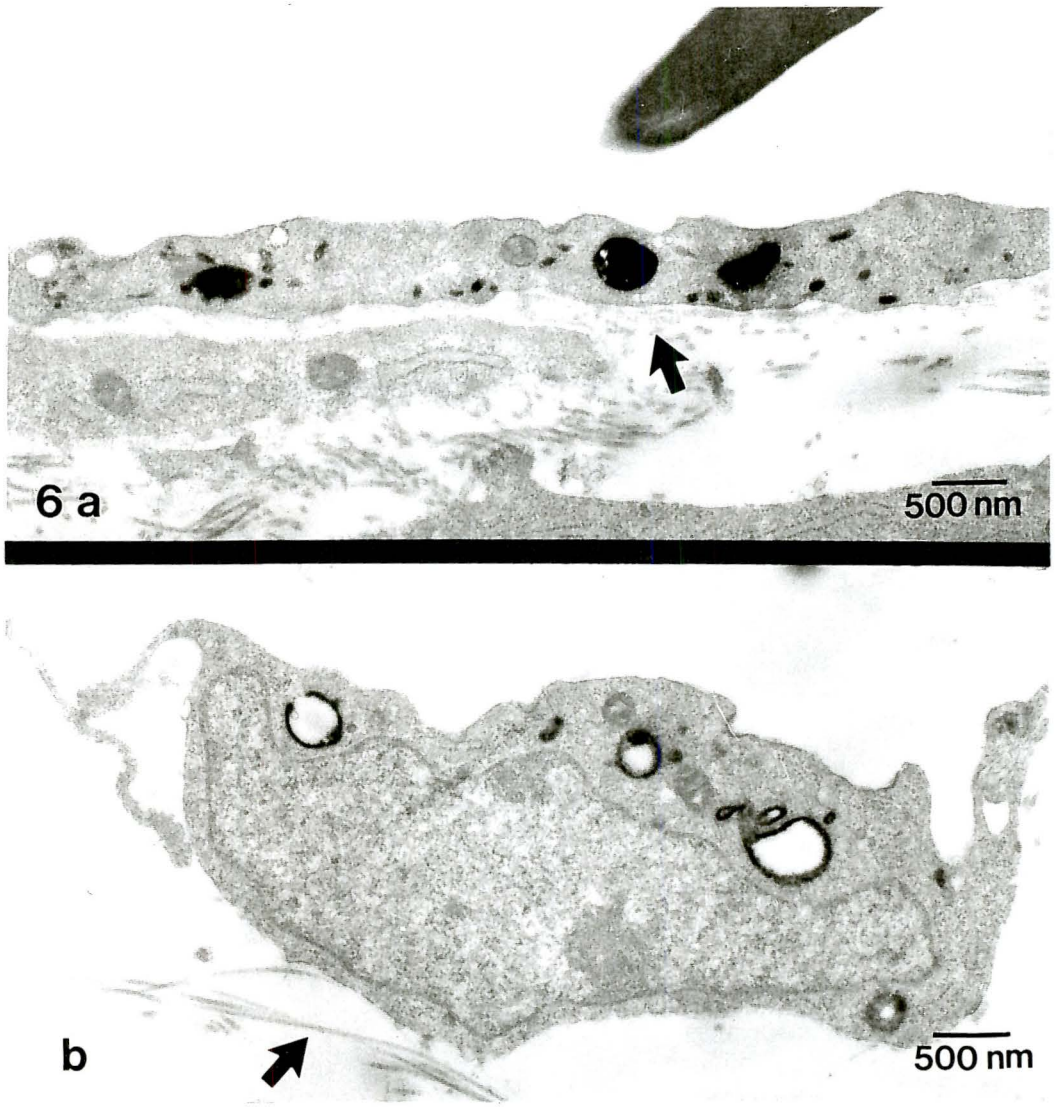
to 500 nm, as did those within the endothelial cells of the venules at the tumour-host interface. These vesicles were found as pinocytotic omega (Ω) profiles at the luminal plasma membrane as well as free within the cytoplasm. Vesicles were found throughout the endothelial cells (Figure 6a), including between the plasma membrane and the nucleus (Figure 6b). As in the tumour-associated blood vessels, there are quite a few large vesicles, unlike within the control endothelial cells where the smaller vesicles are much more common. Fused vesicles were observed, but neither channels nor VVOs were found within this system.

Interendothelial junctions were of both varieties (short, perpendicular and long, diagonal). Underlying the endothelium was a basement membrane and scattered pericytes. These did not appear to be any different from those of the tumour blood vessels. The endothelium possessed underlying collagen fibrils, which were seen in longitudinal and cross-section.

3.2.4 Control 16 Day Old CAM

The blood vessels of the normal CAM of a 16 day old chick embryo were examined by electron microscopy. The endothelium of the venules was flattened and endothelial cells were rarely seen to fold into the lumen of the blood vessels. The vessels were lined with a continuous endothelium as was seen in all of the systems examined. As in the other systems, small and large vesicles were found

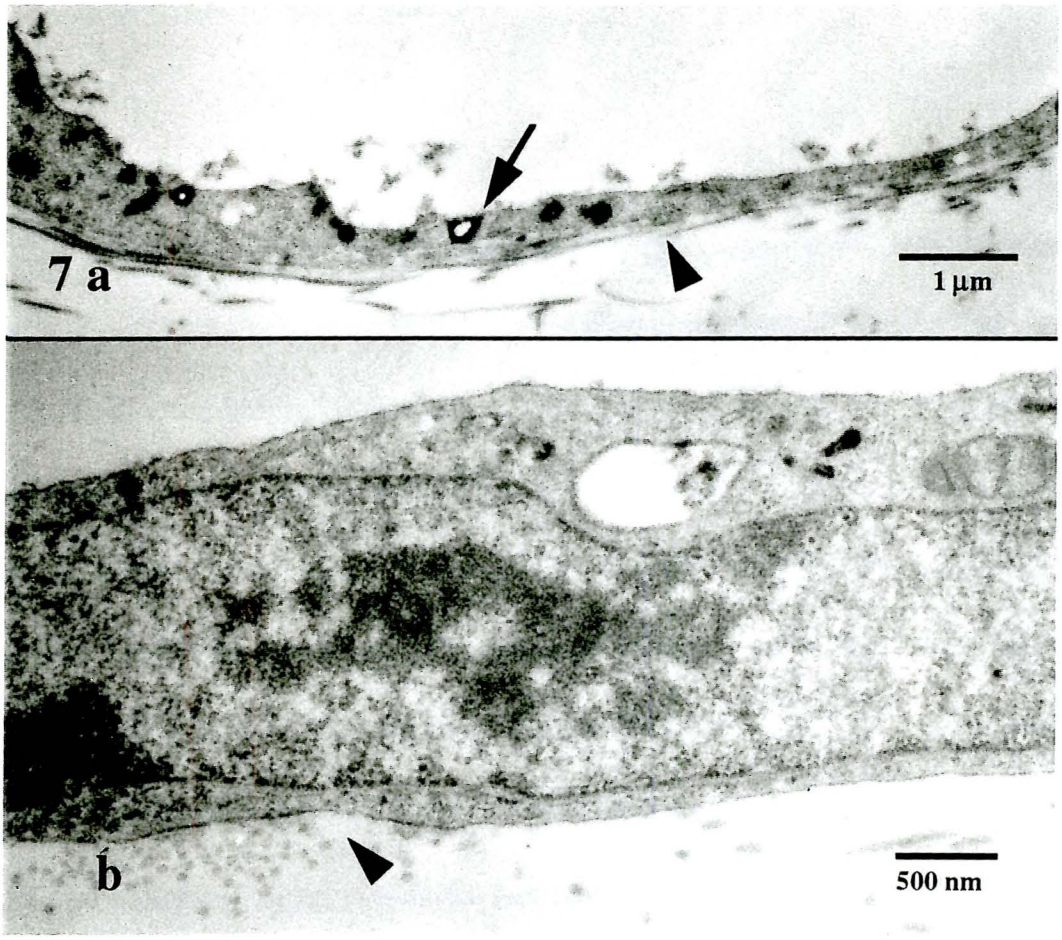
Figure 6: Micrograph of pinocytotic vesicles within endothelial cells of N-fmlp-treated CAM. Vesicles are found free within the cytoplasm (**a**) and between the plasma membrane and the nucleus (**b**). Basement membrane (arrow) underlies the endothelial cells with collagen fibrils subjacent to it.


6 a
500 nm
b
500 nm

within the cytoplasm; small vesicles far outnumbering the larger ones (Figure 7). The vesicles ranged in size from 50 nm to approximately 300 nm. These vesicles were predominantly free structures within the cytoplasm. As in the other systems, the vesicles were seen to pinch off and be found both associated with the nucleus (Figure 7b) and in the cytoplasm located away from the nucleus (Figure 7a). Fused vesicles were observed, but neither channels nor VVOs were ever found within the endothelium.

The interendothelial junctions were both short, perpendicular structures as well as long diagonal ones. Tracer was not seen in these interendothelial spaces. Underlying the blood vessels was a basement membrane as well as some scattered pericytes interspersed between arrays of collagen fibrils.

Figure 7: Micrograph of pinocytotic vesicles within endothelial cells of control 16 day old CAM. **a.** Vesicles are seen free within the cytoplasm and pinocytotic omega (Ω) profiles (arrow) are found at the cell surface as well. **b.** Vesicles are located between the plasma membrane and the nucleus. Both **a** and **b** show an underlying basement membrane with collagen fibrils scattered beneath it (arrowhead).


7 a
1 µm
b
500 nm

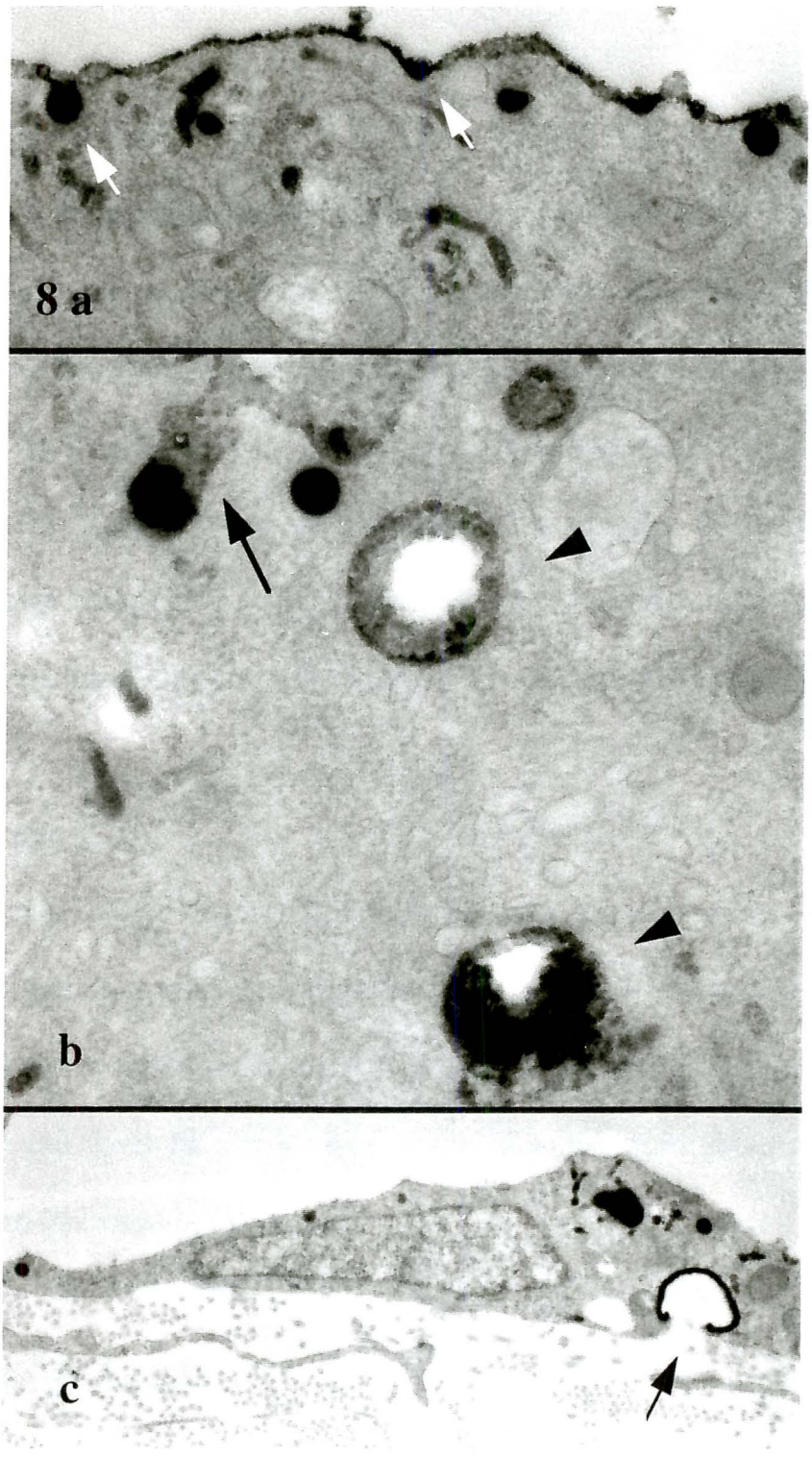
3.3 EXTRAVASATION OF SOLUBLE TRACER (HRP) FROM VENULES/SMALL VEINS

3.3 1 Tumours

Extravasation through the endothelium of blood vessels at the tumour-host interface occurred predominantly by way of membrane-bound vesicles that pinched off at the luminal plasma membrane, travelled through the cytoplasm, and later opened onto the abluminal surface of the same cell. In some cases, smaller vesicles fused to form larger vesicles (Figure 4) which travelled through the cytoplasm and later opened onto the abluminal plasma membrane. Many medium and large-sized labelled vesicles were found within the cytoplasm. Tracer-labelled vesicles were seen at different levels throughout the cytoplasm (Figure 3); i.e. as pinocytotic omega (Ω) profiles at the luminal plasma membrane (Figure 8a), at different depths through the endothelial cell cytoplasm (Figure 8b), and opening onto the abluminal surface (Figure 8c). The larger vesicles are not completely full of tracer, but rather seem to be lined by it on the inside. VVOs were not seen in any sample examined.

Tracer was not found in the interendothelial spaces. In some instances, intercellular junctional complexes were visible (Figure 2).

Figure 8: Micrograph of pinocytotic vesicles at different depths within endothelial cells at the tumour-host interface. Labelled vesicles were found throughout endothelial cells after five minutes of tracer circulation. They were seen as pinocytotic profiles (arrows) and near the luminal surface (**a**) as well as at different depths through the cytoplasm (arrowheads) (**b**). Occasionally, large vesicles were seen opened onto the abluminal surface (arrow) (**c**).


8 a
b
c

3.3.2 Hepatocyte Masses

Vesicles were the predominant means of tracer extravasation in the blood vessels at the hepatocyte mass-host interface. These vesicles were visible at different depths within the cytoplasm (Figure 5) and, occasionally, smaller vesicles were seen to be fused to larger ones. Most of the vesicles were found to be free structures and not associated with any other vesicle in its vicinity VVOs were not found within this system and are thus not involved in tracer extravasation.

Tracer was not found between endothelial cells, in the interendothelial spaces.

3.3.3 N-fmlp-treated CAM

Extravasation of soluble tracer (HRP) occurred by the same means described for tumour and hepatocyte mass-associated blood vessels. The vesicles were predominantly free throughout the cytoplasm of the endothelial cells (Figure 6). VVOs were not seen at any time within this system. Tracer was not found to be located within the interendothelial spaces.

3.3.4 Control 16 Day Old CAM

As was seen in past studies, tracer extravasated through the endothelium of normal tissue to a lesser degree (Dvorak et al. 1984, Heuser & Miller 1986, Underwood & Carr 1972) compared to tumour-associated vasculature. Vesicular

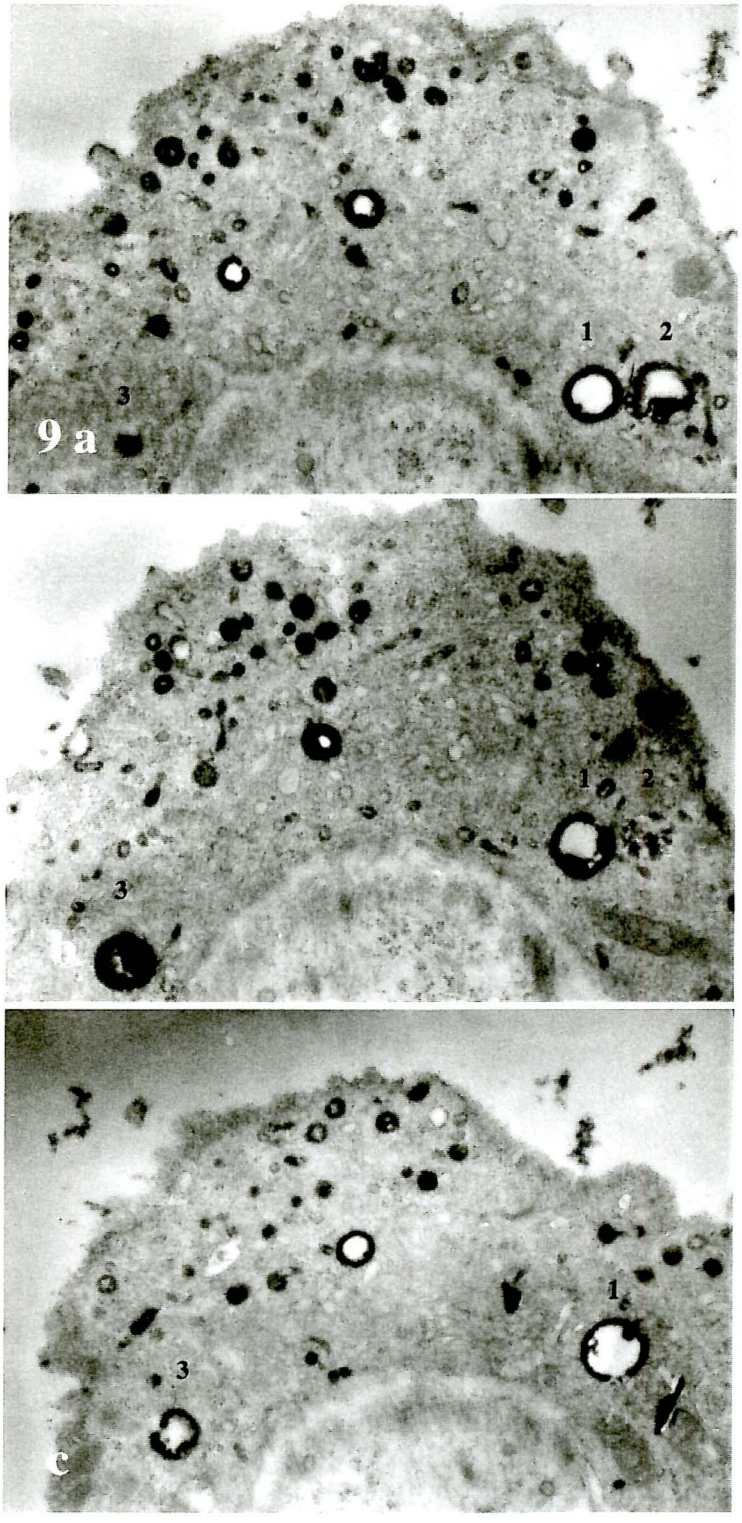
transport was the predominant means of tracer transcytosis. Free vesicles were found throughout the endothelial cell cytoplasm (Figure 7) and, occasionally, fused vesicles were seen. Tracer was not present in the interendothelial spaces. As with the other systems, VVOs were not found in the endothelium of normal CAM blood vessels.

3.4 SERIAL SECTION ANALYSIS OF VESICLES

In order to determine the association, if any, that exists between cytoplasmic vesicles, serial sections of endothelium at the tumour-host interface were evaluated at the electron microscopic level. In Figure 9, it can be seen that within the 400-500 nm thickness examined, the vesicles are not associated with vacuoles in VVO structures as described by Dvorak's group (Dvorak et al. 1996, Kohn et al. 1992). This same group has also determined that VVOs are enormous structures with a median area of approximately 0.12-0.14 μm^2 (Dvorak et al. 1996). Although, the vesicles were seen within areas this large (and even larger), the smaller vesicles are all free within the cytoplasm, and do no form clusters. No caveolae-like structure can be seen to exist between adjacent vesicles. Most of the vesicles were not located close enough together to be joined in any way. The VVOs have been said to contain vesicles with diameters of greater than 200 nm, but vesicles with diameters in the range of 500 nm were not described. In this study, vesicles of this large size were seen quite often. In Figure 9, there is a vesicle shown that has a diameter of approximately 400-450 nm.

The outer limits of the larger vesicles are evident. By doing this, it is apparent that the larger vesicles are indeed coated by the electron-dense tracer (HRP), while

Figure 9: Micrographs of three serial sections of an endothelial cell at the tumour-host interface. Many vesicles are seen throughout the cytoplasm in each image. The smaller vesicles (located predominantly near the luminal surface) only appear in one image; the vesicles that are in **a** are not in **b** or **c**. Thus their limits are not discernible. The limits of the larger vesicles are evident. These vesicles are labelled 1, 2, and 3. **a.** Vesicles 1 and 2 are fully visible, while only the edge of 3 is present. **b.** All three large vesicles are visible with the section being through the edges of 2 and 3 (full of tracer). **c.** This section was made outside of the limits of vesicle 2.


1
2
3
9 a
1
2
3
b
1
3
c

the smaller ones are filled with HRP. Also by this method, it is possible to see that vesicular channels are not present within this section of the endothelium.

3.5 PINOCYTOTIC VESICLE COUNT

In order to determine level of permeability for each test group, pinocytotic vesicles were counted and their cytoplasmic concentration calculated. The vesicle counts do not irrefutably indicate permeability, but rather suggest that this is what is occurring. The data are presented in Table 1.

The control 16 day old chorioallantoic membrane (CAM) vascular endothelial cells showed 0.51 +/- 0.09 vesicles/μm^2. For the five tumours examined, pinocytotic vesicle concentration was calculated to be 0.99 +/- 0.28 vesicles/μm^2, which was determined to be significantly greater in tumour vascular endothelium ($p < 0.05$). The density of labelled vesicles is approximately two times that of control in the chick embryo CAM assay.

The peptide N-formyl-methionine-leucine-phenylalanine (N-fmlp) is known to be a chemoattractant to neutrophils. When placed on the CAM, neutrophils were attracted to the area and the pinocytotic vesicle count was calculated to be 1.04 +/- 0.09 vesicles/μm^2. In comparison to control, vesicle concentration was found to be significantly greater ($p < 0.05$). The vesicle density in the N-fmlp group is approximately twice that of control as it was in the tumour. There is not a statistically significant difference ($p > 0.05$) between N-fmlp permeability (1.04 +/- 0.09 vesicles/μm^2) and that of the tumour group (0.99 +/- 0.28 vesicles/μm^2).

Hepatocyte growth on the CAM resembled that of the Hey-3 tumour cell line, but the masses did not get as large as the tumours; the masses were round and invaded the mesoderm of the CAM. Permeability was found to be very different. The vascular endothelial cells at the hepatocyte mass-host interface contained 0.54 +/- 0.03 vesicles/μm^2 compared to the 0.99 +/- 0.28 vesicles/μm^2 seen in the tumour vasculature. These vesicle concentration differences are statistically significant ($p < 0.05$). As with the tumour group, vesicle density in the N-fmlp samples (1.04 +/- 0.09 vesicles/μm^2) was much greater than in the hepatocyte samples; this difference was also highly statistically significant ($p < 0.05$). However, vesicle counts in the hepatocyte samples (0.54 +/- 0.03 vesicles/μm^2) were very similar to those of the control group (0.51 +/- 0.09 vesicles/μm^2).

Thus, the number of pinocytotic vesicles labelled with HRP between the groups can be summarized as follows: N-fmlp > tumour > hepatocyte > control.

Table 1: Horseradish Peroxidase/DAB - Labelled Pinocytotic Vesicles Within Endothelial Cell Cytoplasm

	Cytoplasmic Area (μm^2)	Vesicle Count	Vesicles/μm^2 (+/- SD)
Control (n=5)	111.71	57	0.51 +/- 0.09
Tumour (n=5)	246.90	234.8	0.99 +/- 0.28 [a,d,e]
N-fmlp (n=3)	191.40	198	1.04 +/- 0.09 [b,d,f]
Hepatocyte (n=4)	259.85	139.5	0.54 +/- 0.03 [c,e,f]

Statistical comparison of vesicles/μm^2 of endothelial cell cytoplasm:

[a] Tumour and Control $p < 0.05$
[b] N-fmlp and Control $p < 0.05$
[c] Hepatocyte and Control $p > 0.05$
[d] Tumour and N-fmlp $p > 0.05$
[e] Tumour and Hepatocyte $p < 0.05$
[f] N-fmlp and Hepatocyte $p < 0.05$

3.6 NEUTROPHIL LOCALIZATION IN THE EXTRAVASCULAR TISSUE

Polymorphonuclear leukocytes (neutrophils) in the extracellular space were identified by morphological features at the light and transmission electron microscopic levels (Hodges 1979).

3.6.1 Tumours

Neutrophils, or heterophils* in the avian circulation, were seen to be a prominent and consistent feature in the extravascular tissue at the tumour-host interface. Figures 10a and 10b show the localization of neutrophils around the tumour mass at the light microscopic level. At the low magnification in Figure 10a, it is apparent that the neutrophils are not evenly distributed around the tumour. The greatest concentration is evidently in closest proximity to the tumour, within the first 100 μm, and seems to encircle the mass. In essence, the neutrophils are forming a ring around the tumour mass. Although it is not possible to determine if these cells are actually moving toward the tumour, this is a possibility, since they were observed in the vascular lumens as well as underlying the endothelium (Figure 12). The CAM around the tumour mass is greatly enlarged, and the mesoderm is filled with embryonic connective tissue.

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* From this point on, the heterophils will be referred to as neutrophils.

Figure 10: Light micrograph of a cross-section of an 8 day old Hey-3 tumour mass on a 16 day old CAM. The edge of the tumour is visible as well as the surrounding CAM (**a**). Neutrophils (b) are located around the entire periphery of the tumour and are concentrated in the first 100μm (arrow). The CAM outside of this area possesses some scattered neutrophils. The CAM mesoderm is filled with embryonic connective tissue and blood vessels.

Figure 11: Light micrograph of a cross-section of an 8 day old hepatic mass on a 16 day old CAM. Neutrophils are found concentrated in the mesoderm underlying the CAM ectoderm adjacent to the hepatic mass (**a**). These neutrophils (**b**) also radiate somewhat around the periphery of the mass, but not to the degree seen around the tumour mass.

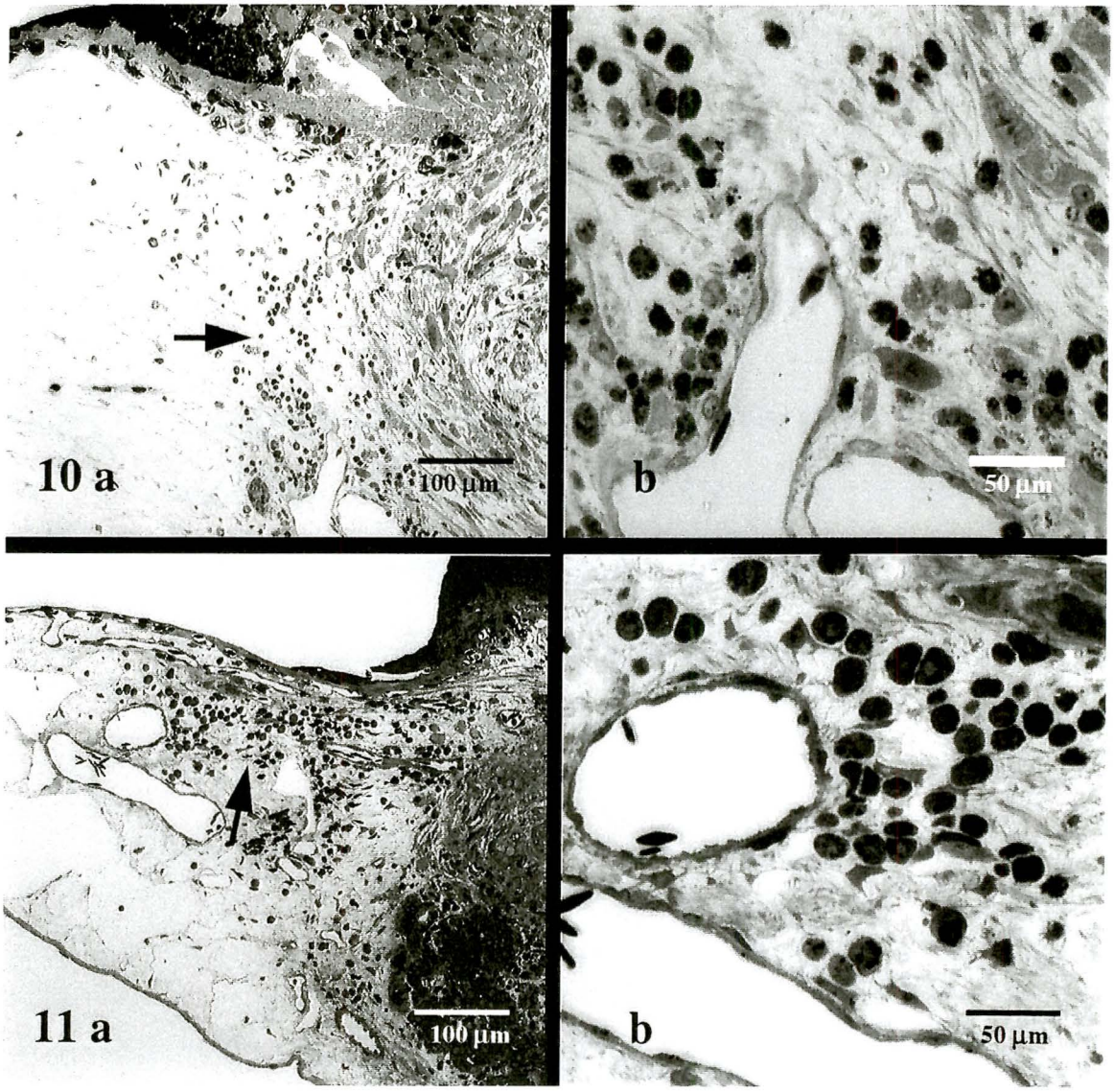
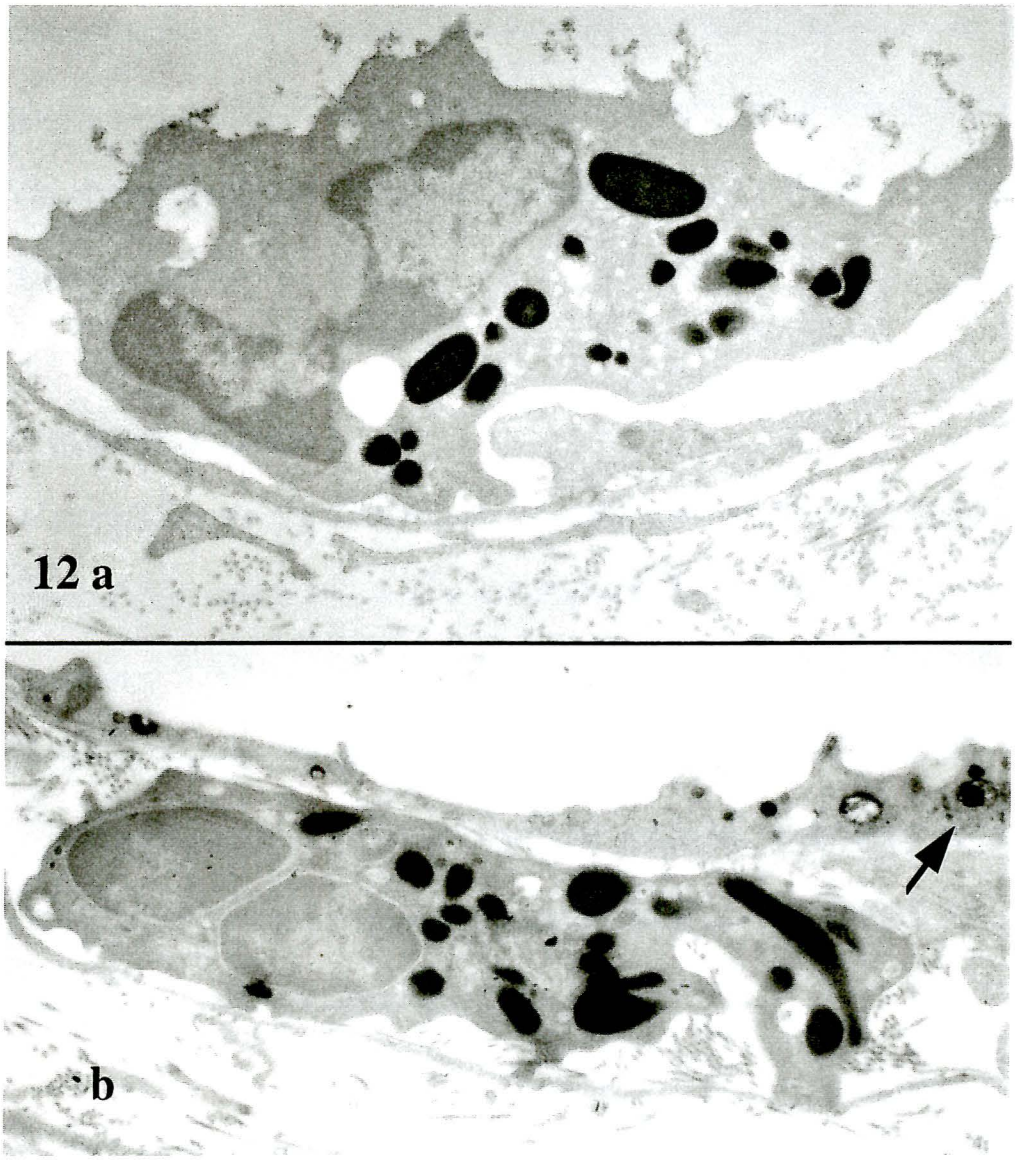

10 a
100 µm
b
50 µm
11 a
100 µm
b
50 µm

Figure 12: Micrographs of chick heterophils (neutrophils) at the electron microscopic level. A neutrophil is seen in contact with the endothelium at the luminal surface (**a**) as well as at the abluminal (**b**). Labelled vesicles are seen in the endothelium as well (arrow).


12 a
b

In Figure 10, neutrophils are seen to be present in the circulation as well as in the extravascular tissue. Although neutrophils were found in great numbers in the extravascular tissue at the tumour-host interface, no other leukocytes were seen to be present. By day 16 of chick embryo gestation, there are other leukocytes within the circulation (Sandreuter 1951), but, as expected, these were not present in the extravascular tissue.

3.6.2 Hepatocyte Masses

Neutrophils were also seen in great numbers at the hepatocyte mass-host interface. Fewer neutrophils were associated with the hepatic mass in comparison to the tumour. The distribution was also quite different from that seen in relation to the tumour masses (Figure 10a with Figure 11a). A large fraction of the leukocytes are found to be gathered around the periphery of the hepatic mass, but they are also greatly concentrated near the ectodermal surface of the CAM. This was not seen in the tumour-host interface.

3.6.3 N-fmlp-treated CAM

The CAM in the N-fmlp-treated area is shown in Figure 13 There are not as many neutrophils in this section as were seen in the tumour-host interface (Figure 10), but when compared to control, there do appear to be more neutrophils present.

The mesoderm appears to be much more packed with connective tissue in comparison to control. This is very different from the CAM around the tumour and hepatic masses, which possessed mesoderm that resembled that of control.

3.6.4 Control 16 Day Old CAM

The CAM of a normal (control) 16 day old chick embryo appears to be very ordered (Figure 14). The thin ectoderm and endoderm border the thick mesoderm at opposite sides. The mesoderm contains larger blood vessels while the smaller ones are localized in the ectoderm. Embryonic connective tissue fills the remaining mesodermal area. A few neutrophils are present in the area around the blood vessels (Figure 14b), but not free within the mesodermal connective tissue.

Figure 13: Light micrograph of a control 16 day old CAM. The ectoderm and endoderm are thin and border a thick mesoderm filled with embryonic connective tissue. There are a few neutrophils (arrow) present, associated with the blood vessels.

Figure 14: Light micrograph of a N-fmlp-treated 16 day old CAM. There are not many neutrophils (arrow) in the area, but those that are there are associated with the blood vessels.

3.7 CHEMOTAXIS EVALUATION

In order to determine whether Hey-3 tumour cells synthesize and secrete a chemoattractant for neutrophils, conditioned media from these cells was obtained and its effect on neutrophils was evaluated in comparison to positive and negative controls. The complete data are presented in the Appendix (Tables A1-A3) and summarized in Table 2.

Chemotaxis was evaluated be measuring the distance travelled by each neutrophil within a mixed ester filter, using the Boyden chamber assay. For each test group, two filters were examined in cross section throughout the complete filter. Table 2 (row 1) is a compilation of the mean distances travelled by the neutrophils within the filters in response to the substance in the lower compartment of the chambers. By examining these numbers, it is evident that the distance the neutrophils have travelled is different depending on the substance that is present in the compartment. Analysis of variance was used to determine the significance of the difference between the distances travelled.

When comparing the distances travelled by the neutrophils in response to conditioned EB_3 media (c-EB_3) to those in the other test groups, it is evident that the mean distance is greater for c-EB_3 than for the positive and negative controls. The positive controls are the different concentrations of the chemotactic peptide

Table 2: Distance Travelled (in micrometers) by Granulocytes (Neutrophils) Through Mixed Ester Filters (Mean +/- SD)

c-EB_3[β] (n=20)	Nfmlp[χ] 10^{-6} M (n=18)	Nfmlp 10^{-5} M (n=18)	Nfmlp 10^{-4} M (n=14)	EB_3 (n=19)	DF[δ] (n=19)	HBSS[ε] (n=20)	HFBS[ϕ] (n=18)	dH_2O (n=16)
44.36 +/- 5.4[a]	36.75 +/- 3.3	32.93 +/- 2.5	34.68 +/- 2.8	16.88 +/- 2.5	21.81 +/- 2.6	22.62 +/- 1.9	20.03 +/- 3.0	7.12 +/- 0.7
110.43 +/-19.6[b]	144.82 +/- 6.6	135.72 +/-10.9	130.58 +/- 9.0	38.96 +/- 9.5	40.70 +/- 5.0	49.13 +/- 2.3	44.90 +/- 6.8	9.86 +/- 1.4
91.17 +/-11.1[c]	128.94 +/-13.2	108.71 +/-11.4	107.90 +/- 9.3	28.91 +/- 5.4	34.58 +/- 4.5	45.09 +/- 2.4	37.87 +/-7.2	8.84 +/- 1.0
p-value[α]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[a] Mean distance travelled by all neutrophils
[b] Mean distance travelled by front = 2 cells
[c] Mean distance travelled by front = 5 cells

[α] P-values refer to the column in question compared to c-EB_3
[β] Conditioned-EB_3 media
[χ] N-Formyl-Met-Leu-Phe (Chemotactic Peptide)
[δ] DMEM/F12 media
[ε] Hanks Balanced Salt Solution (1x)
[ϕ] HBSS with 0.5% FCS (Fetal Calf Serum)

N-formyl-met-leu-phe (10^{-6} M - 10^{-4} M), while the negative controls are EB_3 media, DMEM/F12 media (DF), Hanks Balanced Salt Solution (HBSS), HBSS + 0.5 % Fetal Bovine Serum (HFBS), and distilled water (dH_2O).

The mean distance travelled in response to c-EB_3 (44.36 +/- 5.4 μm) is greater than that for each of the three concentrations of N-fmlp (10^{-6} M, 10^{-5} M, 10^{-4} M), which travelled 36.75 +/- 3.3 μm, 32.93 +/- 2.5 μm, and 34.68 +/- 2.8 μm, respectively. With p-values of less than 0.05 ($p < 0.05$) for each of the three N-fmlp concentrations, these differences are statistically significant. The mean distances travelled by the neutrophils in response to EB_3 (16.88 +/- 2.5 μm), DF (21.81 +/- 2.6 μm), HBSS (22.62 +/- 1.9 μm), HFBS (20.03 +/- 3.0 μm), and dH_2O (7.12 +/- 0.7 μm) are much lower than the distance travelled in response to c-EB_3. The differences are also statistically significant ($p < 0.05$).

Thus, mean distance travelled in response to the substance in the lower compartment of the Boyden chamber can be summarized as follows: c-EB_3 > positive controls (N-fmlp) > negative controls (HBSS > DF > HFBS > EB_3 > dH_2O.

Chemotactic response can also be evaluated by determining the distance moved by the front of neutrophils through mixed ester filters in the Boyden chamber assay. A front has been defined as the distance travelled by the furthest

two neutrophils (Zigmond & Hirsch 1973). The investigators did not examine sections of the filter, but rather looked at the undersurface of the filters through a microscope and focused in on the cells. When the first two cells came into focus in their field of view, this distance was the front.

Table 2 (row 2) is a compilation of the fronts in response to the different substances in the lower compartment of the Boyden chamber. In this row, front is defined as the mean distance travelled by the furthest two cells. By examining the data in this row, it is evident that the different concentrations of N-fmlp (10^{-6} M, 10^{-5} M, 10^{-4} M) induced the neutrophils to travel the furthest, with their fronts being 144.82 +/- 6.6 μm, 135.72 +/- 10.9 μm, and 130.58 +/- 9.0 μm from the surface of the filter, respectively. Conditioned EB_3 (c-EB_3) media induced a front that is 110.43 +/- 19.6 μm from the surface and significantly different from the front of each concentrations of N-fmlp ($p < 0.05$) for each. The negative controls had fronts that had not travelled nearly as far as the one for c-EB_3. The fronts of cells in response to EB_3, DF, HBSS, HFBS, and dH_2O were 38.96 +/- 9.5 μm, 40.70 +/- 5.0 μm, 49.13 +/- 2.3 μm, 44.90 +/- 6.8 μm, and 9.86 +/- 1.4 μm from the surface of the filter, respectively. These distances were significantly different from the distance the front of neutrophils travelled in response to c-EB_3 ($p < 0.05$ for each). A front being defined by two cells is not satisfactory, though, so the

data was also compiled with a front being defined as the mean distance travelled by the leading five cells in each section of the filter examined. This data is presented in Table 2 (row 3) and it is evident that the relationships are similar to those in row 2.

The distances travelled by the fronts of neutrophils, in response to the different concentrations of N-fmlp (10^{-6} M, 10^{-5} M, 10^{-4} M), are still the greatest; 128.94 +/- 13.2 μm, 108.71 +/- 11.4 μm, 107.90 +/- 9.3 μm from the surface, respectively. Again, this is greater and significantly different from the distance travelled in response to c-EB_3 (91.17 +/- 11.1 μm) ($p < 0.05$ for each). The negative controls had fronts that were less than and significantly different from that of c-EB_3 ($p < 0.05$ for each). The mean fronts for EB_3, DF, HBSS, HFBS, and dH_2O were 28.91 +/- 5.4 μm, 34.58 +/- 4.5 μm, 45.09 +/- 2.4 μm, 37.87 +/- 7.2 μm, and 8.84 +/- 1.0 μm from the surface, respectively.

Thus, the mean distance travelled by the neutrophils and the distance travelled by the front of cells (where front = 2 cells and front = 5 cells) present slightly different sets of data. The data for the mean distance travelled can be summarized as follows: c-EB3 > positive controls (N-fmlp) > negative controls (HBSS > DF > HFBS > EB_3 > dH_2O). The data for distance travelled by fronts of neutrophils

can be summarized as follows, regardless of definition: positive controls (N-fmlp) > c-EB3 >> negative controls (HBSS > HFBS > DF > EB3 > dH2O).

4.0 DISCUSSION

The goal of the present investigation was to determine what, if any, role polymorphonuclear leukocytes (PMNs), or neutrophils, play in tumour vascular hyperpermeability. Interstitial neutrophilia was observed early on at the Hey-3 tumour-host interface in the chick embryo choriaoallantoic membrane (CAM) assay. Thus, the hypothesis was that these granulocytes themselves were responsible, directly or indirectly, for increasing tumour vascular permeability.

It is a well-established finding that blood vessels supplying tumours are hyperpermeable to circulating macromolecules (Brown et al. 1988a, Brown et al. 1988b, Dewey 1959, Heuser & Miller 1986, Song & Levitt 1971, Underwood & Carr 1972). Various mechanisms of vascular permeability within normal and neoplastic tissue have been proposed, most dealing with vesicular transcytosis. In recent years, Dvorak's group has postulated that the pathway of macromolecular tracer transport across the blood vessels at the tumour-host interface involves the upregulation of vesiculo-vacuolar organelles (VVOs) (Dvorak et al. 1996, Kohn et al. 1992). These investigators have also suggested that vascular permeability

factor (VPF), which is released by tumour cells, regulates VVO function (Qu-Hong et al. 1995).

4.1 VASCULAR PERMEABILITY IN THE CAM ASSAY

A substantial difference in the permeability of blood vessels supplying tumour and normal tissues, as well as hepatic masses and N-fmlp-treated CAM, was shown in the present study. It is well known that the tumour vasculature shows an increased vascular permeability to circulating macromolecules (Brown et al. 1988a, Brown et al. 1988b, Dewey 1959, Heuser & Miller 1986, Song & Levitt 1971, Underwood & Carr 1972). In addition, Dvorak's group found that it was venules and small veins at the tumour-host interface, as well as those in the connective tissue separating and surrounding tumour nodules, that were permeable to macromolecular tracers (Dvorak et al. 1988). Within normal tissue, it is also accepted that it is the post-capillary venules that are permeable to circulating macromolecules (Curry & Joyner 1988). Thus, it was these venules and small veins that were examined throughout this study.

4.1.1 Tumour Hyperpermeability

Permeability was defined as the number of vesicles per square micrometre of endothelial cell cytoplasm (vesicles/μm^2). This was the number of labelled vesicles that accumulated in the five minutes that the tracer was allowed to perfuse through the chick embryo's circulation. Thus, tumour vascular permeability was determined to be 0.99 +/- 0.28 vesicles/μm^2, which is almost twice that of control at 0.51 +/- 0.09 vesicles/μm^2. The differential data on tumour hyperpermeability in the present study using the chick embryo CAM was consistent with previous findings within other species. For instance, Heuser and Miller (1986) investigated tumour vascular permeability in rat Walker 256 carcinosarcoma and found that there was a significantly lower fluorescent intensity in the interstitium of normal (control) compared to tumour implanted cremaster muscle. One minute following injection of fluorescein isothiocyanate-labelled rat serum albumin (FITC-RSA), fluorescent intensity in Walker 256 tumours was 24 +/- 3.0 as compared to 4 +/- 1.5 in the control. This difference was found to increase over time, as the fluorescent tracer accumulated in the interstitium.

4.1.2 Response to Hepatic Mass Growth on the CAM

Interestingly, in the present study, neutrophils were a prominent feature at the tumour-host interface. Although they were also found within normal CAM tissue,

they were not as numerous as in the tumour stroma. For this reason, it was proposed that these neutrophils may be playing a role in the increased tumour vascular permeability. In order to determine whether the presence of neutrophils at the site was an immune response to foreign cells, murine hepatocytes were placed on the CAM and grown into masses. When examined histologically, there was also a visible increase in neutrophil number as was seen in the tumour tissue, but the distribution pattern was different. The neutrophils did not accumulate around the entire periphery of the hepatic mass as they did with the tumour; rather they were more localized in the CAM's upper mesoderm, underlying the ectoderm, as well as in the upper periphery of the mass. Permeability was determined to be 0.54 +/- 0.03 vesicles/μm^2 in the venules and small veins at the hepatocyte mass-host interface, which was similar to that of the control (0.51 +/- 0.09 vesicles/μm). Thus, the different distribution of neutrophils around the hepatic mass as well as the significantly lower permeability observed compared to that at the tumour-host interface suggests that the hyperpermeability associated with tumour growth is not simply an immune response, but could actually be specifically due to the presence of the tumour.

4.1.3 Response of the CAM to Chemoattractant

N-formyl-methionine-leucine-phenylalanine (N-fmlp) is a standard neutrophilic chemotactic factor used in various chemotactic assays (Davis et al. 1982, Ribaudo & Kreutzer 1985). The most common chemoattractants for neutrophils are bacteria and bacterial products; some of these peptides possessing the N-formyl-methionine terminal amino acid (Cotran et al. 1989, Murphy 1976). Thus, the commercially available N-fmlp is meant to simulate the presence of bacteria in the area, thereby attracting the neutrophils. In the present study, N-fmlp was applied onto the CAM in order to attract neutrophils to the area in order to determine whether these leukocytes could induce hyperpermeability when chemotactically stimulated. The permeability of the blood vessels in this tissue was determined to be 1.04 +/- 0.09 vesicles/μm^2, which is similar to that of vessels at the tumour-host interface (0.99 +/- 0.28 vesicles/μm^2). This would suggest that neutrophils can, directly or indirectly, induce vascular hyperpermeability.

4.2 VASCULAR PERMEABILITY IN INFLAMMATION

In inflammation due to injury, one of the major components of the process is an increased vascular permeability which leads to the hyperpermeable of circulating macromolecules into the extravascular interstitium (Cotran et al. 1989).

Contrary to what was observed in this study, the endothelium becomes hyperpermeable during inflammation due to gap formation between adjacent cells. There are five basic mechanisms of increased vascular permeability in inflammation: (i) endothelial cell contraction, (ii) junctional retraction, (iii) direct injury, (iv) leukocyte dependent leakage, and (v) regenerating endothelium. Each mechanism leads to the formation of gaps in the endothelium through which macromolecules can pass. In the present study, the plasma membranes of adjoining endothelial cells were observed to be tightly apposed, and the endothelium was not found to be injured at any point. This was also the case for tumour-associated vasculature as well as that of the control, N-fmlp-treated, and hepatic mass-associated vessels. The regenerating endothelium mechanism of increased vascular permeability during inflammation needs to be considered. During repair, endothelial cells proliferate and new blood vessels form (angiogenesis) (Cotran et al. 1989). Angiogenesis is also a prominent feature of solid tumour growth (Cotran et al. 1989, Folkman 1984, Folkman 1990, Klagsbrun et al. 1977). In the present study, those vessels that were examined and that contributed to the tumour vascular hyperpermeability were observed to possess collagen fibrils underlying the basement membrane (in the adventitia layer), which is indicative of mature vessels (Junqueira et al. 1992). As well, there were no visible interendothelial gaps which would be present during neovascularization.

Thus, angiogenic vessels were not contributing to the tumour vascular hyperpermeability

The leukocyte dependent transport mechanism in inflammation refers specifically to injury caused to the endothelium by adhering leukocytes (Cotran et al. 1989). Leukocytes, when activated, produce oxygen radicals and proteolytic enzymes that may cause endothelial injury and detachment, leading to increased vascular permeability In the present study, the increased vascular permeability observed was due to the upregulation of endogenous pinocytotic activity based on evidence with HRP Tracer was not observed between endothelial cells in the intercellular spaces, rather only within cytoplasmic vesicles and pinocytotic omega (Ω) profiles at the plasma membrane. Therefore, the lack of interendothelial gaps in the vasculature at the tumour-host interface rules out any of the described mechanisms that have been implicated in inflammatory vascular hyperpermeability Increased transcytosis via vesicles across the cytoplasm is known to be another mechanism of hyperpermeability and has been demonstrated specifically in blood vessels within tumours (Cotran et al. 1989).

4.3 NEUTROPHIL-INDUCED VASCULAR PERMEABILITY

Humans with malignant tumours sometimes exhibit varying degrees of leukocytosis (Cotran et al. 1989). In the present study, neutrophils were found to be a prominent feature in the tumour stroma, specifically at the tumour-host interface. By examining the hepatic mass, histologically, it was determined that the presence of these cells was not merely an immune reaction by the chick embryo in response to foreign cells. As well, the chick embryo has been shown to be immunologically immature until the end of gestation (Glick 1976). The CAM was shown to accept tissue xenografts and allow their growth on the CAM up until day 18 of gestation (Murphy 1914) at which time the graft is rejected. Thus, in the present study, the established fact that the chick embryo is immunoincompetent raised the question as to why neutrophils were in the tumour area and whether they possessed the ability to increase vascular permeability without damaging the endothelium.

4.3.1 Hey-3 Tumour Cell-Induced Neutrophil Chemotaxis

The possibility that the Hey-3 tumour cells (an ovarian adenocarcinoma cell line) were producing a chemotactic factor that attracted neutrophils to the area was examined in the present study. Chemotaxis is the directional movement of cells,

along an increasing chemoattractant gradient toward the area of highest concentration (Ribaudo & Kreutzer 1985). In order to test whether the Hey-3 tumour cells were producing a chemotactic factor for neutrophils, the Boyden chamber assay (Boyden 1962) was used. It was assembled in such a way as to place the human neutrophils in the upper chamber, while the chemotactic substance was injected into the lower chamber. These two compartments were separated by a mixed ester filter (Wilkinson 1974, Wilkinson 1982), the most common types used in these assays. It was necessary to use a filter with a pore size between 3 and 5 μm so that the neutrophils could actively migrate without falling through. This pore size would also keep out larger cells such as macrophages that may be present in the neutrophil suspension.

Conditioned serum-free media (EB_3) from confluent Hey-3 cells was the test chemotactic substance. The response of neutrophils to conditioned EB_3 media was compared to that of both positive and negative controls. Positive controls consisted of different concentrations of N-formyl-methionine-leucine-phenylalanine (N-fmlp) (10^{-6} M, 10^{-5} M, 10^{-4} M), a standard test chemotactic factor for neutrophils. Negative controls were EB_3 media, DMEM/F12 media, Hank's balanced salt solution (HBSS), HBSS with 0.5 % fetal bovine serum (HFBS), and distilled water (dH_2O). Each was used for a different reason; EB_3 and DMEM/F12 in order to determine whether the media itself contained any

chemoattractants; HFBS (within which the neutrophils were suspended) was used to demonstrate chemokinesis; and HBSS and dH_2O to observe any random movement that may occur.

The method of measuring the chemotactic response is also very important (reviewed by Wilkinson 1974, Wilkinson 1982). Two different methods were employed in the present study: a modified "in-filter count" as well as a measurement of the distance travelled by the leading front of cells within the filter. For the first, the distance travelled by individual cells was measured within a designated area (1.5 mm) and the mean calculated. In response to the conditioned EB_3 media, the overall mean distance travelled (+/- standard deviation) by the neutrophils through the filter was calculated to be 44.36 +/- 5.4 μm. This was greater than the mean calculated for each concentration of N-fmlp. The neutrophils travelled an overall mean distance of 36.75 +/- 3.3 μm, 32.93 +/- 2.5 μm, and 34.68 +/- 2.8 μm in response to 10^{-6} M, 10^{-5} M, and 10^{-4} M N-fmlp, respectively. The mean distance travelled by each of the negative controls was significantly lower than that of the conditioned EB_3 media. Thus, this data suggests that the conditioned EB_3 media contains a factor, not present in EB_3, that is comparably chemoattractive for neutrophils as is the N-fmlp peptide.

The second method of measurement used in the present study defined the leading front of neutrophils as the distance travelled through the filter by the two

leading cells in each section. This method was used by Zigmond and Hirsch (1973), but was not considered a significant indication of chemoattraction in the present study. Thus the front was also defined as the distance travelled by the five leading cells. When the data for each was compared, though, it was found to be quite similar. When the front was equal to two cells, each concentration of N-fmlp had leading fronts that had moved farther than that of the conditioned EB_3 media. The conditioned media-stimulated neutrophils travelled an overall mean of 110.43 +/- 19.6 μm, while 10^{-6} M, 10^{-5} M, and 10^{-4} M N-fmlp-stimulated cells moved 144.82 +/- 6.6 μm, 135.72 +/- 10.9 μm, and 130.58 +/- 9.0 μm, respectively. The negative controls were seen to move only a fraction of this distance. When the front was defined as the leading five cells, the N-fmlp-stimulated neutrophils moved farther than the conditioned EB_3-stimulated cells. Overall, the front did not move as far through the filter as it did when the front was equal to the leading two cells.

The data from the chemotaxis experiments suggests that the Hey-3 tumour cells are indeed synthesizing and secreting a factor that is a chemoattractant for neutrophils. The tumour cell derived chemoattractant has an effect on the cells that is similar to that of the N-fmlp concentrations that were used. Where the N-fmlp is able to induce a leading front that travels farther, the tumour-derived factor stimulates the neutrophils to move a greater overall distance through the filter.

Although, this data explains why the neutrophils are localized at the tumour-host interface, the question still remaining is whether neutrophils are capable of increasing vascular permeability.

A variety of human tumour cells have been found to produce monocyte chemoattractants (Negus et al. 1995, van Damme 1992). In fact, monocyte chemoattractant protein - 1 (MCP-1) has been detected and localized in human ovarian carcinomas (Negus et al. 1995). Interleukin - 8 (IL-8), a chemotactic cytokine secreted by a variety of cells (i.e. monocytes, endothelial cells, fibroblasts) (reviewed by Nathan & Sporn 1991), is a known chemoattractant for neutrophils. Recently, it was shown that the IL-8 gene is expressed in human transitional cell carcinomas, as well as renal cell carcinomas (Abruzzo et al. 1992). Yoshida et al. (1992) also found that IL-8 as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) were produced by a cell line from thyroid carcinoma. The conditioned media from these cells was shown to have extensive chemotactic activity for neutrophils. GM-CSF was found to be produced by a number of tumour cell lines; such as, human melanoma (Lilly et al. 1987), human bladder carcinoma (Morioka et al. 1990), and human thyroid squamous cell carcinoma (Okabe et al. 1982). Thus, many tumour cell lines have been found to produce chemoattractant factors for neutrophils.

4.4 NEUTROPHIL-DEPENDENT VASCULAR PERMEABILITY

During acute inflammatory reactions, neutrophils can cause endothelial cell injury as well as damage to the surrounding tissue. Neutrophil granules contain many factors that can cause damage if they are allowed to escape from the cell by enzymatically digesting tissue or by acting directly on cellular and extracellular targets (Henson 1972). The neutrophilic granules of various species have also been found to contain proteins that can increase vascular permeability. Moses et al. (1964) isolated a so-called granulocyte substance (material obtained from the in vitro incubation of granulocytes) from rabbits. They found that this granulocyte substance induced leukocyte adhesion and migration, as well as the vascular transport of protein-bound dye in the area of injection. The effects of this substance were found to be temperature dependent when suspended in either saline of physiologic media. In addition, basic (cationic) proteins of neutrophils have been found to increase vascular permeability (Golub & Spitznagel 1965, Janoff & Zweifach 1964, Moses et al. 1964, Seeger & Janoff 1966). This is most likely the active fraction that Moses found in his granulocyte substance, since the basic proteins have also been found to stimulate neutrophil adhesion as well as increase vascular permeability (Janoff et al. 1965). The cationic protein fraction from

neutrophil lysosomes was not found to possess any kinin-like, serotonin, or histamine activity (Janoff & Zweifach 1964), which are known to increase vascular permeability. Ranadive & Cochrane (1968) isolated four neutrophilic cationic proteins from lysosomal granules that increase vascular permeability. These four proteins have been isolated from both rat and rabbit neutrophils (Ranadive & Cochrane 1968, Ranadive & Cochrane 1970). One was found to be a mastocytolytic agent (caused mast cell degranulation) (Janoff et al. 1965), and thus was not the same granulocyte substance described by Moses (Moses et al. 1964). Upon mast cell degranulation, histamine, a vasodilator, is released, causing increased vascular permeability through junctional retraction (Seeger & Janoff 1966, Yi & Ulich 1992). Thus, one of these acts by causing the degranulation of mast cells, while the mechanism of action of the remaining three was not elucidated. Differences do exist in the quantity and quality of mast cell disruption from one species to the next, thus the same may be the case for the remaining three. The neutrophils of a variety of species contain cationic proteins that act by histamine dependent and independent mechanisms, so it is possible that human neutrophilic granules may also contain proteins that are similar to these. This is one of the proposed pathways by which neutrophils may be acting on the vasculature at the tumour-host interface, inducing hyperpermeability.

Cytokines may also play a role in the process. The main characteristics of acute inflammation are the migration of neutrophils out of the post-capillary venules, as well as the increased vascular permeability leading to edema. Through studies that involved the injection of interleukin - 1 (IL-1), tumour necrosis factor (TNF), and lipopolysaccharide (LPS), it was observed that each of the three could increase vascular permeability (Yi & Ulich 1992). Together with hyperpermeability, neutrophils were found to accumulate in great numbers at the site of injection. By further investigation with neutropenic rats, it was established that the IL-1, TNF, and LPS-induced vascular hyperpermeability was neutrophil-dependent. IL-1 and TNF are both proinflammatory cytokines capable of increasing vascular permeability, but it is not known if neutrophils induce their production and release into the area. The effects of IL-1 and TNF being neutrophil-dependent suggests that neutrophils do, in fact, play a role in their synthesis; directly or indirectly.

Once chemotactically activated, neutrophils adhere to the endothelium and emigrate through the interendothelial spaces down the chemoattractant gradient toward the area of highest concentration (Ribaudo & Kreutzer 1985). This process leads to the neutrophilic release of lysosomal granular contents and respiratory burst (production of oxygen radicals) (reviewed by Fantone & Ward 1982,

Palmblad 1984, Peterson et al. 1995, Warren & Ward 1986), which requires chemoattractant concentrations greater than that needed for neutrophil migration (Palmblad 1984). Among the many components present within granules, those of interest are myeloperoxidase and the elastases found within the primary (azurophil) granules. In experiments with whole animals and cell cultures, human neutrophil elastase has been found to contribute to increased epithelial permeability (Peterson et al. 1995). This group found that neutrophil elastase was capable of increasing permeability without causing injury to the cells and that it could act cooperatively with other neutrophilic components in the process.

Myeloperoxidase is an important component in the production of oxygen radicals (Palmblad 1984). Those produced in neutrophils are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$). It has been found that some oxygen radical species play a direct role in increasing vascular permeability. In cultured endothelial cells, H_2O_2 (10^{-5} M) was found to increase fluid-phase endocytosis (Sundquist & Liu 1993). The same investigators also determined that the phorbol myristate acetate (PMA)-stimulated endogenous production of reactive oxygen species, specifically H_2O_2 and O_2^-, caused an increased uptake of macromolecules that could be blocked by catalase and superoxide dismutase (SOD). Through confocal laser microscopy, it was determined that the increased permeability was indeed due to the vesicular uptake

of macromolecules and that these vesicles were either arranged as channels or individual clusters of cells as described by Simionescu (1983).

The superoxide anion (O_2^-), produced by activated neutrophils, has also been found to be involved in the inhibition of nitric oxide (NO) synthesis (Gryglewski et al. 1986, Rubanyi & Vanhoutte 1986). NO is a vasodilator released by the vascular endothelium. It also functions to inhibit leukocyte adhesion to the endothelium possibly through interfering with bond formation, or by suppressing CD11/CD18 expression (Kubes et al. 1991). The inhibition of NO synthesis results in the rapid increase in vascular permeability. This inhibition also promotes the increased adhesion of leukocytes and which leads to inflammation (Kubes et al. 1991, Kubes & Granger 1992). Hence the process is self-perpetuating; with the increased adhesion of leukocytes, more neutrophils migrate and release their granular contents, leading to the increased production of reactive oxygen species. The presence of catalase and SOD results in the transformation of O_2^- to H_2O_2 which actually increases NO release (Rubanyi & Vanhoutte 1986).

Thus, neutrophils do possess the potential to increase vascular permeability. Many animal species have been found to contain four cationic proteins within their neutrophilic granules that can induce vascular hyperpermeability through histamine-dependent and independent pathways. Another mechanism of action may be through the action of cytokines like IL-1 and TNF, either directly or

indirectly. Contained within the primary granules of the neutrophils are elastases and reactive oxygen species. Oxygen radicals have been shown to possess the capabilities to increase vascular permeability, both by acting directly on the endothelium and through NO suppression.

4.4.1 Vascular Permeability Factor

Vascular permeability factor (VPF) cannot be forgotten or ignored, even though neutrophils do not play a role in it's actions on the endothelium. VPF is a protein produced by tumour cells that has been implicated in tumour-associated vascular hyperpermeability. Much of the work that had lead to a better understanding of the actions of VPF has been done by Dvorak and his colleagues (Dvorak et al. 1991, Dvorak et al. 1992, Dvorak et al. 1995, Dvorak et al. 1995, Nagy et al. 1995, Senger et al. 1983, Senger et al. 1986, Senger et al. 1993). Their findings have suggested that the changes in endothelial cells which lead to hyperpermeability are of the kind due to a cytokine-like factor (Senger et al. 1993), but the evidence that VPF is this cytokine is entirely circumstantial. The findings can be summarized as follows (reviewed by Senger et al. 1993): (i) VPF mRNA is found to be expressed by tumour cells in vivo and in vitro; (ii) the VPF protein is synthesized and secreted by tumour cells in vivo and in vitro; (iii) VPF protein

is found in ascites fluid and it increases vascular permeability; and (iv) co-localization evidence (VPF immunostaining and colloidal carbon exudation).

Contrary to the findings of the present study, Senger et al. (1983) found that after a single injection into guinea pig skin, VPF induces interendothelial gap formation in normal tissue. This is similar to the action of histamine and other inflammatory mediators (Janoff & Zweifach 1964, Seeger & Janoff 1966, Yi & Ulich 1992). Since then, Senger's colleagues, Qu-Hong et al. (1995), found that VPF regulates VVO function. Thus, the action of VPF has not been inconclusively established. The role of VPF in tumour vascular hyperpermeability is not being ruled out, rather it is being stressed that other mechanisms may be involved.

4.5 CHARACTERISTICS OF TUMOUR VASCULAR HYPERPERMEABILITY

The data presented in this study confirm and further extend earlier studies that identified venules and small veins at the tumour-host interface as the predominantly hyperpermeable vessels in solid tumours (Dvorak et al. 1988). Horseradish peroxidase (HRP) was the macromolecular tracer of choice and it was observed to exit the circulation by membrane-bound cytoplasmic vesicles. As

well, the HRP tracer was not seen to move through the endothelium by way of the interendothelial spaces.

Contrary to the findings of Dvorak's group (Dvorak et al. 1996, Kohn et al. 1992), tumour venules were not seen to possess the described vesiculo-vacuolar organelles (VVOs); three-dimensional aggregates, comprised of approximately 12 individual vesicles and vacuoles. In this study, macromolecular tracer (HRP) was found inside individual vesicles within the vascular endothelial cells. These vesicles were found as pinocytotic omega (Ω) profiles along the luminal plasma membrane, as well as free within the cytoplasm. In some instances, pairs of attached vesicles were found, with one being smaller than the other and the lumens of the two being continuous. These appeared as overturned eight (∞) configurations, where each loop was a different size. VVOs were described as clusters of interconnected ~70 nm vesicles and vacuoles that span the thickness of the endothelial cell, and which communicated with each other by way of stomata (Dvorak et al. 1996). The vesicles and vacuoles seen by Dvorak and his group were close enough together that it is possible to see how they could be connected. The vesicles seen in the present study were not located in such clusters and they were not closely apposed. In fact, there was never any evidence to suggest the presence of VVOs within the vascular endothelium at the tumour-host interface.

One explanation for the differences seen between this study and that of Dvorak and his colleagues deals with species variations. The present study utilized the chick embryo chorioallantoic membrane assay while Dvorak and his group conducted their experiment with rodents such as mice and guinea pigs. Thus, the differences seen in the tumour endothelium could be due to a species variation. Variations were found in the endothelium of normal tissue, as well.

The findings in normal tissue from the present study are consistent with those of other investigators who followed the passage of various macromolecular tracers through the vascular endothelium in many species. Through these studies, various mechanisms of transendothelial transport have been proposed, but it has not been established as yet whether the vesicles are permanent or transient, free or joined structures. Unlike findings in the present study, Dvorak's group found VVOs within the endothelial cells of normal tissues. In studies of normal vascular permeability, it has been established that cellular and intercellular transport of macromolecules occurs primarily across post-capillary venules (Curry & Joyner 1988). It is also agreed that many vesicles are visible in the normal vascular endothelial cell cytoplasm (Bennett et al. 1959, Bruns & Palade 1968, Majno 1963, Palade 1961, Simionescu et al. 1974). These have been seen as single vesicles, fused vesicles, and vesicles opening onto the luminal and abluminal surface (Bruns & Palade 1968, Simionescu et al. 1974). One hypothesis to explain

the mechanism of transendothelial vesicular transport states that vesicles do not exist as free structures within the cytoplasm. Instead, they are arranged in static, fused clusters with vesicles that communicate with each other, and the extracellular space at the luminal and abluminal surfaces (Bundgaard et al. 1979, Bundgaard et al. 1983, Frokjaer-Jensen 1980). These investigators also believe that macromolecular exchange occurs between cells rather than through them. This is very similar to what was described by Dvorak, except for the route of macromolecular exchange. Unfortunately, most investigators do not believe this to be the mechanism of transendothelial vesicular transport.

The endocytotic uptake of macromolecules has been observed by many groups who have also provided evidence for the processing and release of the macromolecules by the endothelium (Davies et al. 1984, Kataoka & Tavassoli 1984, Milici et al. 1987, Tavassoli et al. 1986, Williams et al. 1984). Palade, Simionescu, and Simionescu (1979, 1983, 1988) have contributed even more evidence in support of the concept of endocytotic uptake and vesicular transport of macromolecules by the endothelium. If endocytosis is occurring, then Bundgaard and Frokjaer-Jensen's theory cannot be true. Three remaining hypotheses for the transendothelial vesicular transport of macromolecules exists (reviewed by Michel 1992): (i) "shuttle" or "ferryboat" system (Palade 1960, Simionescu 1983); (ii) fusion-fission hypothesis (Clough & Michel 1981, Loudon

et al. 1979); and (iii) channel hypothesis (Milici et al. 1987, Simionescu et al. 1975). All of these involve the endocytotic take-up of macromolecules by the plasma membrane. Evidence was found in the present study to support each of these mechanisms, both in the vascular endothelium at the tumour-host interface and within normal CAM tissue. Although the structures were found to be much more common within the tumour-associated vasculature, similar structures were also seen within the normal 16 day CAM.

The first hypothesis deals with the movement of single vesicles from the luminal to the abluminal plasma membrane (Palade 1960, Simionescu 1983). The free, individual vesicles, in the vascular endothelium at the tumour-host interface, observed continuously throughout this study support this hypothesis. When a section of endothelial cell was examined through serial sections at the electron microscopic level, these same vesicles were seen to be free at various depths within the cytoplasm. In fact, as stated, they were too far apart for any connections to exist between them. Thus, this suggests that macromolecular tracers moved through the endothelium by way of the "shuttle" or "ferryboat" system. On the other hand, the fused vesicles that were seen, on occasion, suggest that the vesicular "shuttle" system is not the only mechanism of macromolecular transcytosis.

The second hypothesis of transendothelial vesicular transport of macromolecules states that once a vesicle is formed at the luminal surface, it travels part way through the endothelium where it fuses with another vesicle in order to transfer its contents; fusions continue until the contents can be expelled at the abluminal surface of the plasma membrane (Clough & Michel 1981, Loudon et al. 1979). This hypothesis can also be supported by the observations made in the present study. Large and small vesicles were seen in pairs, fused in a somewhat distorted "∞" configuration. No membrane was found to exist between the two fused vesicles, suggesting that the smaller vesicle was joining with the larger one and pooling their contents. Thus, the data from the present study suggests that macromolecular tracer is also being moved through the endothelium by the fusion-fission mechanism of vesicular transcytosis. The fused vesicles that were seen within the endothelium could also be part of a channel that connects the lumen of the vessel with the extravascular space.

The third hypothesis of transendothelial vesicular transport of macromolecules states that vesicles are permanently fused forming a channel that extends from the luminal to the abluminal surface (Milici et al. 1987, Simionescu et al. 1975). These chains of vesicles were first observed by Palade (Bruns & Palade 1968, Palade & Bruns 1968). In Figure 4b, two vesicles are joined by a tube-like structure. This image as well as Figure 4a shows what could be part of a chain of

vesicles forming a channel. The fused vesicles seen in this study could be a fraction of the whole structure. The specimen block could have been cut in such a way as to only have part of the channel present in the section that was examined. Thus, the macromolecular tracer could also be moving through the endothelial cells by way of transcellular channels.

Other explanations may exist for the increased permeability observed in the vasculature at the tumour-host interface. Inflammatory mediators have been observed to induce endothelial cell contraction that results in interendothelial gap formation (Majno et al. 1969). Since the HRP tracer was not seen in the interendothelial spaces, this is not believed to be occurring. In many instances, as well, cell junctions were visible, suggesting that endothelial cell contraction was not being induced and thus, gaps were not formed between the cells. Endothelial cell injury was also not observed in the Hey-3 tumours and thus, this could not be the cause of the increased vascular permeability. As mentioned, the endothelium at the tumour-host interface was of the continuous type and for that reason, the presence of a fenestrated endothelium could also not be the cause. Regardless, it has been found that there is no difference in tracer extravasion between continuous and fenestrated endothelia (Brown et al. 1988a, Brown et al. 1988b).

5.0 SUMMARY

In summary, the role of neutrophils in tumour vascular hyperpermeability may be greater than have ever been believed. Interstitial neutrophilia is a prominent feature associated with Hey-3 ovarian tumour growth on the chick embryo chorioallantoic membrane (CAM) as is a significantly increased vascular permeability. Through chemotaxis assays with the Boyden chamber, it was shown that Hey-3 cells in culture are producing a chemotactic factor that is an attractant for human polymorphonuclear cells (neutrophils). Once in the area, neutrophils do possess the potential to increase vascular permeability. This could be accomplished by the production of cationic protein or through consequences of respiratory burst and the actions of oxygen radicals. Whatever the agent of action may be, it increases the vesicular transport of macromolecules through the vascular endothelium. Vesiculo-vacuolar organelles (VVOs) are not involved in vascular permeability in the normal or neoplastic tissue, contrary to Dvorak's findings. The neutrophilic mechanism of action does not injure the endothelial cells or cause junctional retraction. Whatever the action of neutrophils on the endothelium in

tumour vascular permeability, these granulocytes are in the area due to a chemoattractant.

6.0 APPENDIX

Table A1: Distance Travelled by Granulocytes (Neutrophils)Through Mixed Ester Filters

Table A2: Distance Travelled by Front of Granulocytes (Neutrophils) Through Mixed Ester Filters, Where Front = 2 Cells

Table A3: Distance Travelled by Front of Granulocytes (Neutrophils) Through Mixed Ester Filters, Where Front = 5 Cells

Table A1: Distance Travelled (in micrometers) by Granulocytes (Neutrophils) Through Mixed Ester Filters

c-EB_3[β]	Nfmlp[χ] 10^{-6} M	Nfmlp 10^{-5} M	Nfmlp 10^{-4} M	EB_3	DF[δ]	HBSS[ε]	HFBS[ϕ]	dH_2O
38.22	37.55	36.71	35.48	16.50	23.74	19.35	21.63	7.46
47.40	42.75	29.82	34.73	16.71	21.33	26.57	19.31	7.51
43.90	37.43	32.03	32.45	15.13	24.12	22.52	21.52	9.06
38.71	33.21	32.21	39.46	17.22	24.66	23.74	13.73	6.36
37.50	41.78	34.90	35.36	17.85	21.76	23.11	20.54	8.43
34.41	33.07	32.45	33.22	18.49	18.00	21.66	16.20	7.24
39.26	38.13	30.36	36.82	20.08	22.13	23.27	21.43	7.63
43.56	35.95	30.94	32.51	20.41	22.35	23.77	15.98	6.47
46.54	34.71	32.29	35.24	16.26	27.48	24.51	19.11	6.85
42.72	40.93	28.49	32.92	18.90	18.20	22.68	16.18	6.75
54.65	40.15	29.52	32.98	14.19	22.45	20.65	25.60	6.92
42.62	36.15	32.39	36.84	16.04	20.68	23.43	21.36	6.72
52.72	31.47	34.63	28.90	12.51	25.82	22.96	20.39	6.81
45.51	34.24	36.43	38.60	14.66	18.49	26.25	23.22	6.46
52.93	37.39	35.09	--	17.76	19.08	23.89	19.43	6.78
45.44	32.31	33.59	--	14.48	19.67	19.67	24.13	6.55
47.64	38.00	36.39	--	16.82	20.60	22.97	20.03	--
48.20	36.37	34.47	--	14.28	21.38	22.28	20.76	--
40.61	--	--	--	22.51	22.49	21.65	--	--
44.74	--	--	--	--	--	20.22	--	--
Mean +/- SD								
44.36 +/- 5.4	36.75 +/- 3.3	32.93 +/- 2.5	34.68 +/- 2.8	16.88 +/- 2.5	21.81 +/- 2.6	22.62 +/- 1.9	20.03 +/- 3.0	7.12 +/- 0.7
p-value[α]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[α] P-values refer to the column in question compared to c-EB_3

[β] Conditioned-EB_3 media

[χ] N-Formyl-Met-Leu-Phe (Chemotactic Peptide)

[δ] DMEM/F12 media

[ε] Hanks Balanced Salt Solution (1x)

[ϕ] HBSS with 0.5% FCS (Fetal Calf Serum)

Table A2: Distance Travelled (in micrometers) by Front of Granulocytes (Neutrophils) Through Mixed Ester Filters, Where Front = 2 Cells

c-EB_3[β]	Nfmlp[χ] 10^{-6} M	Nfmlp 10^{-5} M	Nfmlp 10^{-4} M	EB_3	DF[δ]	HBSS[ε]	HFBS[ϕ]	dH_2O
88.97	137.27	143.59	145.70	32.99	37.52	46.12	49.05	11.01
94.84	148.30	140.84	136.37	31.72	33.93	48.38	43.92	10.89
112.38	125.66	150.00	135.10	31.87	47.06	45.91	51.75	12.80
90.11	148.89	138.28	134.31	37.92	45.92	50.00	31.18	9.23
79.06	150.00	129.80	128.57	33.28	47.39	49.45	43.89	11.68
106.53	149.44	145.61	126.26	28.83	39.59	51.67	37.10	9.88
107.15	141.34	125.69	129.57	43.33	36.30	50.19	48.45	11.45
149.46	134.30	110.72	116.44	31.64	44.88	49.09	48.18	8.66
119.67	143.79	129.45	124.63	34.96	39.87	52.22	39.94	9.18
89.79	141.96	133.28	119.73	51.11	34.84	49.46	31.46	8.61
105.56	148.92	119.18	139.17	35.74	34.98	48.57	56.71	10.89
105.00	148.94	123.60	121.88	66.19	33.80	48.57	51.01	8.66
149.50	148.49	136.68	125.74	35.19	43.27	53.57	45.45	8.66
100.19	148.96	147.33	144.61	36.37	35.89	45.62	48.29	8.02
135.40	149.47	137.07	--	42.40	41.01	48.57	43.62	9.73
108.55	143.67	144.19	--	29.45	46.39	49.62	43.13	8.45
123.92	148.43	148.47	--	50.93	39.43	49.24	43.01	--
127.72	148.97	139.14	--	36.82	42.94	52.25	51.99	--
94.30	--	--	--	48.43	48.25	49.26	--	--
120.54	--	--	--	--	--	44.77	--	--

Mean +/- SD

110.43 +/- 19.6	144.82 +/- 6.6	135.72 +/- 10.9	130.58 +/- 9.0	38.96 +/- 9.5	40.70 +/- 5.0	49.13 +/- 2.3	44.90 +/- 6.8	9.86 +/- 1.4

p-value[α]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[α] P-values refer to the column in question compared to c-EB_3

[β] Conditioned-EB_3 media

[χ] N-Formyl-Met-Leu-Phe (Chemotactic Peptide)

[δ] DMEM/F12 media

[ε] Hanks Balanced Salt Solution (1x)

[ϕ] HBSS with 0.5% FCS (Fetal Calf Serum)

Table A3: Distance Travelled (in micrometers) by Front of Granulocytes (Neutrophils) Through Mixed Ester Filters, Where Front = 5 Cells

c-EB_3[β]	Nfmlp[χ] 10^{-6} M	Nfmlp 10^{-5} M	Nfmlp 10^{-4} M	EB_3	DF[δ]	HBSS[ε]	HFBS[ϕ]	dH_2O
80.42	103.83	123.85	105.22	26.52	34.79	43.48	38.37	9.88
86.72	123.76	99.49	117.43	28.22	27.04	45.63	33.10	9.73
88.17	119.43	113.86	111.99	26.20	41.54	41.12	38.70	10.88
83.11	146.07	88.47	112.63	28.31	43.88	43.23	24.92	8.10
79.06	147.00	102.86	105.32	28.16	32.96	47.27	38.79	10.31
88.66	135.26	102.15	111.21	26.16	34.39	46.67	25.81	9.32
80.96	120.76	112.25	112.65	34.12	32.54	46.80	38.55	9.18
99.62	118.05	90.82	104.12	30.62	39.14	46.91	33.35	8.04
83.82	108.62	113.02	106.43	27.65	36.72	48.35	38.21	8.59
77.11	133.40	103.90	109.64	30.00	28.39	47.29	27.36	8.10
105.56	147.31	114.98	128.34	20.04	33.27	43.05	52.59	9.23
80.51	127.51	96.68	97.70	30.96	29.91	44.27	45.50	8.04
110.13	136.12	109.70	90.44	24.81	36.35	44.05	42.20	8.04
96.22	142.69	124.38	97.47	21.60	30.44	43.20	45.72	7.46
96.66	136.13	99.31	--	41.03	31.83	47.34	37.24	9.16
100.92	118.84	119.47	--	21.35	33.51	46.21	42.20	7.44
86.96	137.94	126.88	--	37.09	33.79	48.65	34.98	--
111.41	118.12	114.83	--	33.52	35.69	41.58	44.08	--
81.62	--	--	--	32.98	40.92	45.85	--	--
105.79	--	--	--	--	--	40.75	--	--

Mean +/- SD

91.17 +/- 11.1	128.94 +/- 13.2	108.71 +/- 11.4	107.90 +/- 9.3	28.91 +/- 5.4	34.58 +/- 4.5	45.09 +/- 2.4	37.87 +/-7.2	8.84 +/- 1.0

p-value[α]	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

[α] P-values refer to the column in question compared to c-EB_3

[β] Conditioned-EB_3 media

[χ] N-Formyl-Met-Leu-Phe (Chemotactic Peptide)

[δ] DMEM/F12 media

[ε] Hanks Balanced Salt Solution (1x)

[ϕ] HBSS with 0.5% FCS (Fetal Calf Serum)

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