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UMI
In Vitro Characteristics of Intimal Cells at Intercostal Ostia in the Porcine Thoracic Aorta

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

Graduate Department of Cellular and Molecular Pathology
University of Toronto

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Abstract

In humans, atherosclerotic plaques tend to develop immediately downstream of branches in areas of eccentric intimal thickening. Organ cultures of porcine thoracic aorta intercostal branches were used to study the reaction of branch point intimal cushion regions as well as unbranched regions to injury. Preliminary studies using both bromodeoxyuridine (BrdU) labeling and proliferating cell nuclear antigen (PCNA) localization revealed a significantly higher degree of intimal cell proliferation at the flow divider (BrdU: 9.5 ± 2.7%; PCNA: 12.2 ± 2.7%; n=3) than on its opposing wall (BrdU: 3.9 ± 2.9%; PCNA: 6.3 ± 4.0%; n=3) at 4 days. Further experiments have confirmed this difference in day 4 culture and have determined it due to increased endothelial (p < .05; n=14) as opposed to smooth muscle cell proliferation. However, endothelial cell number did not differ significantly between opposing walls of the branch in 4-day cultures, suggesting increased cell death at the flow divider. This was confirmed quantitatively by the live/dead assay and supported by TUNEL labeling. Tissue culture studies suggest that endothelial cells isolated from branch regions have a decreased capacity to effect wound repair, which may be due in part to decreased migration as opposed to proliferation, although cytoskeletal analysis revealed no obvious differences in wounded cells. These findings may indicate the existence of endothelial cells that differ in terms of their intrinsic responses to injury in organ and tissue culture, characteristics which may play a role in vivo, in the development of atherosclerosis-prone regions in the arterial vasculature.
Acknowledgements

I would like to take this opportunity to thank my supervisor Dr. Avrum Gotlieb for all the time he spent with me and for his words of encouragement during the writing of this thesis and over the duration of my training at the Vascular Research Laboratory. I have met many wonderful people during these past few years and made many friends whom I will always remember.

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Finally, I wish to acknowledge my family, who have always encouraged and supported me in my academic endeavors. I dedicate this thesis to them.
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<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ET-1</td>
<td>endothelin-1</td>
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<tr>
<td>EthD-1</td>
<td>ethidium homodimer</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FD</td>
<td>flow divider</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FGFR-1</td>
<td>fibroblast growth factor receptor-1</td>
</tr>
<tr>
<td>FrdU</td>
<td>fluorodeoxyuridine</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IEL</td>
<td>internal elastic lamellae</td>
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<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
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<tr>
<td>ICE</td>
<td>interleukin 1-β converting enzyme</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<tr>
<td>IL-1</td>
<td>interleukin-1</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NB</td>
<td>unbranched aorta opposite the intercostal branch</td>
</tr>
<tr>
<td>NBBS</td>
<td>unbranched aorta above the first intercostal branch</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OxLDL</td>
<td>oxidized low density lipoprotein</td>
</tr>
<tr>
<td>OFD</td>
<td>upstream wall opposite the flow divider</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PECAM-1</td>
<td>platelet endothelial adhesion molecule-1</td>
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<tr>
<td>PGI$_2$</td>
<td>prostacyclin</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PLSD</td>
<td>protected least sum difference</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>SSRE</td>
<td>shear stress response element</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl tranferase-mediated dUTP nick end labeling</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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CHAPTER 1

LITERATURE REVIEW

1.1 ATHEROSCLEROSIS

1.1.1 Introduction

Atherosclerosis is a chronic, inflammatory disease beginning in the intima of elastic and large muscular arteries. Endothelial and smooth muscle cells of the artery wall change structure and function over time to promote the formation of a stenotic, fibrofatty plaque (Ross, 1993). Such plaques have a characteristic architecture consisting of a core of extracellular lipid and necrotic debris, lipid-laden foam cells, inflammatory cells, and smooth muscle cells, all covered by a thick cap of collagen-rich fibrous tissue containing more smooth muscle cells (Davies and Woolf, 1993).

The pathogenesis of atherosclerosis is considered to be multifactorial in nature with respect to both the initiation of cell dysfunction and progression from precursor or early lesions to fibrofatty plaque formation. Despite recent advances in the diagnosis and treatment of this disease, its clinical consequences, including stroke, myocardial infarction, peripheral vascular disease, and aneurysm formation remain as the major causes of death in the Western hemisphere (Davies and Woolf, 1993).
1.1.2 The Intima, Intimal Thickening and Vessel Remodeling in Response to Hemodynamics

In humans, the tunica intima is defined as the region of the arterial wall extending from and including the endothelium to the internal elastic lamina. Thickening of this layer of the vessel is a normal, physiological feature of human arterial development (Velican and Velican, 1976). In segments of muscular arteries with thickening of the intima, 2 layers may be distinguished. The abluminal layer is referred to as the musculoelastic layer because of the presence of abundant collagen, elastic fibers, and contractile smooth muscle cells. The luminal layer, also called the proteoglycan layer, predominantly contains proteoglycan ground substance. Smooth muscle cells in this layer are more randomly arranged and are thought to serve for both synthesis and contraction.

The intima, along with the rest of the arterial wall, can undergo remodeling in response to changes in pressure and flow rates, and arterial geometry (Glagov and Zarins, 1989). Remodeling represents attempts by vascular tissue to offset mechanical stresses in vivo, such as variations in flow, shear, and wall tension (Langille, 1993). For example, a decrease in wall shear coupled with increased wall tension, appear to correlate with regions of adaptive thickening. Under such conditions, thickening of the intima and media serve to strengthen the vessel wall and thus to resist tensile stress. Moreover, thickening decreases lumen diameter, which increases flow velocity, thereby readjusting wall shear (Glagov and Zarins, 1989). Such factors play a significant role in wall remodeling, especially at bifurcations and branch points which typically feature both high and low shear regions of flow. These regions exhibit a more randomized distribution of stress and hence show differential degrees of intimal thickening and
subsequent atherosclerotic changes compared to relatively straight arterial segments where mechanical stresses are evenly organized and intimal thickening is generally uniform. Patterns of intimal thickening thus fall into two general variants, eccentric and diffuse thickening.

Eccentric intimal thickening is a focal increase in the thickness of the intima which has been observed mainly at the branch points of human coronary, carotid, and cerebral arteries from the first weeks of life and thereafter (Stary, 1987). Although individual variation exists, the structure is usually a crescent-shaped increase in intimal thickness extending for a short distance along the length of the artery in the half of the circumference opposite to the flow divider, a U-shaped, lip-like structure that defines the downstream margin of aortic branches that is formed by the junction of aortic and branch tissues (Stary, 1987).

Diffuse intimal thickening is less prominent than eccentric thickening though more extensive in terms of area (Stary, 1989). Unlike eccentric thickening, there appears to be no correlation between the extent of diffuse thickening and anatomic landmarks within vessels (fig. 2.1).

1.1.3 Intimal Thickening and its Relationship to Atherosclerosis

Adaptive intimal thickenings, resulting from reorganization of the vessel wall in response to fluid dynamics, exist in arteries obtained from healthy human subjects of all ages. They do not obstruct the vascular lumen nor do they show atherosclerotic changes. As mentioned earlier however, some adaptive intimal thickenings, namely those of the eccentric type, do occur in locations at which atherosclerotic lesions develop later in life. These areas are referred to as the atherosclerosis-prone regions
Cells within such regions as the intimal cushion, an area of thickening present on the downstream margins of branch point openings, have been found to differ in vivo with respect to both function and physiology compared to cells from adjacent, thinner areas of the intima. Caplan and Schwartz in 1973, observed focal patterns of increased cell proliferation localized with high permeability to Evans Blue dye at flow divider intimal cushions in the intercostal ostia of normocholesterolaemic young pigs. The turnover of endothelial cells in guinea pigs (Wright, 1968) has also been shown to be increased in such areas. Numerous other investigators (Davies et al, 1986; Sumpio et al, 1987; DePaola et al, 1992) have implicated turbulent flow as a stimulus for endothelial cell turnover in vitro. Cell proliferation, particularly of intimal smooth muscle cells, is a key event in the formation and development of atherosclerosis. The intimal cushion is of interest in this respect because of the relatively large proportion of intimal smooth muscle cells inherently resident in this region compared to other areas of the normal vessel intima. Patterns of smooth muscle cell proliferation (Stary and McMillan, 1970) similar to those observed in endothelial cells at branch ostia, and an increased permeability of the endothelial lining to plasma blood lipids (Schwenke and Carew, 1988) have also been observed in rabbit models of atherosclerosis.

1.1.4 Atherosclerotic Lesion Types: Classification and Pathogenesis

The initial lesions and exact sequence of events that precede advanced plaque development remain largely speculative. Intimal changes either in the form of intimal thickening or fatty streak lesions predispose to atherosclerotic plaque formation and it is generally agreed upon that plaque development begins early in life (Stary, 1987). Indeed by the third decade, advanced plaques are almost ubiquitous in Western
populations. Hence, there appears to be a long presymptomatic phase before the disease manifests itself in a clinical setting.

Mechanistically speaking, large plaques most assuredly evolve from smaller plaques. The time course for this progression however is unknown. Detailed studies made on the arteries of young subjects, from infancy to over 30 years of age (Stary, 1989), have since provided the basis for the correlation of particular lesion types with age. In human subjects from birth to middle age, Stary described 3 morphologically characteristic precursors of advanced lesions which he termed types I, II, and III. Both type I and type II lesions appear as fatty dots or streaks, barely raised above the intimal surface, containing lipid-laden foam cells. Type II lesions were distinguished from type I by a more organized structure of macrophage foam cells and newly resident lipid-containing smooth muscle cells. Such lesions were mainly found in infants and young children but also in adults with very little atherosclerosis. Type III lesions, also known as intermediate or preatheroma lesions, referred to a transitional form between type II and the advanced lesion (type IV). Advanced lesions were also classified into 3 histologically characteristic types based on the extent of luminal narrowing, necrotic debris, fibrous connective tissue deposition, and the presence of complications such as hemorrhage (Stary, 1995).

Whether fatty streaks are the precursors of more advanced plaques is still a matter of contention. Advanced plaques appear to develop in sites where intimal thickening and fatty streaks are most prevalent. Indeed type IV lesions, when found in young people, are located mainly in areas of eccentric thickening. However, it is clear that in certain populations, advanced atherosclerosis is not an inevitable outcome
despite the presence of large numbers of fatty streaks in the infant population (Restrepo and Tracy, 1975).

In fatty streaks that do go on to develop into atherosclerotic plaques, progression is associated with an inflammatory response of the intima to lipid injury. Herrmann et al (1994), have observed elevated low density lipoprotein (LDL) permeability in regions of intimal thickening at branch sites in normocholesterolaeamic rabbits. Native plasma lipids, in particular low-density lipoproteins, are known to freely enter the vessel intima and do not initiate any form of physiological/immunological response. However, when these molecules are oxidized or otherwise altered, they become chemotactic for monocytes and macrophages, induce migration of smooth muscle cells, and initiate further immune responses (Berliner et al, 1995). Monocytes/macrophages, smooth muscle cells and even bovine vascular endothelial cells (Sawamura et al, 1997) possess scavenger receptors for oxidized-LDL, which are not down-regulated by intracellular lipid concentrations. Hence cells may become filled with lipid and become foam cells.

Although it is generally accepted that endothelial dysfunction is a necessary step in atherogenesis, smooth muscle cell accumulation, together with macrophage and lymphocyte recruitment, are the central cellular features of atherosclerotic lesion development. And, in addition to their capacity to synthesize large amounts of connective tissue, smooth muscle cells can also accumulate lipid. They are present in early fatty streaks, in which macrophages are the principle cell type, but then become more dominant as streaks progress to fibrofatty lesions (Stary et al, 1992). Proliferation and migration from the media into the intima is largely controlled by growth factors released from a number of cells including inflammatory cells such as macrophages, as
well as platelets, endothelial cells, and other smooth muscle cells (D'Amore, 1993; Bonin, 1994). Inflammatory reactions readily increase growth factor production (FitzGerald et al, 1984), and oxidized-LDL itself may even induce growth factor release from smooth muscle cells (Ananyeva et al, 1997), setting into motion a cascade that may lead to more rapid plaque growth and development at lesion prone sites within the vasculature.

1.1.5 Inflammation and the Immune Response in Atherosclerosis

It is clear that components of the immune system are involved in but do not necessarily elicit the atherosclerotic disease process. Moreover, the fact that immunocompetent cells are recruited to the arterial intima early in the development of atherosclerosis-like lesions in laboratory animals (Hansson et al, 1991), suggests that components of the fatty streak, itself not clinically significant, may be involved in later events that do lead to clinically significant disease. Such events are directly related to the oxidation of lipids in low-density lipoproteins that become trapped in the fibrous network of extracellular matrix in the subendothelial space (Neivelstein et al, 1991). In fatty streak susceptible mice on an atherogenic diet for instance, more oxidized lipid accumulation was observed compared to fatty-streak resistant mice on the same diet (Liao et al, 1993). Additionally found in fatty streak susceptible mice was the activation of an NFκB-like transcription factor and expression of genes with NFκB binding sites including JE, the mouse homologue of monocyte chemoattractant protein-1. Thus, it has been proposed that oxidized lipids may mediate the immune response via a mechanism involving NFκB activation of genes, the protein products of which induce an inflammatory response which initially involves predominately monocyte
recruitment (Berliner et al, 1990; Giuffre et al, 1997) via endothelial production of monocyte chemoattractant protein-1 and monocyte colony stimulating factor (Faruqi and DiCorletto 1993). A concomitant upregulation of the monocyte tethering and adhesion molecules P-selectin (Vora et al, 1994) and L-selectin (Guiffre et al, 1997) respectively, has also been described.

The potential for LDL lipid oxidation increases tremendously once newly recruited monocytes are converted to macrophages. It is at this stage that foam cells begin to populate the arterial intima as LDL lipids are modified such that they are now recognized by scavenger receptors on resident cells. Eventual smooth muscle cell proliferation and recruitment into the developing lesion may be accounted for in part by platelet-derived growth factor released by monocytes and macrophages (Ross, 1993). The products of lipoprotein oxidation have also been shown to affect other events associated with atherogenesis. The secretion of both interleukin-1 (Ku et al, 1992) and basic fibroblast growth factor (Ananyeva et al, 1997) is stimulated by oxidized lipids. Lysophosphatidylcholine, a product of oxidation, is a chemoattractant for monocytes and T lymphocytes, induces platelet derived growth factor mRNA expression in both endothelial and smooth muscle cells, and can induce the adhesion molecules VCAM-1 and ICAM-1 (Kume et al, 1992). Highly oxidized LDL can inhibit endothelial cell migration and repair (Murugesan et al, 1993) and has been shown to be toxic to endothelial cells and macrophages (Reid and Mitchinson, 1993). Macrophage death, in turn, may contribute not only to the amplification of the inflammatory process but to the formation of the necrotic core found in advanced lesions.
1.1.6 The Response to Injury Hypothesis

In 1974, Russell Ross proposed that the lesions of atherosclerosis resulted from an inflammatory, fibroproliferative response to endothelial dysfunction or disruption, initially meant to protect the artery wall from various forms of insult. Such insults for instance may include hyperlipidemia, hemodynamic forces, immune complex deposition, irradiation, exposure to exogenous chemicals, and physical trauma (Minick et al, 1979; Davies et al, 1986; Schwartz, 1994). Depending on the duration of injury however, this protective response might eventually prove excessive, contributing ultimately to the development of the atherosclerotic plaque.

Recent work suggests that injury to the vascular endothelium begins at specific sites along the vascular tree, resulting in the recruitment of peripheral blood monocytes and T-lymphocytes (Gerrity, 1981; Hansson, 1993; Walpola et al, 1993; Berliner et al, 1995; Giuffre et al, 1997). These cells adhere to and migrate through the endothelium via upregulated adhesion molecules such as ICAM-1 and VCAM-1, and invade the artery wall where they may become activated, liberating cytokines and various growth regulatory molecules which effect cell replication, migration and the further recruitment of blood leukocytes. These factors, in turn, promote the accumulation of intimal smooth muscle cells. Smooth muscle cells have been shown to undergo phenotypic modulation from a contractile to a synthetic state once activated (Dilley et al, 1987; Takaichi et al, 1993). The importance of the phenotypic expression of these cells lies in the ability of the synthetic-state cells to migrate into the arterial intima where they produce collagen, elastin and proteoglycan ground substance. This results in a substantial degree of intimal thickening.
1.2 NEOINTIMAL FORMATION

1.2.1 Neointimal Formation

It is important to distinguish between intimal thickening caused by physiological adaptation and neointimal, or "new intima" formation, which refers to the intimal hyperplasia that results from a general physiologic response to acute tissue injury. This phenomena, most often described in the context of the restenotic lesion as a key event contributing to the failure of angioplasty in humans, is characterized by intimal thickening resulting from smooth muscle cell proliferation and/or migration from the media and extracellular matrix production. Many animal models that have been developed to study neointimal formation as it relates to atherosclerosis were also quite useful when the study of restenosis became important after the failure of many angioplasties. However, it is important also to recognize that neointimal formation is a process in and of itself, which may differ in its pathogenesis, dependent upon such things as the state of the vessel wall, that is, normal versus diseased or previously injured (Strauss et al, 1994; Strauss et al, 1996), the nature and degree of injury stimulus (Andersen et al, 1996; Indolfi et al, 1995), and the species of animal (Muller et al, 1992; Schwartz, 1994).

Neointimal formation occurs in all arteries as a response to a wide variety of injuries including air drying (Fishman et al, 1975), stent placement (Bai et al, 1994; Miller et al, 1995), nylon filament denudation (Reidy and Schwartz, 1981), endotoxin injection (Gerrity et al, 1975), adventitial removal (Barker et al, 1994), and dilatation of arteries with an embolectomy balloon catheter (Steele et al, 1985; Okamoto et al, 1992; Indolfi et al, 1995). Similar changes are even seen in transplanted veins that undergo
arterialization. As such, it is important to determine the relative contribution of neointimal formation to the pathogenesis of atherosclerosis and restenosis.

Compared with the decades usually required for the development of clinically significant atherosclerotic lesions, the restenotic lesion develops so rapidly, it has been described as a form of accelerated atherosclerosis induced by injury (Ip et al, 1990). As such, three phases of wound healing have been implicated in its pathogenesis:

1. an inflammatory phase, involving the coagulation of blood, aggregation of platelets and mononuclear cells followed by the subsequent release of growth factors (ie fibroblast growth factor) effecting mesenchymal cell migration, proliferation and matrix production.
2. a granulation phase, highlighted by endothelial, smooth muscle cell, and fibroblast proliferation.
3. an extracellular matrix remodeling phase which continues over the course of months and describes the gradual replacement of the initially deposited fibronectin matrix with proteoglycans secreted by newly resident mesenchymal cells. (Forrester et al, 1991).

Some animal models used to study restenosis as well as other aspects of the pathogenesis of intimal thickening are summarized below.

The contribution of migrating smooth muscle cells to the development of intimal hyperplasia has been well studied in the rat. The rat makes an excellent model for such study owing to the fact that they do not inherently possess intimal SMC’s in their artery walls unlike pigs, rabbits, and humans. In balloon ed carotid arteries for instance, significant numbers of unlabelled SMC’s in thickened intima exposed to $[^3]H$ TdR
throughout its development show that these cells have migrated from the media (Clowes and Schwartz, 1985). Moreover, the proliferative capacity of these cells appears to correlate with the degree of injury to the vessel via a pathway involving the activation of phospholipase C and protein kinase C (Hishikawa et al, 1994).

The use of double-injury models (Strauss et al, 1994; Strauss et al, 1996), have successfully induced lesions in the rabbit carotid without the use of a high cholesterol diet. Such studies have demonstrated increases in the synthesis of major extracellular matrix components, such as elastin, proteoglycans and collagen in particular, at 4 weeks over the course of restenotic lesion growth.

The regenerating endothelium may possibly play a role in the development of intimal thickening in animals which undergo balloon catheterization and cholesterol feeding by enhancing native LDL oxidation \textit{in vivo} (Kisanuki et al, 1992). Antioxidant treatment has proven effective in reducing the degree of intimal thickening and the extent of monocytic infiltration observed in both normo and hypercholesterolemic rabbits. A putative role for lipoprotein A, a potent promoter of smooth muscle cell proliferation and migration, has been described in primates (Ryan et al, 1997).

The importance of cell proliferation in the development of neointimal thickening is made apparent through the use of anti-proliferatives, which effectively reduce the proliferative response after arterial injury in both rats and rabbits (Muller et al, 1992). Agents such as halofuginone have also been successful in the inhibition of bovine aortic endothelial cell proliferation \textit{in vitro} (Nagler et al, 1997). In human trials however, anti-proliferatives have been largely unsuccessful in reducing either the occurrence or extent of restenosis. This raises the possibility that neointimal formation does not play
as important a role in the growth of the restenotic lesion, or that injury/response processes in smaller animals differ significantly from those in humans.

The pig has become widely used in models of experimental atherosclerosis since they spontaneously develop the disease without the need for cholesterol feeding as in the rabbit. And, contrary to results obtained in rat arterial injury models, pig coronary models appear to better reflect the pathogenesis of human restenosis (Muller et al, 1992; Ferrell et al, 1992). This may be due, in part, to the similarity between human and pig platelet-coagulation systems. Smooth muscle cells are also found normally in the intimas of pigs, as is the case in humans. Such similarity has allowed the study of various pathophysiological responses to procedures such as angioplasty and its associated consequences including platelet deposition, mural thrombus formation, and restenosis (Steele et al, 1985).

Our laboratory has previously characterized an in vitro porcine model of neointimal formation in which explants were harvested from the thoracic aorta and cultured, full thickness for up to one month. Smooth muscle cell number within the intima was shown to double over the first 7 days in culture, then double again over the next 7 days whereupon an equilibrium was reached for the duration of the culture period (Koo and Gotlieb, 1991). Furthermore, media collected from 4 day, as opposed to 24 day, cultures was able to significantly effect smooth muscle cell proliferation compared to standard growth medium. This led to the assumption that a soluble proliferation-effecting mediator was being liberated into 4-day conditioned media. The observation that explants denuded of their surface endothelium did not form a neointima in culture but the fact that this could be overcome by the addition of conditioned media from non-
denuded explants, further led credence to the hypothesis that the injured endothelium was responsible for releasing these factors. Analysis of conditioned media from both denuded and non-denuded explants revealed no appreciable differences in the level of growth factors present however, basic fibroblast growth factor was found in the greatest quantity in both sets of explants. It was later determined that fibroblast growth factor receptor-1 was being up-regulated in intact as opposed to denuded explants and that receptor levels were being reciprocally regulated by the level of soluble fibroblast growth factor present in the culture media (Daley and Gotlieb, 1996).

1.2.2 Cell Proliferation and Cell Death in the Vessel Wall

Healthy vessels under normal, in vivo conditions are stable structures that show a very low level of cell proliferation and turnover (Folkman and Klagsbrun, 1987). Intimal cells in these vessels exist in a quiescent, differentiated state that is maintained by interaction with extracellular matrix components in the subendothelial space (Pauly et al., 1992). The mechanisms which underlie this stringent growth control are not well understood however, it is clear that the vessel wall is remarkably adept at responding to injury once activated. Thus, in the damaged vessel, cells are constantly being lost while others dedifferentiate, migrate and proliferate as needed for repair, sometimes at the expense of vessel and/or plaque stability (Ross, 1993).

Intimal cell necrosis due to the accumulation of toxic products in the plaque core (Guyton et al., 1990) and degradation of the connective tissue matrix by macrophage-derived matrix metalloproteases (Galis et al., 1994) are considered to be the primary causes of plaque rupture and instability. Recent evidence from ex vivo analysis of advanced plaques in humans (Geng and Libby, 1995; Bjorkerud and Bjorkerud, 1996)
and cell culture studies (Bennett et al, 1995) have suggested that apoptosis may play a large role in this respect as well.

Apoptosis is a form of physiological cell death whereby cells follow cell-death pathways mediated by genes such as ICE (interleukin 1-β converting enzyme)(Geng and Libby, 1995) and bcl-2 in mammals (Vaux, 1993). It is usually confined to isolated cells as opposed to contiguous patches of tissue and dying cells are phagocytosed by their neighbors, thus preventing an inflammatory response. The DNA of cells undergoing apoptosis is rapidly broken down at internucleosomal sites by endonucleases released by the dying cell (Wyllie et al, 1981) forming a characteristic ladder when analyzed by gel electrophoresis. Alternatively, dying cells may be detected by morphological characteristics which include cell shrinkage, nuclear condensation, the loss of cell-cell contacts, and blebbing of the plasma membrane (Cohen et al, 1992). Cleavage of the DNA into oligonucleosomal fragments may also be detected in situ using the TUNEL (terminal deoxynucleotidyl tranferase (Tdt)-mediated dUTP nick end labeling) method (Gavrieli et al, 1992). The Tdt enzyme catalyzes the polymerization of the nucleotide dUTP to free 3'-OH ends of the DNA in a template-independent manner, thus labeling breaks in high molecular weight DNA strands (Batistatou and Greene, 1993).

In cell culture, the removal of serum growth factors induces apoptosis in human arterial smooth muscle cells (Bennett et al, 1995). Plaque smooth muscle cells were also observed to be more sensitive than smooth muscle from healthy vessels. Crisby et al (1997), have since detected DNA fragmentation in 9-20% of T cells, macrophages and smooth muscle cells in human carotid atherosclerotic plaques however, necrosis
was observed to occur in greater abundance as determined by electron microscope analysis. The exact role that various forms of cell death play in arterial vessels may vary according to age (Vaux et al, 1994), location within the vascular tree (Gerrity et al, 1975), and in health and disease states (Orrenius, 1995 Review), however apoptosis remains an integral physiological response to both acute and chronic vessel injury.

1.2.3 The Role of the Smooth Muscle Cell in Vascular Pathology

Interactions between endothelial cells and smooth muscle in the vascular wall are known to be important in determining vessel diameter, thickness, SMC proliferation and phenotype (Chamley-Campbell and Campbell, 1981; Langille and O'Donnell, 1986; Zarins et al, 1987). In the acutely damaged vessel, superficial damage to the endothelium that results in the death or loss of endothelial cells results in minimal vessel involvement. Such lesions are repaired via the proliferation and migration of neighboring endothelial cells (Ettenson and Gotlieb, 1994). More extensive damage however, involving the disruption of the basement membrane and underlying media constituents, results in the aggregation of platelets and the subsequent release of autocrine and paracrine factors which effect intimal cell proliferation and smooth muscle cell migration into the vessel intima. For instance, injury to cultured endothelial cells leads to the release of fibroblast growth factor-2, normally confined within cells due to lack of secretive mechanisms, via tears in the plasma membrane and leakage of cytoplasmic constituents (McNeil et al, 1989). This potent smooth muscle cell mitogen and chemoattractant has been shown to induce the production of proteases which may degrade the extracellular matrix further, mobilizing more FGF-2 from stores in the
extracellular matrix (Bashkin et al, 1989). Such events result in the loss of normal vessel architecture and formation of the neointimal lesion (Ross, 1986).

Smooth muscle cell proliferation in general depends largely on cell phenotype. It has become evident during recent years that arterial smooth muscle cells may exhibit a broad spectrum of properties, some conferring tensile strength to the cells themselves, while others, the ability to synthesize components of the vessel. Synthetic-state cells are found in arteries during embryogenesis and soon after birth, where they take part in the formation of the vessel wall through proliferation and extracellular matrix production. Contractile-state cells predominate in the vessels of adults and are primarily involved in the control of blood pressure and flow. These cells express high levels of the muscle-specific actins, in particular α-actin, and intermediate filaments such as vimentin, the main functions of which are the maintenance of vessel tone (Ross and Glomset, 1973). Contractile cells have the ability to return to a synthetic phenotype however, and this has great importance to the development of intimal thickening because of the enhanced proliferation, migration and synthetic capabilities of these cells. Indeed, synthetic-state cells are found in many regions of intimal thickening and in atherosclerotic lesions themselves (Campbell et al, 1988; Shanahan et al, 1993), leading researchers to postulate that this modulation is a primary event in atherosclerotic lesion development.

Many structural and functional changes indicating the phenotypic modulation of smooth muscle cells from a contractile to a synthetic phenotype have been observed by electron microscopic studies and the induction of experimental intimal lesions in animals (Kocher et al, 1991; Babaev et al, 1993; Pauletto et al, 1994; Groves et al, 1995; Bochaton-Piallat et al, 1996). For instance, synthetic smooth muscle cells may be
clearly distinguished from contractile cells by an increase in the size of the rough endoplasmic reticulum and the Golgi complex (Kocher et al, 1984). A shift in differentiated properties during atherogenesis and experimental lesion formation is also reflected in the expression of cytoskeletal proteins. In both cases, there is a transition from α to β-actin content, a decrease in smooth muscle myosin, and a diminished desmin to vimentin ratio (Kocher et al, 1991). Similar observations have also been made in vitro (Chamley-Campbell et al, 1979). Chamley-Campbell et al (1981), have since determined that phenotypic modulation may occur in vitro without subsequent DNA synthesis or cell proliferation in the absence of growth factors, suggesting that modulation is a necessary, but not sufficient, requirement for smooth muscle cell proliferation in vitro.

Closely associated with the phenomenon of phenotypic modulation in response to injury is the concept of heterogeneity within specific cell populations of the vessel wall. At the smooth muscle level, heterogeneity of cells in experimental intimal thickenings versus those in the media has been observed in the rat (Bochaton-Piallat et al, 1996) and the rabbit (Okamoto et al, 1992). Frid et al (1994), have identified at least 4 smooth muscle cell populations expressed simultaneously within the mature bovine pulmonary artery media. Babaev et al (1990), have also identified heterogeneous smooth muscle cells in human aortic atherosclerotic plaques. Moreover, cells cloned from the aortas of rat pups can differ widely in terms of morphology, proliferation rates and the production of growth factors and matrix molecules (Lemire et al, 1994). Clearly, a vast majority of intimal smooth muscle cells differ from those resident in the normal vessel media. To date, approximately 80 genes have been identified that show
constitutive differences in expression levels between intimal versus medial rat smooth muscle cells (Schwartz et al, 1995). Since heterogeneity in cell populations infers heterogeneity of function, it is worthwhile to imagine a scenario whereby a single population of cells may respond to a particular stimulus, and expand to numbers that may eventually affect the normal function of the vessel or its reaction to injurious stimuli.

1.2.4 The Role of the Endothelium in Vascular Pathology

Vascular endothelial cells line the entire surface of all blood vessels in the body. As such, they play an important role in the maintenance and regulation of permeability, coagulation events (Gertler and Abbott, 1992), vessel tone (Furchgott and Vanhoutte, 1989), leukocyte adhesion in inflammation (Vane et al, 1990), and vascular smooth muscle cell growth (Reidy, 1988; Peiro et al, 1995). Originally thought to play only a passive role in such events, the vascular endothelium is now known to be a dynamic tissue, secreting and modifying vasoactive substances (Furchgott and Zawadski, 1980), influencing the behaviour of other cell types (Piero et al, 1995), and regulating extracellular matrix production and composition (Langille, 1993; Strauss et al, 1994).

In addition to the variety of extracellular matrix proteins that are produced by the endothelium, endothelial cells produce several growth promoting substances such as platelet derived growth factor, basic fibroblast growth factor, and insulin-like growth factor-1 (Nabel, 1991), which is balanced by the production of nitric oxide (NO) (Garg and Hassid, 1989), prostacyclin (Shirotani et al, 1991), and certain extracellular substances such as collagen V, and glycosaminoglycans (Castellot et al, 1981), which
inhibit cellular proliferation. This balance can be easily upset in dysfunctional endothelium.

In 1990, Ip and colleagues proposed a pathophysiologic classification of vessel wall injury to better understand the role of the endothelium in the pathogenesis of vascular disease. Type I injury was described as functional alterations of the endothelium without substantial morphologic changes. Type II injury was more severe and was associated with endothelial denudation and intimal damage without injury to the internal elastic lamellae. Finally, type III injury included frank endothelial denudation with accompanying damage to the underlying intima and media. Morphologically, endothelial cell denudation in vivo is not generally apparent, even over advanced atherosclerotic lesions, although the general orientation of endothelium, normally aligned in the direction of blood flow, is lost over the plaque (Nabel, 1991). This is not to say that type II and type III lesions can not occur over atherosclerotic lesions. When they do, it results in the loss of anticoagulant activity, the accumulation of platelet aggregates, and smooth muscle cell migration into the intima from the vessel media.

The use of tissue culture systems has provided ample evidence to support a role for the extracellular matrix and soluble growth regulators in influencing the modulation and/or state of proliferation of not only smooth muscle cells but of vascular endothelial cells as well, although the factors that regulate cell growth during remodeling and in response to injury remain unknown. One of the first observations that pointed to the importance of the extracellular matrix in modulating vascular endothelial function was the fact that cells plated on glass or plastic proliferated until they formed a characteristic
monolayer, whereas endothelial cells cultured on a reconstituted gel of basement membrane proteins ceased proliferating, aligned, and formed capillary-like structures within 24 hours (Kubota et al, 1988). Another study (Form et al, 1986), found that the proliferation of microvascular endothelial cells was greater on the matrix molecule laminin than on comparable substrates of fibronectin, collagen IV, or the interstitial collagens I and III. Laminin may also effect the differentiation of human endothelium in vitro (Grat et al, 1989) while fibronectin and its receptors are known to be required for normal vascular development in the mouse (Brooks et al, 1994). In vivo, the profile of rabbit capillary-associated proteoglycans also differs between proliferating endothelium versus those of stable and quiescent capillaries (Ausprunk et al, 1981).

Heterogeneity in terms of both morphology and function is evident in vascular endothelium. Morphological differences in microvascular endothelium correlate with vascular permeability -- the endothelium of high endothelial venules and those constituting the blood brain barrier, being prime examples. Capillary endothelial cells in general have been found to differ from endothelium in large vessels in terms of monocyte endothelial cell marker (OKM5) expression, intercellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex (MHC) molecule class I and II expression (Page et al, 1992). Moreover, fibroblast growth factor receptors appear to be expressed only by large vessel endothelium (Peters et al, 1992) and the endothelium of their vasa vasora (Edelman et al, 1992; Hughes et al, 1993; Daley and Gotlieb, 1996). The important implication of these observations is that vascular endothelium from vessels of different sizes and from different compartments may express different phenotypic properties and as such, may play different roles both in normal and disease
states. The fact that endothelial cells also share many phenotypic and functional properties with antigen presenting cells (Hirshberg et al, 1980), reinforces the notion that they play a key role in the immune reaction, especially in response to cytokine activation.

Changes in the endothelium, specifically in endothelial junctions, largely influence the morphogenesis and physiology of the vessel wall (Del Maschio et al, 1996). The functional importance of these junctions lies in the association of diseases such as atherosclerosis with alterations in endothelial adhesion (Frostegard et al, 1991). Moreover, changes in the endothelial expression of adhesion molecules affect extravasation and/or intravasation of inflammatory cells (Albeda et al, 1994). The adhesion molecule PECAM-1 (platelet endothelial cell adhesion molecule-1/CD31), is an important adhesion molecule expressed by vascular endothelial cells, platelets, monocytes, neutrophils, and naïve T lymphocytes (Albeda et al, 1991). This transmembrane glycoprotein is a member of the IgG gene superfamily of adhesion molecules and is responsible for mediating leukocyte-endothelial adhesiveness via homophilic (CD31-CD31) interaction (Pialli et al, 1995). PECAM-1 sites on the endothelium are also becoming increasingly recognized as important modifiers of platelet adhesion and aggregation when injury has not been sufficient to denude the endothelium (Rosenblum et al, 1996). Stewart and colleagues (1996), have recently demonstrated that the inflammatory cytokine tumor necrosis factor-α alone, or in combination with interferon-γ, can significantly decrease PECAM-1 steady state mRNA levels while inducing ICAM-1 mRNA in a bovine aortic endothelial cell model. Alternatively, PECAM protein levels following cytokine treatment were not found to be
decreased but rather redistributed away from intercellular junctions in human endothelial cells (Romer et al, 1995) via a mechanism thought to involve PECAM-1 dephosphorylation (Lu et al, 1996). In these cases, cell junctions are weakened in response to these inflammatory mediators, while leukocyte adhesion molecules are upregulated, thus promoting monocyte attachment and subsequent transmigration through the endothelial barrier.

Studies in animal models have shown that atherosclerosis also induces an early selective impairment of endothelium-derived relaxation (Rubanyi and Vanhoutte, 1986; Davies and Hagen, 1993). Such modulation occurred in rabbits on a cholesterol diet before any visible changes in the vessel wall could be detected and may involve inactivation of the vasodilator nitric oxide, through its interaction with oxidized low density lipoproteins (Rubanyi and Vanhoutte, 1986). The capacity of vascular tissue to synthesize prostacyclin also decreases with the progression of atherosclerosis (Vane et al, 1990). Associated with its declining levels, is a marked decrease in the cholesterol ester metabolism of smooth muscle cells and increased accumulation of cholesterol in macrophages. Loss of prostacyclin mediation of these events coupled with perturbed NO production, leads ultimately to a loss of thromboregulation and greatly exacerbates lesion development.

The endothelium is subject to many mechanical forces including alterations in fluid shear stress (both laminar and turbulent), pressure and stretch. The identification of shear stress responsive elements (SSRE) in the promoter regions of a number of growth factors which influence vascular cell growth, leukocyte migration, vascular tone and homeostasis, suggests that hemodynamic force either directly or indirectly, via
intermediary effectors such as NFκB or AP-1 (Depaola et al, 1992), activates gene transcription to affect the vessel response to its surrounding environment (Malek et al, 1993; Resnick and Gimbrone Jr, 1995). These same mechanisms may be involved in the vessel response to the absence of flow.
1.3 STATEMENT OF PURPOSE

Endothelial cell injury or dysfunction is recognized to play an important role in the pathogenesis of atherosclerotic lesions. Atherosclerotic lesion formation at specific sites of the vascular tree, namely at branch points and bifurcations, suggests that shear stress may induce changes in the vessel wall, specifically at the endothelial interface, which predisposes to neointimal formation and subsequent plaque development. Branch regions have been shown in vivo to be different from other areas of the vessel in terms of permeability to blood lipids and rates of cell turnover. Furthermore, heterogeneous populations of smooth muscle cells are known to exist within atheromatous plaques of human aorta as suggested by double-labeling immunofluorescence of cytoskeletal proteins (Babaev et al 1990). These observations imply that I may be able to detect characteristic differences in the properties of cells from different regions within vessels.

Neointimal formation, located at branch points in vivo, is considered to be a vascular change that predisposes to atherosclerotic plaque formation. To analyze this further, I used an aortic organ culture system, utilized previously in the laboratory, and adapted it to study the porcine thoracic intercostal branch point and its cellular response to injury. In this model, there is non-specific perturbation to the vessel wall as the artery is placed into static culture conditions. Parameters relating to the ability of the endothelium to repair itself were studied, since dysfunctional repair is thought to be important in the pathogenesis of at least some atherosclerotic plaques. In my characterization of branch point thoracic aortic organ cultures, I hypothesized that a decreased capacity for repair would be observed in atherosclerosis-prone regions around branches. In particular, I studied the extent of cell turnover, cell proliferation, and cell
death in regions of intimal thickening at the intercostal branch point, compared to unbranched regions within the thoracic aorta, in short term organ culture.

In addition, cell cultures were used to study the endothelial cells harvested from branched and unbranched thoracic aorta to assess their growth characteristics and ability to repair linear denuding wounds in the absence of influence from other components of the vessel. Such studies may provide important insight regarding the characteristics of cells at areas predisposed to plaque formation.
CHAPTER 2
Intimal Cushions at Porcine Thoracic Intercostal Branches Show Enhanced Proliferation and Cell Death in Aortic Organ Culture

2.1 Introduction

The intimal cushion present on the flow divider wall at branches in vivo is thought to be a normal physiological response to modifications in flow dynamics that may eventually become a precursor lesion of atherosclerosis. Modifications of flow implicated in lesion development include increased or decreased flow velocity or wall shear stress, flow separation, and departures from unidirectional laminar flow, including both orderly, non-linear flow patterns and turbulence (review, Glagov et al, 1988). It has been previously reported that fluctuations in blood flow in experimental animals, particularly at sites of branching, can affect endothelial cell morphology (Reidy and Bowyer, 1977; Langille and Adamson, 1981), effect separation of intercellular junctions (Baker et al, 1991), and cause the denudation of cells with the subsequent adherence of platelets (Thorgeirsson and Robertson, 1978). Such factors may account for the preferential location of intimal plaques at the entrances of branching vessels.

Clearly, the ability of the vessel to respond rapidly to alterations in its fluid environment is key to the maintenance of vascular homeostasis. Cell turnover thus may be regarded as an important parameter of the vessel's ability to respond to injurious events. The aim of the following experiments were the quantitative evaluation of intimal cell proliferation and cell death in branched and unbranched regions of the porcine thoracic aorta in response to organ culture.
2.2 Materials and Methods

2.2.1 Porcine Aortic Organ Culture

Porcine thoracic aortas were obtained from a local slaughterhouse within 15 minutes of death. They were transported to the laboratory in sterile phosphate buffered saline containing antibiotics, and gently cleaned of fat and adventitia.

To characterize the branch region, representative paraffin sections from the proliferation studies were stained with hematoxylin and eosin and Movat's pentachrome stain to visualize the histology of the vessel wall and branch sites. Ham-56 and α-SMC actin staining were performed to identify macrophages and smooth muscle cells in the intima of cultures. Additionally, to identify the presence of the endothelial monolayer, the expression of PECAM-1 was assessed over the organ culture period by en face immunofluorescence staining of organ cultures at times 0 (fixed at the abattoir), 5 hours, 1 and 4 days following the onset of incubation in culture.

To assess the degree of proliferation at the onset of culture, approximately 4 hours following harvest, three aortas were fixed with 10% neutral buffered formalin for 24 hours, paraffin embedded, then stained for proliferating cell nuclear antigen (PCNA). Four regions in aortas were analyzed, including the intercostal branch flow divider (fd), the area distal to the intercostal opening (ofd), unbranched tissue opposite the branch point (uboi), and unbranched tissue above the first intercostal but below the aortic arch on the branch side (ubai) (fig. 2.2).

To investigate the extent of cell proliferation and cell death in organ culture, 28 additional aortas were opened down their length along the vessel opposite the branch point, exposing the luminal surface. Segments approximately 2 cm square were
harvested from the first three single intercostal ostia and adjacent unbranched tissue opposite selected ostia (fd, ofd, uboi). Tissue was placed in sterile Falcon trays with medium M199 (Gibco, Grand Island, NY), 5% fetal bovine serum (Gibco), 1% penicillin/streptomycin and 1% fungizone (Gibco) for 1 and 4 day time points.

Wounding experiments were also performed by creating an acute mechanical injury to the endothelium of vessel branches in order to provide an additional stimulus for the formation of intimal hyperplasia. Lesions created in porcine models (Steele et al, 1985), have shown partial endothelial regrowth by 4 days and almost full regrowth by 7 days, hence these times were among those chosen for study. A total of 6 branch points were harvested from each of 3 aortas, and the intima of 3 from each were wounded by drawing a 3.0 nylon filament across the branch region in a direction opposite to blood flow (fig. 2.3A). Eighteen segments in total, 9 normal and 9 wounded, were separated into 3 groups of 6 (3 normal and 3 wounded) and cultured as described above for 1, 4, and 7 days. Unbranched tissue was not analyzed in these wounding experiments.

2.2.1 Platelet Endothelial Cell Adhesion Molecule (PECAM-1) Expression

Eight thoracic aortas collected within 15 minutes of death from a local abattoir. To observe the endothelial cells as soon as possible after death, 3 were fixed at the abattoir in cold methanol (30 minutes) and transported to the lab where they were immersed in cold acetone for 15 minutes. Fixed aortas were rinsed with PBS, opened longitudinally, and 2-cm square pieces were removed from each of the 4 areas sampled on each aorta.
Five aortas transported to the laboratory in sterile PBS containing antibiotics were prepared for organ culture as previously described. The first three single intercostal branches from each aorta were harvested and randomly placed in incubation for 3 time periods — onset of culture (5hrs after harvest), 1 day, and 4 days. Three pieces of unbranched aorta opposite from each intercostal and 3 pieces from above the first intercostal from each aorta were also harvested and subjected to the same culture and assay protocol as intercostal branches.

Organ cultures were fixed in methanol and acetone, washed 3 times with PBS, then incubated with anti-human PECAM-1 (Newman et al, 1990) monoclonal mouse antibody (1:20; R & D Systems, Barton Lane, Abingdon) for 1 hour. Following 3 more PBS washes, tissue was incubated with FITC-conjugated goat anti-mouse antibody (1:50; Sigma Chemical Co., St. Louis, MO) and ethidium homodimer (1μM; Molecular Probes, Eugene, Oregon) for 30 minutes. Samples were washed with PBS, inverted and placed in Probe Clip mounting chambers (Grace Bio-Labs, Sunriver, Oregon) with a 1:1 solution of PBS/glycerol, covered with a glass slide, and viewed en face under laser scanning confocal microscopy. Five fields on either side of intercostal branch margins were scanned at 60X magnification. Five fields were also scanned at random from unbranched tissue taking care to avoid the edges. On average, ten 1.5 μm optical slices were scanned per field for both branched and unbranched tissue.

2.2.3 Proliferation Assay

Cell proliferation was quantified in tissue sections using monoclonal antibodies to localize proliferating cell nuclear antigen (Tsukada et al, 1991; McCormick et al, 1992) and 5-bromo-2'-deoxyuridine (BrdU) (Gratzner et al, 1982; Coltera et al, 1991).
Prior to day 1 organ culture incubation, 5-bromo-2'-deoxyuridine labeling reagent containing 5-fluoro-2'-deoxyuridine (FrdU) in a 1:10 ratio, (BrdU/FrdU; Amersham Corp., Arlington Heights, IL) was added to the media (1:1000) and the mixture sterilized by filtration (.22 μm; Nalgene, Rochester, NY). The addition of FrdU provided a more accurate reflection of proliferation by allowing BrdU incorporation in the absence of competition by endogenous thymidine. Segments of aorta were incubated with BrdU labeling reagent for 24 hours prior to harvest (for 1 and 4 day incubations) in a humidified chamber (5% CO₂, 95% air) at 37°C. Media was changed in day 4 cultures at 24 and 72 hours following the onset of culture.

In mechanically wounded cultures, day 4 and day 7 branches were also fed at 24 and 72 hours following the onset of culture and labeling reagent was added to the cultures 24 hours prior to harvest at 1, 4 and 7 days.

Aortas were fixed in 10% buffered neutral buffered formalin for 24 hours before standard processing and paraffin embedding. Sections of aorta were cut at 4 μm thickness from tissue embedded on edge (fig. 2.3B). Immunohistochemistry was performed on unstained tissue collected on 3-aminopropyltriethoxysilane-coated (Sigma Chemical Co., St. Louis, MO) glass slides (fig. 2.3C). Immunologic reactions were developed using standard avidin-biotinylated alkaline phosphatase complex procedures (Vector Laboratories, Burlingame, CA).

Briefly, aortic sections were cleared of paraffin in 4 washes of xylene and then rehydrated in 5 changes of ethanol, followed by 2 washes with phosphate buffered saline. A 20% normal horse serum/PBS block was applied for 20 minutes in order to block non-specific binding then slides were incubated in a humidified chamber with
either of 2 monoclonal mouse primary antisera for 2 hours in the case of anti-BrdU (ready to use; Amersham Corp., Arlington Heights, Il) or overnight as for anti-PCNA clone PC10 (dilution 1:200; Boehringer Manheim, Indianapolis, Id). Following 2 PBS washes, sections were incubated with biotinylated horse anti-mouse immunoglobulins (dilution 1:200; Vector Laboratories, Burlingame, Ca) for 40 minutes and then with an alkaline phosphatase solution (Vectastain ABC, Vector Laboratories, Burlingame, Ca) for 45 minutes, with intervening PBS washes. All antisera were diluted in PBS with 5% normal horse serum. Vector Black chromagen (Vector Laboratories) in a Tris-HCl buffer, pH 9.5, was applied and development was monitored under light microscopy for approximately 8 minutes. Sections were counterstained with Nuclear Fast Red (Zymed Laboratories, Miss. Ont.) and aldehyde fuschin to demonstrate nuclei and elastin respectively. Slides were then mounted in Crystal Mount, an aqueous mounting medium (Biomedia, Foster City, Ca).

Sections of porcine small intestine incubated with BrdU labeling reagent in media M199 for 24 hours served as a positive control for both PCNA and BrdU immunohistochemistry. Slides incubated with non-immune mouse IgG\textsubscript{1} (30 μg/ml; Serotec Inc., Bankside, Station Field, Ind) in place of primary antisera were used as a negative control.

Proliferating endothelial and intimal smooth muscle cells were counted on each side of the branch point beginning on the flow divider wall where the medial-adventitial border became perpendicular to the intercostal branch (fig 2.4A, lower arrowhead). A total of 9 fields were counted at 20X magnification covering a total of 3mm on one side. This distance included the intimal cushion (fig. 2.4A, upper arrowhead) of the flow
divider wall in all samples. The wall opposite the flow divider and unbranched tissue were counted in a similar manner.

No attempt was made to separate endothelial and smooth muscle cell counts when counting wounded and control branches in the 3 aortas subjected to mechanical injury. Cell counts otherwise were performed as described above.

2.2.4 Tdt-mediated dUTP Nick End Labeling (TUNEL)

To assess the degree of DNA fragmentation caused by cell death and/or necrosis in organ cultures, unstained, BrdU-labeled tissue sections from earlier proliferation experiments were analyzed using an in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany). Slides were cleared of paraffin to PBS as described above then incubated with proteinase K (2μM) in a 10mM Tris-HCL buffer, pH 7.6, for 10 minutes. The TUNEL reaction mixture was applied for 1 hour after which time, a converter alkaline phosphatase was added for 30 minutes, followed by the Vector Black chromagen (6 minutes). From this point, the protocol described above for BrdU and PCNA staining was followed. Slides incubated with DNase I (10μg/ml, 10 minutes) in DN buffer were used as a positive control while the TUNEL label without the terminal transferase enzyme was used as a negative control. All incubations, with the exception of DNase I and proteinase K pre-digestion (24°C), were carried out at 37°C. The proportions of TUNEL positive and non-positive endothelial and smooth muscle cells were counted as described previously in the analysis of proliferation.

2.2.5 Live Dead Cell Assay
To quantify the extent of cell death occurring in the organ culture system, calcein AM ester (live reagent) and ethidium homodimer (dead reagent), were incubated with tissue samples, then viewed en face under confocal microscopy. Optimal dilutions were determined for the reagents: Calcein AM (2μM) EthD-1 (1μM) (Molecular Probes, Eugene, Oregon). Eight porcine thoracic aortas were prepared and segmented as described previously. The first three single intercostal branches from each aorta were used and randomly chosen for incubation at 0, 1 and 4 days. Three pieces of contiguous tissue from above the 1st intercostal were also harvested and subjected to the same culture and assay protocol as branched samples.

Following harvest after culture, samples were trimmed to fit into 24-welled Falcon trays then washed with warmed media for ~5 min. At this point, the live/dead reagents were mixed in 5 ml of standard media, warmed to 37°C and 1 ml was added to each well. The tray was then placed in an incubator for 30 minutes after which time, samples were washed 3 times with fresh media, inverted, and placed in Probe Clip mounting chambers, covered with a glass slide and viewed under laser confocal microscopy.

Live and dead cells were counted en face for 10 randomly chosen fields in each of 3 regions (fd, ofd, ubai) by the use of a 1cm x 1cm ocular grid under 60X magnification. All dead cell nuclei within this grid, and visible to the observer upon slight focus adjustment, were counted. Similarly, live cells in the same field were counted by switching filter sets. The proportion of dead cells was determined as a percentage of total cells and averaged for the 10 fields in each region.
A second study was later conducted using 6 additional aortas in which a fourth region, the unbranched area opposite the branch point (uboi) was analyzed. Live and dead cells were counted en face for 5 randomly chosen fields in unbranched tissue (uboi, ubai) and along the flow divider (fd) and its opposite wall (ofd) in branch containing samples. Each field in branch regions was analyzed by counting only endothelial cells over 10 superimposed optical sections to an average depth of 15 μm at 60X magnification. Unbranched tissue was scanned to depths of 5-6 μm. Live cells in the same field were counted by switching filter sets. The proportion of dead cells was determined as a percentage of total cells and averaged for the 5 fields in each region.

2.2.6 Statistics

Positive cells in all experiments were expressed as a percentage of total cells. Differences between the mean percentage labeled in each were compared using a factorial ANOVA to determine significance. If significance was observed, then a Fisher's PLSD post hoc test was run. (Statsview 4.5, Power Macintosh 7200/90).

2.3 Results

2.3.1 Intimal Cushion Morphology

Intimal cushions at the flow divider and its opposite, upstream wall at intercostal ostia were easily distinguished in tissue sections by the sharpness of the branch opening at its downstream end and the prominence of the intimal cushion at the flow divider (fig. 2.4). Hematoxylin & eosin and Movat staining revealed prominent extracellular matrix in flow divider cushions and a relatively fragmented internal elastic lamina at the branch. A continuous endothelium was present at the cushions and also in unbranched
tissue. Cushions upstream from the flow divider were smaller than those on the flow divider and \(\alpha\)-smooth muscle cell actin staining was abundant throughout the intima and media of tissue sections.

2.3.2 PECAM-1 Expression

Continuous, peripheral PECAM-1 expression at cell-cell contacts on both the flow divider and opposing wall of the branch point and also in unbranched areas opposite to and above the intercostal openings in time zero cultures was observed (figs. 2.5A-F, 2.6A through 2.9A). Cells at the apex and lips of the branch point flow divider (fig. 2.5A, C, E) were oriented in the direction of flow. Cells of the lip opposite the divider showed less orientation to flow (fig. 2.5B, F), while cells within the middle of the wall were elongated in the direction of flow (fig. D). Cell morphology in unbranched regions showed no preferred orientation and maintained a cobblestone-like morphology with minimal disruption in the monolayer. Cell nuclei, as demonstrated by ethidium homodimer, appeared spherical at all time points.

While PECAM-1 expression did not appear to differ between any of the 4 regions sampled at time zero, by 4 hours focal loss along branch margins (figs. 2.6B, 2.7B) and punctate staining in unbranched regions (figs. 2.8B, 2.9B) were seen. Thereafter, PECAM-1 expression appeared to increase at 24 hours (figs. 2.6C, through 2.9C) and was fully re-established by 4 days (figs. 2.6D through 2.9D). Of interest was relatively dense pattern of PECAM-1 expression particularly evident between endothelial cells at the flow divider.
In terms of cell orientation, the pattern of alignment with flow described earlier persisted along branch margins through 4-hour cultures but was not apparent by 1 and 4 days when endothelial cells adopted a more cobblestone-like morphology.

2.3.3 Proliferation Assays

PCNA staining of thoracic aortas at the onset of culture (~5 hrs) did not reveal any significant differences in endothelial cell proliferation between branched (fd, ofd) and unbranched regions (uboi, ubai; fig. 2.10). Endothelial cell number was similar between regions at approximately 100 cells. Smooth muscle cell proliferation in unbranched tissue opposite the branch was greater than that within the flow divider but was not significantly different compared to other regions of the vessel. Smooth muscle cell number was consistent between regions at approximately 35 ± cells per total fields counted (fig. 2.10).

Analyses of proliferation and cell number following organ culture at 4 days using BrdU immunohistochemistry demonstrated a significantly higher degree of endothelial cell proliferation along with a corresponding increase in cell number in unbranched tissue (uboi) but not at branches, after 1 day in culture (figs. 2.4B, C & 2.11). Smooth muscle cell proliferation was observed to increase marginally over time in all three regions sampled however, no differences between regions was observed.

Following 4 days in culture, there was marked endothelial cell proliferation in the flow divider cushion to levels similar to that observed in unbranched tissue opposite the branch. Despite this increase in proliferation however, endothelial cell number remained constant in this region at 1 and 4 days (fig. 2.12), suggesting a role for cell death in the culture system.
The extent of PCNA localization at branch point intimal thickenings was enhanced in mechanically wounded cultures (fig. 2.13). A vast majority of PCNA positive cells in both the flow divider wall region (fig. 2.13A) and in the wall opposite the flow divider (fig. 2.13B) were endothelial cells. However, no significant differences between proliferation in the flow divider wall and proliferation in the wall opposite the flow divider were observed. Proliferation at 4 days on both sides of the branch subsided to approximate day 1 levels by day 7. In non-wounded, control branches, the percentage of positive intimal cells in the flow divider region was significantly increased as compared to the region opposite the flow divider, at 4 and 7 days ($p < .05$) using PCNA staining. This increase in proliferation was confirmed at day 4, but not at day 7, when proliferating cells were labeled using BrdU immunohistochemistry ($p < .05$) (fig. 2.14).

### 2.3.4 TUNEL Labeling

Two general trends were evident in the analysis of TUNEL labeled sections over 4 days. Greater than 50% labeling at 5 hours was noted (fig 2.15) which decreased to 25% by 24 hours (figs. 2.15; 2.16A, B, E), but was elevated again by 4 days in all 3 areas analyzed (fd, ofd, uboi; figs. 2.15; 2.16 C, D, F) for both endothelial and smooth muscle cells. A relatively unlabeled tunica media at branches at 4 days culture was observed (fig. 2.16 C, D) while the media in unbranched tissue had much greater labeling at 4 days (fig. 2.16 F). At no time point however, was there a difference in labeling between the flow divider and its upstream wall. Statistical differences between regions were found at 5 hours only, between the 2 branch regions and the unbranched region opposite the intercostals. This was the case for both endothelial and smooth
muscle cell populations. TUNEL labeling was greatest in non-branching endothelium but lowest in intimal smooth muscle cells at 5 hours.

2.3.5 Identification of Live and Dead Intimal Cells

Using the live/dead assay, approximately 20% intimal cell death was observed in en face preparations measured by immunofluorescence at the onset of culture (fig. 2.17). Dead cell nuclei appeared spherical and stained bright red with ethidium homodimer while calcein labeled cells were bright green. There were no significant differences observed in cell death between the 2 walls of the branch opening, at the onset of culture (fig. 2.18A, B) or following 1 day in culture (fig. 2.19A, B); however, death was markedly lower in unbranched areas at both these time points (figs. 2.18C, D & 2.19C, D). Intimal death at 4 days culture (fig. 2.20) was observed to be lowest in unbranched tissue (fig. 2.20C, D) and significantly greater at the flow divider (fig. 2.20A) than on the opposing side of the branch (fig. 2.20B).

Intimal cell death data (fig. 2.17) was consistent with data taken from the second series of aortas that were analyzed using optical slices from the confocal microscope to distinguish between endothelial and smooth muscle cells (fig. 2.21), suggesting that endothelial cell turnover was responsible for the observed pattern of cell death.
Chapter 2

Figures
fig. 2.1 -- Schematic of the porcine thoracic aorta intercostal branch. The general extent and location of eccentric and diffuse intimal thickening are shown. Areas of interest in this study are also marked (boxed regions).
fig. 2.2 -- Schematic of the porcine thoracic aorta. The 4 regions under study are marked.
Reference Points in the Porcine Thoracic Aorta

Legend
- ofd: upstream, opposite flow divider
- fd: flow divider
- uboi: unbranched, opposite intercostals
- ubai: unbranched, above intercostals
fig. 2.3 — A, Section of thoracic aorta showing intercostal branch. The wall opposite the branch has been opened, exposing the luminal surface. B, Section of cut aorta showing how sections were cut through the branch region. (dashed lines) C, Orientation of tissue as it appears on a glass slide.
Slide Preparation from Organ Cultures

A

 direction of wounding in wounded cultures

flow divider

flow

B

section at 5 μm

flow divider

endothelium

flow divider

C
fig. 2.4 — A, Photomicrograph of the branch region at 4 days. Large arrow represents the direction of flow. The flow divider intimal cushion is marked (upper arrowhead), as is the point where the medial-adventitial border begins to become perpendicular to the thoracic aorta (lower arrowhead). A horizontal line was followed to the endothelial surface, and counting began here. Mag. 1x, Bar = 0.4mm. B, Intimal cushion in A at 4 days showing BrdU-labeled endothelial and smooth muscle cells (arrowheads). Mag. 20x, Bar = 20μm. C, Wall opposite the flow divider at 4 days showing a BrdU positive smooth muscle cell (arrowhead). Mag. 20x, Bar = 20μm.
fig. 2.5 — Confocal photomicrographs of branch point PECAM-1 expression at time zero. A, C, E, flow divider lip, apex, and lip respectively. B, D, F, opposite flow divider upper edge, middle and lower edge. Mag. 60x, Bar = 25μm
fig. 2.6 — Confocal photomicrographs of PECAM-1 expression at the flow divider (fd). A, Time zero. B, 4 hours. C, 1 day culture. D, 4 days culture. Mag. 60x, Bar = 25μm
**fig. 2.7** -- Confocal photomicrographs of PECAM-1 expression opposite the flow divider (ofd). A, Time zero. B, 4 hours. C, 1 day culture. D, 4 days culture. Mag. 60x, Bar = 25\(\mu\)m
**fig. 2.8** -- Confocal photomicrographs of PECAM-1 expression opposite the branch (uboi). **A,** Time zero. **B,** 4 hours. **C,** 1 day culture. **D,** 4 days culture. Mag. 60x, Bar = 25μm
fig. 2.9 -- Confocal photomicrographs of PECAM-1 expression above the intercostals (ubai). A, Time zero. B, 4 hours. C, 1 day culture. D, 4 days culture. Mag. 60x, Bar = 25μm
Fig. 2.10 -- Mean percentage of PCNA positive endothelial and intimal smooth muscle cells at the onset of culture. Lower panel, endothelial and smooth muscle cell number in the intima for total fields counted for each region. Error bars represent standard errors.

* $p < .05$ unbranched versus branched
** $p < .05$ flow divider versus unbranched region opposite the branch

$n = 3$ aortas
Cell Proliferation and Number at the Onset of Culture

* $p < 0.05$

**fig. 2.10**

- **PCNA positive cells**
  - % PCNA positive cells
  - Bars for different groups:
    - ofd
    - fd
    - uboi
    - ubai

- **Intimal cell number per total fields counted**
  - Bars for different groups:
    - Endothelial cells
    - Smooth muscle cells

Graphs showing the percentage of PCNA positive cells and intimal cell number per total fields counted for endothelial and smooth muscle cells across different groups.
**fig. 2.11** -- Mean percentage of endothelial and smooth muscle cell PCNA and BrdU positive cells in organ cultures over 4 days. Tissue at the onset of culture stained with anti-PCNA, while day 1 and day 4 cultures were labeled with anti-BrdU. Error bars represent standard errors.

* p < .05  flow divider and opposite flow divider versus unbranched opposite branch

** p < .05  opposite flow divider versus flow divider and unbranched opposite branch

*** p < .05  flow divider versus unbranched opposite branch

n = 14 aortas
Endothelial and Smooth Muscle Cell Proliferation in Organ Culture

* p<.05

** Graph showing proliferation of endothelial cells and smooth muscle cells in organ culture.

- **Endothelial cells**
  - Culture onset: No significant difference.
  - Day 1: Significantly higher proliferation in UBoi compared to Ofd and Fd.
  - Day 4: Significantly higher proliferation in all groups.

- **Smooth muscle cells**
  - Culture onset: No significant difference.
  - Day 1: No significant difference.
  - Day 4: Significantly higher proliferation in UBoi compared to Ofd and Fd.

Legend:
- Ofd
- Fd
- UBoi
fig. 2.12 -- Mean percentage of endothelial and smooth muscle cell numbers per total fields counted in organ culture over 4 days. Error bars represent standard errors.

- $p < .05$ unbranched opposite branch versus flow divider and opposite flow divider

$n = 14$ aortas
Endothelial and Smooth Muscle Cell Number in Organ Culture

* p<.05

** endothelial cells **

- cell number per fields counted

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** smooth muscle cells **

- cell number per fields counted

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**Fig. 2.13** -- Mean percentage of PCNA and BrdU labeling in cells of the flow divider wall and opposite wall regions of wounded thoracic aortas and non-wounded control aortas. Error bars represent standard errors.

* p < .05 flow divider versus opposite flow divider
n = 3 aortas
Intimal Cell Proliferation in Wounded and Non-wounded Intercostal Branches

* p < 0.05

% PCNA positive intimal cells

% BrdU positive intimal cells

wounded

non-wounded

day
day
fig. 2.14  --  A, Photomicrograph of PCNA-labeled intimal cells at the flow divider wall in 4 day wounded aorta. 20x, Bar = 20 μm.  B, Photomicrograph of PCNA-labeled intimal cells in the wall opposite the flow divider of 4 day wounded aorta. 20x, Bar = 20 μm.
**Fig. 2.15** -- Mean percentage of TUNEL positive endothelial and smooth muscle cells in organ culture over 4 days.

* p < .05 unbranched opposite branch versus flow divider and opposite flow divider

** p < .05 opposite flow divider versus unbranched opposite branch

*** p < .05 flow divider versus unbranched opposite branch

n = 3 aortas
Endothelial and Smooth Muscle Cell DNA Fragmentation in Organ Culture

* p<.05

**% TUNEL positive cells**

**Endothelial Cells**

- * p<.05

**Smooth Muscle Cells**

- ** ofd
- fd
- uboi

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**fig. 2.16** -- Photomicrographs of TUNEL-labeled sections. A, wall opposite the flow divider, 1 day culture. B, flow divider, 1 day culture. C, wall opposite the flow divider, 4 day culture. D, flow divider, 4 day culture. E, unbranched wall opposite intercostal, 1 day culture. F, unbranched wall opposite intercostal, 4 day culture. Mag. 20x, Bar = 40µm
fig. 2.17 -- Intimal cell death over 4 days, live/dead assay
(see page 34 methods)

* $p < .05$ unbranched opposite branch versus flow divider and opposite flow divider

** $p < .05$ flow divider versus opposite flow divider and unbranched opposite branch

$n = 8$ aortas
Total Intimal Cell Death in Intercostal Branch Organ Cultures

* p<.05

% intimal cell death

days in culture
**Fig. 2.20** — Confocal photomicrographs of live (green) and dead (red) cells in 4 regions of the thoracic aorta labeled by the live/dead assay following 4 days in organ culture. **A,** flow divider. **B,** wall opposite the flow divider. **C,** unbranched aorta opposite the intercostal branch. **D,** unbranched aorta above the first intercostal. Mag. 60x, Bar = 25 μm
fig. 2.18 -- Confocal photomicrographs of live (green) and dead (red) cells in 4 regions of the thoracic aorta labeled by the live/dead assay at 5hrs (time of culture). A, flow divider. B, wall opposite the flow divider. C, unbranched aorta opposite the intercostal branch. D, unbranched aorta above the first intercostal. Mag. 60x, Bar = 25 μm
fig. 2.19 — Confocal photomicrographs of live and dead cells in 4 regions of the thoracic aorta labeled by the live/dead assay following 1 day in organ culture. A, flow divider. B, wall opposite the flow divider. C, unbranched aorta opposite the intercostal branch. D, unbranched aorta above the first intercostal. Mag. 60x, Bar = 25 μm
**fig. 2.21** -- Endothelial cell death over 4 days, live/dead assay  
(see page 35 methods)

* $p < 0.05$ flow divider versus opposite flow divider and both unbranched regions

$n = 6$ aortas
Endothelial Cell Death In Organ Cultures

* p<.05

% endothelial cell death

culture onset 1 4

days in culture

fig. 2.21
2.4 Discussion

The proliferation of intimal cells of the arterial vessel is significant in the early stages of atherogenesis. Growth of lesions may be attributed both to smooth muscle cell migration and proliferation within the intima, which is directly influenced by factors released from the overlying endothelium (Griendling and Alexander, 1996). Intrinsic injury response characteristics in endothelial cells may therefore play a major role in the ability of the vessel wall to resist early changes induced by injurious stimuli which predispose to atherosclerosis. Analysis of proliferation and cell death kinetics within specific regions in the intercostal branches of porcine thoracic aortas, using PCNA, BrdU, TUNEL, and the live/dead assay, have provided *in vitro* evidence for intimal cells that differ with respect to their rates of turnover in different regions of the porcine thoracic aorta in response to organ culture injury. Such differences in the ability of intimal cells to respond to injury may ultimately contribute to the development of atherosclerosis-prone regions in the arterial vasculature.

Immunocytochemical analysis of human atherosclerotic lesions (Tsukada et al, 1991; Katsuda et al, 1992) report the extent of PCNA localization to be between 1% and 2%. Investigators suggest that such low levels are consistent with the observation that the development of clinically significant lesions occurs only after several decades or longer. Such lesions were not studied in the porcine model, however a high degree of proliferation (5-15%) within all regions in both model systems (wounded and non-wounded) was observed. These increases may be due to inherently higher proliferation in porcine aortas compared with human lesions. Alternatively, Barker et al, (1994) propose that *in vivo* - removal of the adventitia initiates hypoxia-induced
intimal proliferation until such time as a neoadventitia is formed. The preparation of both model systems included the removal of the vessel adventitia, which may have played a role in the intimal response to organ culture injury. Sims et al. (1993) also report that a substantial elastic lamina is important in limiting the progression of atherosclerotic changes. The internal elastic lamina of branch regions in particular was highly fragmented, although fragmentation appeared to be equally extensive on both walls. The state of the internal elastic lamina in vitro may not be a critical factor however, since the IEL in unbranched tissue was more complete than at branches yet this region was observed to undergo extensive proliferation over the culture period. In any case, proliferation induced by adventitia removal or an incomplete IEL, can not account entirely for the differences in proliferation noted between the flow divider wall, unbranched tissue opposite from the branch, and the wall opposite the flow divider by four days culture. Additionally, approximately parallel rates of replication shown by expression of the cell-cycle-related protein PCNA, as well as BrdU-uptake followed by anti-BrdU immunohistochemistry, provide further evidence that estimates of cell turnover were indeed correct.

The proliferation data collected from the wounded model system demonstrates again that in unwounded controls, there exists the predilection towards an inherently higher degree of intimal cell proliferation within the flow divider region compared to that of the opposite wall. Furthermore, in wounded cultures, there is no longer a difference in proliferation between opposing sides of the branch due to increased proliferation of intimal cells opposite to the flow divider. These results are provocative in that they suggest that this intimal proliferation may result from the
release of soluble factors from the mechanically injured intima over and above those induced by the injury of culture itself.

The exact nature of the soluble factors which permit intimal cell quiescence to be perturbed, with subsequent proliferation as a result, remain undetermined. The difference in cell turnover rates, present between unwounded flow divider walls and walls opposite the flow divider, becomes insignificant in wounded culture systems due to a substantial increase in intimal proliferation within the wall opposite the flow divider. While there exist a vast number of well-characterized regulators of cell growth that may be responsible for this observed increase, wounded or dying endothelial cells are known to release both acidic and basic fibroblast growth factor, (D’Amore, 1992; Ettenson and Gotlieb, 1993) the latter being a potent stimulator of large vessel endothelial cell (EC) proliferation in vitro (D’Amore and Smith, 1993; Reidy and Lindner, 1990). The role of bFGF in the progression of atherosclerosis has been questioned however, because of the observation that it is absent in human carotid plaques (Brogi et al, 1993). Previous study in our laboratory has determined that other molecules liberated from the vessel wall in organ culture, including platelet-derived growth factor, transforming growth factor-β, tumor necrosis factor-α, and epidermal growth factor, do not directly affect neointimal formation (unpublished observations). Interestingly, levels of bFGF reciprocally regulated levels of its high affinity receptor FGFR-1 in cultures over 7 days (Daley and Gotlieb, 1996). Mechanical wounding of organ cultures may thus have allowed the escape of bFGF from EC’s at the branch, effecting local endothelial cell proliferation however, as earlier experiments would indicate, bFGF alone, or in combination with other factors, is not enough to directly
promote neointimal proliferation \textit{in vitro}. In this model, the presence of endothelium is known to be necessary for neointimal proliferation since complete denudation of the surface endothelium reduces intimal proliferation (Koo and Gotlieb, 1989). Taken together, these results suggest that endothelial cells, by way of their ability to become activated, by injury and/or factors liberated from dead or dying cells, express growth factors and their receptors, which may indirectly influence the short term proliferation of intimal cells in organ culture systems.

There also exists the possibility that mechanical wounding of the intima, coupled with inadvertent damage to the underlying media effected the release of a chemoattractant factor which promoted intimal cell proliferation and medial smooth muscle cell migration into the intima. This population could then proliferate once resident in the subendothelium (Yoshida et al, 1988). Smooth muscle cells are stimulated to migrate and proliferate in response to local FGF's, (Reidy, 1993) which, coupled with the easily traversed internal elastic lamina, typically fragmented at the intercostal branch, may account for the localization of focal accumulations of SMC's, \textit{in situ}, in the normal intima at branch sites in pigs.

While cell proliferation data, at 4 days in organ culture, showed an increased capacity of the endothelium to proliferate at the flow divider and at unbranched tissue opposite the branch, in terms of cell number, unlike unbranched (uboi) aorta, there appeared to be no accumulation of endothelial cells at the flow divider. Results from the live/dead assay confirmed that in addition to the increase in endothelial cell proliferation at the flow divider seen at 4 days, there was also an increase in endothelial cell death within this region. The relative contribution of apoptosis versus
cell necrosis to cell death however, was not determined. Condensed nuclei were noted in organ cultures, especially in flow divider endothelial cells following 4 days in culture, while the vast majority of dead cells observed in the 3 other regions possessed intact, spherical nuclei. Even a low incidence of apoptosis may be significant however, owing to the fact that the process is of short duration and therefore difficult to capture at any given moment in time (Kerr et al, 1972). Indeed, apoptotic bodies reportedly are seen for only a few hours before they are phagocytized by neighboring cells (Bursch et al, 1990). The fact then that we noted a significant proportion of morphologically-appearing apoptotic cells at the flow divider at any one time in 4 day cultures may infer that there is substantial enough apoptotic cell death occurring to offset the increase in proliferation also observed in this region. Whether increased cell death is the cause of increased proliferation or vice versa remains to be determined.

TUNEL labeling of breaks in the DNA strand is a method known to result in the preferential labeling of apoptosis as opposed to necrosis. Gold et al (1994) however, caution that DNA which has been degraded by lysosomal enzymes in necrotic cells may also be targeted by the tdt enzyme. Alternatively, DNA fragmentation has been found not only in histologically defined apoptotic cells, but also in morphologically intact cells going through the process of programmed cell death (Umansky, 1982; Motyka and Reynolds, 1991). The substantial degree of TUNEL labeling which we observed at the onset of culture and at 4 days may therefore represent 2 conditions. Due to the sensitive nature of the assay, it is possible that some of the labeling is partly due to DNA fragmentation associated with cell proliferation (Hirose et al, 1996). This may well account for the increase in labeling at
4 days culture. This reasoning may also explain why there is such extensive TUNEL labeling in unbranched tissue as opposed to unbranched tissue subjected to the live/dead assay. It is also entirely possible that spherical nuclei identified via the live/dead assay are in the process of dying via some programmed cell death pathway. Differences in cellular sensitivities to various stimuli are known to exist (Geng et al, 1995). If this is indeed the case, then the proportion of cell death induced by apoptosis, inferred from the live/dead cell assay data by way of cell morphology, may have been underestimated.

The observation that the tunica media of branch regions was relatively unlabeled by TUNEL in contrast to unbranched aorta opposite the intercostals at 4 days, may support the notion that apoptosis occurs in a heterogeneous fashion between different regions within the vessel. This difference may be a function of cellular phenotype, which may confer differing sensitivities to inducers of apoptosis (Geng et al, 1995). The finding that intimal smooth muscle cells show much greater TUNEL labeling than medial smooth muscle, also suggests a potential difference in apoptosis regulation between these 2 populations. Striking differences in gene expression between intimal and medial smooth muscle cells are well known (Lemire et al, 1994; Schwartz et al, 1995), and increased growth rates of atherosclerotic intimal smooth muscle cells compared to cells from the media have been observed in cell culture studies (Yoshida et al, 1988). Moreover, apoptosis has been identified as playing a figurative role in the development of intimal thickening in the rat (Bochaton-Piallat et al, 1995). Hence, in the macroscopically normal aorta, early differences in apoptosis
regulation and cell turnover have intriguing implications in terms of the later propensity of the vessel wall to develop atherosclerotic lesions.

PECAM expression was observed to be sparse and punctate in retracted cells at 4 hours. Major alterations in PECAM expression were observed early during the culture period and gradually recovered over 4 days in organ culture, dependent mainly on cell morphology as cells adapted to the culture environment. By 4 days culture, PECAM-1 expression was similar to its time zero distribution although expression along cell boundaries, especially in endothelial cells at the flow divider, were markedly thickened. Such thickening may reflect a clustering of PECAM molecules that migrate to the cell periphery as the cell attains its cobblestone morphology and re-establishes contact with neighboring cells; however, changes in PECAM expression such as those documented herein over the organ culture period have not been described in the literature. Maschio and colleagues (1996), report an unchanged PECAM-1 distribution at cellular contacts following polymorphonuclear leukocyte adhesion to TNF-α activated endothelial cells from 5 through 60 minutes in vivo. They do note however, a loss of the vascular endothelial (VE)-cadherin/catenin complex and redistribution of VE-cadherin itself, as early as 1 minute following adhesion which suggests that PMN adhesion to the endothelial cell could mediate intracellular signaling events leading to adherens junction disorganization. Similar disorganization of PECAM junctions mediated by PECAM dephosphorylation upon β1-integrin engagement, has also been described in human umbilical vein endothelial cells (Lu et al, 1996). These observations are important owing to the fact that PECAM-1 has been suggested to play a dual functional role in leukocyte-endothelial
interactions, acting both as an agonist receptor mediating outside-in signal transduction events, and as an adhesion receptor able to respond to phosphorylation and other signaling events in order to modulate transendothelial migration (Newman, 1997). PECAM's tyrosine phosphorylation state has also recently been demonstrated to play a role in vasculogenesis in the murine conceptus (Pinter et al, 1997).

In support of PECAM's ability to act as an agonist receptor, PECAM-1 engagement or dimerization in T cells has been shown to increase their adherence to the \( \beta_1 \) integrin substrates VCAM-1 and fibronectin (Tanaka et al, 1992). Affinity modulation of \( \beta_2 \) integrins in response to PECAM-1 dimerization has also recently been demonstrated in natural killer cells (Berman et al, 1996). Upregulation of leukocyte integrin affinity, thereby facilitating interaction with endothelial cell counter-receptors, in turn, more than likely increases cytosolic transduction events, some of which phosphorylate PECAM-1 and otherwise modulate its affinity. Lu et al (1996), report that PECAM-1 is dephosphorylated by the engagement of integrins on cultured endothelial cells while others (Jackson et al, 1996) report increased tyrosine phosphorylation of PECAM-1 in aggregating human platelets upon integrin interaction. The reasons for such disparate observations remain unclear but it is likely that the relative balance of kinase and phosphatase activity plays a large role in determining the phosphorylation state of PECAM-1 and most likely other cellular receptors as well. Decreased PECAM-1 staining at 5 hours post culture may therefore result from cellular events initially transduced through PECAM-1 signaling itself. The recovery of PECAM-1 expression in day 4 cultures is interesting in that expression resembles a band-like pattern, similar in appearance to the dense peripheral band
formed by F-actin in quiescent, confluent endothelial cell monolayers in culture (Wong and Gotlieb, 1986). In contrast to tissue culture however, there are many more cell layers in the organ culture model, with superimposed remodeling occurring relatively early over the culture period. Hence, cell boundaries may overlap, causing the appearance of thickening around the cell periphery when cultures are stained for PECAM-1. This may indeed be the case at branch points especially, where more dramatic remodeling events were observed to be occurring.
CHAPTER 3

Characterization of Cells Derived from Specific Regions within the Porcine Thoracic Aorta

3.1 Introduction

The ability of the vessel endothelium to repair itself rapidly following injury is important in the maintenance of its barrier function (Gotlieb, 1992) and in limiting the development and progression of atherosclerosis (Ross, 1986). In situ, the differentiated phenotype of endothelial cells in blood vessels is maintained, at least in part, by their interaction with extracellular matrix and by autocrine and paracrine factors (Pauly et al, 1992). When injured in vivo, endothelial cells are known to dedifferentiate to a highly mobile, proliferative state to allow new blood vessel formation, to repair damage, and to sustain other functions of the vasculature (Piero et al, 1995). Changes in phenotype may also occur in cultured endothelial cells.

Our laboratory has previously shown that the disruption of vascular integrity in vitro promotes cytoskeletal reorganization, cell spreading, migration, and proliferation to effect rapid and efficient wound repair (Gotlieb et al, 1984; Wong and Gotlieb, 1988; Coomber and Gotlieb, 1990; Ettenson and Gotlieb, 1994; Lee and Gotlieb, 1996). Cell migration followed by cell proliferation is critical to the initiation of endothelial wound repair in both large and small vessels. Once considered to be an event secondary to and dependent upon cell migration, cell proliferation is now known to be able to act as a compensatory primary mechanism of wound healing should cell migration become impaired (Ettenson and Gotlieb, 1994). Inhibition of cell proliferation interestingly, does not impair the efficiency of cell migration to effect wound healing in porcine endothelial cells (Ettenson and Gotlieb, 1994).
The following experiments represent further *in vitro* attempts to define differences in the properties of intimal cells residing at the flow divider and those on the upstream wall of the intercostal branch compared to unbranched regions. Based on data collected from organ culture experiments which showed a lesser degree of proliferation in endothelial cells harvested from the wall opposite the flow diver, endothelial cells were isolated from 4 regions of the porcine thoracic aorta and used to test the hypothesis that the response to cellular injury between regions would again be lower in cells collected from this region, compared with cells from the other 3 locations of the vessel. Linear denuding wound studies were analyzed over 48 hours in terms of their ability to effect closure of the wound. The contribution of cell proliferation to wound closure was studied indirectly in growth curve measurements of plated cells.

Data collected from wound repair time course studies and growth curve experiments suggests that the capacity of cells harvested from branch regions to repair wounds may not be as rapid as cells from unbranched regions. This may be due, in part, to dissimilar rates of cell motility related to cytoskeletal remodeling. To address this issue, the cytoskeleton of wounded endothelial cultures at various time points post-injury were examined in terms of F-actin and α-tubulin distribution, both of which are very important to endothelial repair (Wong and Gotlieb, 1984).

3.2 Materials and Methods

3.2.1 Endothelial Cell Cultures

In order to harvest endothelial cells from the 4 selected regions (fd, ofd, uboi, ubai) of the thoracic aorta, aortas were collected and prepared for cutting into
segments as previously described but gently scraped instead using a no. 22 scalpel blade. Each of the first three intercostals of 3 aortas were scraped along with unbranched areas opposite to and above the intercostals. Each scrape was washed from the blades into separate wells on 24-welled Falcon trays with medium M199 containing 5% FBS and 2% antibiotics using a 10-ml syringe and 21 gauge needle. Cell cultures were fed twice weekly thereafter until cultures were established. They were then plated into 35-mm dishes and grown to confluence at which time, cells harvested from the same region for each of the 3 aortas were pooled and then plated into 100-mm dishes. Confluent cells were then passaged once more into 35-mm dishes in preparation for wounding. Analyses of wound repair kinetics and growth curves were performed on harvested endothelial cells between passages 3 and 5.

Immunofluorescent diI-conjugated acetylated low-density lipoprotein is taken up by macrophages and smooth muscle cells but much more readily by endothelial cells in culture. Acetylated-LDL (Biomedical Technologies Inc., Stoughton, MA) was incubated with harvested cells for 2 hours at 5μg/ml in culture media to demonstrate cells were endothelial in nature. Collagenase-harvested porcine thoracic endothelial and smooth muscle cells served as controls. Immunofluorescent rhodamine phalloidin (Molecular Probes, Eugene, Oregon) staining to demonstrate F-actin and α-SMC actin staining of confluent cell monolayers were also performed on harvested and control cells to confirm their endothelial characteristics.

3.2.2 Wound Closure Experiments

Cells were grown to confluency on coverslips in 35-mm dishes at which time a linear wound measuring approximately 2-2.5 mm was made down the center of the
coverslip using a plastic spatula. An orienting scratch and 3 guide scratches were made on the periphery of the coverslip with a diamond-tipped pencil then wounded cultures were rinsed with warm PBS and fed with fresh media M199 containing 5% FBS and 2% antibiotics. Cells were not fed for the duration of the experiment. The distance between the 2 edges of the wounds were measured under phase microscopy using a 1 mm micrometer at each of 3 scratch marks for each of 3 dishes per location at the time of wounding, and following 24 and 48 hours in culture. Collagenase-harvested endothelial cells were used as controls. Results shown were performed in triplicate and analyzed using a factorial ANOVA to determine significance. If significance was observed, then a Fisher’s PLSD post hoc test was run. (Statsview 4.5, Power Mactintosh 7200/90).

3.2.3 Growth Curves

Growth curves over a 7 day period were carried out for pooled early passage cells (fd, ofd, uboi, & ubai). Confluent cells from the 4 locations scraped were washed twice with warmed PBS, then 2 mls of trypsin was added. Once cells became detached from the culture plate, 3 mls of media was added to neutralize the trypsin. Cells were washed off by gently pipetting the media/trypsin around the dish. A 0.5 ml sample was drawn for counting then the remaining 4.5 mls were collected and centrifuged. The media/trypsin was then aspirated, leaving less than 0.5 ml in each tube. Fresh media was added to each tube in proportions that would achieve a final concentration of 15,000 cells per 300 µl aliquot and cell pellets were resuspended. Ninety-six 35-mm dishes without coverslips (24 per location) were plated with 15,000 cells initially. A total of 3 dishes per location for each of 8 time points (5hrs, 1 day, 2,
Cells were fed every 2 days with standard media M199 containing 5% fetal bovine serum and 2% streptomycin/fungizone.

Three dishes were selected at random from each of the 4 locations at each time point. They were washed twice with warm PBS and 1 ml of warmed trypsin was then added to each dish and incubated at 37°C for 2 minutes. Cells were dispersed well by pipetting, and a 0.5-ml aliquot was withdrawn for counting on a coulter counter (Coulter Industries Inc., model 7163Zf, Hialeah, Florida). Each sample was counted 3 times and an average value taken. Results shown represent experiments performed in triplicate and analyzed using a factorial ANOVA to determine significance.

3.2.4 F-actin and Tubulin Expression in Wounded Cultures

Wounded cells on glass coverslips at 0, 24 and 48 hours post-wounding were rinsed with warm PBS, then fixed for 20 minutes with warm 3% paraformaldehyde, after which time they were rinsed and permeabilized with 1% triton for 3 minutes. Cells were double-labeled using monoclonal mouse anti-α-tubulin (Sigma Chemical Co., St. Louis, MO) applied in a 1:500 dilution for 1 hour, followed by a goat anti-mouse FITC-conjugated secondary (1:100), and rhodamine phalloidin (1:25, 30 minutes) to demonstrate microtubules and F-actin respectively. Coverslips were inverted and mounted in 1:1 glycerol/PBS and cells from the leading edge of wounds were analyzed under laser scanning confocal microscopy. Cells at the leading edge were scanned to depths of 2.5 μm.
3.3 Results

3.3.1 Identification of Harvested Cells

Under phase contrast microscopy, all cell scrapes were observed to grow as islands of cells, typical of endothelial cell growth in culture (fig. 3.1A). They attained cobblestone morphology upon reaching confluency (fig. 3.1B) unlike smooth muscle cell controls (fig. 3.1C). DiI-conjugated acetylated LDL uptake was observed to occur to the same extent in control endothelial cells (fig. 3.2A) and cell scrapes (fig. 3.2C through F) at 2 hours, but to a much lesser degree in control smooth muscle cells (fig. 3.2B). F-actin staining showed the presence of the dense peripheral band in control endothelial cells and cell scrapes but not in control smooth muscle cells. Alternatively, α-SMC actin staining was only observed in control SMC cultures (not shown).

3.3.2 Wound Closure

Percent wound closure refers to the degree to which wounds have closed by the time point measured, with 100% meaning that both sides of the wound edge are touching. The extent of wound closure for pooled early passage endothelial cells is shown in figure 3.3. Significantly lower wound closure was measured in cells derived from either side of the intercostal branches (fig. 3.5A), compared to cells from unbranched areas (fig. 3.5B) and control endothelial cells (fig. 3.5). Wound edges of cells harvested from the flow divider and wall opposite the flow divider closed by 22 ± .084 μm and 22 ± 1.3 μm respectively (n=3) versus control endothelial cells (30 ± 2.2 μm) at 24 hours. By 48 hours, this difference was even more pronounced, with pooled cells from branch regions again showing a significantly decreased degree of wound closure (fd: 48 ± 3.4 μm; ofd: 47± 3.6 μm) compared to control endothelial cells (ec:...
61 ± 3.4 μm). Wound closure in cells derived from unbranched regions was comparable to control endothelial cells at both 24 and 48 hours.

3.3.3 Growth Curves

Cell culture growth activity over 7 days for pooled early passage cells are shown in figure 3.4. Plating efficiency averaged 99% at 5 hours and cell numbers grew exponentially from day 2 (30,211 ± 3,577; avg. of 4) and thereafter through day 4 (126,294 ± 16,092; avg. of 4) after which time, proliferation slowed by 50% presumably due to cell quiescence as monolayers approached confluency. No differences were observed between cells on any given day over 7 days culture (final count: 275,140 ± 24,354; avg. of 4) suggesting that differential rates of cell proliferation may not account for the differences in wound closure observed in earlier experiments.

3.3.4 F-actin and α-Tubulin Expression

At the time of wounding, a dense peripheral band of actin microfilaments was noted around cell borders and cells contained few central microfilament bundles in endothelial cells from controls (fig. 3.6). Microtubules spanned the entire area of cells in a fibril-like network in cells along the wound edge and centrosomes were observed to be located randomly around the cell nucleus. Cells from branched and unbranched areas were similar in terms of morphology, F-actin, and α-tubulin distribution (figs. 3.6A (fd), and 3.6B (uboi) respectively).

Loss of the dense peripheral band in cells along the wound edge was observed in all cells at 24 hours post wounding along with prominent central microfilaments
oriented perpendicular to the wound edge in elongated cells. Cells appeared larger than at the time of wounding, and microtubule distribution, which again spanned the length of cells and extended into lamellipodial extrusions, was more condensed. Centrosomes in cells at the leading edge of the wound were oriented towards the wound edge. At 48 hours, cells were similar to those at 24 hours in terms of actin microfilament orientation though slightly smaller. Microtubule distribution resembled that seen at 24 hours. Specific colocalization between microtubules and actin microfilaments was not observed in lamellipodium or within the perinuclear region of the cells at any time point.
Chapter 3

Figures
fig. 3.1 -- Phase contrast photomicrographs showing cell morphology of: A, flow divider-harvested endothelial cells at 4 days following plating. B, control endothelial cells at confluence. C, control smooth muscle cells. Mag. 10x, Bar = 10 μm
**Fig. 3.2** — DiI-conjugated acetylated low density lipoprotein uptake by cell scrapes to determine their endothelial characteristics. A & B, Photomicrographs of collagenase-harvested endothelial and smooth muscle cells respectively, stained for acetylated-LDL. C & D, Photomicrographs of flow divider and opposite the flow divider-harvested endothelial cells respectively, labeled with acetylated-LDL. E & F, Photomicrographs of cells scraped from unbranched tissue opposite the intercostals (uboi) and unbranched tissue above the intercostals (ubai) respectively. Mag. 60x, Bar = 25 μm.
**fig. 3.3** — Extent of wound closure for early passage endothelial cells pooled from the scrapes of 3 aortas.

* *p* < .05 opposite flow divider versus unbranched areas and control endothelial cells
** *p* < .05 flow divider versus unbranched areas and control endothelial cells

c: collagenase-harvested endothelial cell control

*n* = 3 experiments
Endothelial Cell Wound Closure over 48 hours

* p<.05
fig. 3.4 — Growth curve for early passage endothelial cells pooled from the scrapes of 3 aortas.

n = 3 experiments
Endothelial Cell-Scrapes: Growth over 7 days Culture

cells per dish

days in culture
fig. 3.5 — Phase contrast photomicrographs of wounded control endothelial cell cultures showing the extent of cell migration of one side of the wound. A, time zero following wounding. B, 24 hours post-wounding. C, 48 hours post-wounding. Mag. 10x, Bar = 10μm
fig. 3.5A -- Phase contrast photomicrographs of wounded branch-harvested endothelial cell cultures showing the extent of cell migration of one side of the wound. A, C, E, times zero, 24 and 48 hours post wounding of flow divider-harvested (fd) cells. B, D, F, times zero, 24 and 48 hours post wounding of opposite the flow divider-harvested (ofd) cells. Mag. 10x, Bar = 10μm
**fig. 3.5B** -- Phase contrast photomicrographs of wounded endothelial cell cultures harvested from unbranched regions showing the extent of cell migration of one side of the wound. A, C, E, times zero, 24 and 48 hours post wounding of opposite the intercostal-harvested (uboi) cells. B, D, F, times zero, 24 and 48 hours post wounding of above the intercostal-harvested (ubai) cells. Mag. 10x, Bar = 10μm
**fig. 3.6** — Confocal photomicrographs of wounded control endothelial cell cultures stained for F-actin and α-tubulin. A, C, E, times zero, 24 and 48 hours post wounding of F-actin stained cells. B, D, F, times zero, 24 and 48 hours post wounding of cells showing α-tubulin expression. Mag. 60x, Bar = 25μm
fig. 3.6A — Confocal photomicrographs of wounded flow divider-harvested (fd) cells stained for F-actin and tubulin. A, C, E, times zero, 24 and 48 hours post wounding of F-actin stained cells. B, D, F, times zero, 24 and 48 hours post wounding of tubulin-stained cells. Mag. 60x, Bar = 25μm
**fig. 3.6B** -- Confocal photomicrographs of wounded cells harvested from unbranched aorta (uboi) stained for F-actin and tubulin. A, C, E, times zero, 24 and 48 hours post wounding of F-actin stained cells. B, D, F, times zero, 24 and 48 hours post wounding of tubulin-stained cells. Mag. 60x, Bar = 25 µm
3.4 Discussion

Analysis of wound healing indices between endothelial cells harvested from different regions of the porcine thoracic aorta indicated that cells from the branch region showed a lesser capacity to repair wounds than did cells harvested from unbranched aorta or control endothelial cells. At present, the mechanism for this difference is undetermined however, it is important to recognize that the repair of wounds in vitro results from a combination of both migration and proliferation.

The integrin family of cell surface receptors are well known for their role in mediating cellular adhesion to extracellular matrix proteins but are also thought to be directly involved in migration through the stimulation of secondary messengers such as cytosolic free calcium and intracellular pH (Jaconi et al, 1991; Ingber et al, 1990). Schwartz and colleagues (1993) have since demonstrated that endothelial adhesion to matrix triggers an influx of extracellular calcium via voltage-independent calcium channels which has the potential to directly or indirectly regulate cytoskeletal structure and consequent cell motility. Cytoskeletal responses to integrin ligation may also include the local accumulation of vinculin, talin, α-actinin and F-actin along the cell membrane (Lewis and Schwartz, 1995).

Postinjury elevations in magnesium concentration with concomitantly decreased calcium have also been shown to occur in early rat and porcine wounds and may affect both migration and proliferation (Grzesiak and Pierschbacher, 1995). Elevated magnesium, as a cofactor for adenylate cyclase and cAMP-dependent phosphodiesterase, may increase the rate of cell migration by altering cAMP levels (Banai et al, 1990). Decreases in calcium meanwhile, may enhance migration by
destabilizing other Ca^{2+}-dependent complexes secondary to the integrins, some of which are responsible for cell-cell adhesion such as E-cadherin (Clark, 1985). Certain of the importance of divalent cation concentrations to integrin function and migration, it is possible that wounds in endothelial cell cultures were exposed to appreciably different concentrations of Mg^{2+} and Ca^{2+} over 24 and 48 hour time points.

Analysis of F-actin microfilament distribution and the associated tubulin network in actively migrating endothelial cells at the wound edge did not reveal obvious distinctions between harvested cells. Loss of the dense peripheral band and the appearance of central stress fibers oriented perpendicular to the wound edge, conditions which are characteristic to porcine endothelial cell migration, appeared to be similar amongst cells at both 24 and 48 hours. It is possible that differences in cytoskeletal reorganization might have occurred; however, the qualitative analysis of the cytoskeleton as well as the presence of only modest migration differences in the quantitative data may preclude detection of subtle cytoskeletal differences. These observations, in addition to wound closure data showing significant closure by 24 hours in unbranched and control cells but not cells harvested from branch regions, suggests that events of interest may be occurring in the first 24 hours following injury. Lee and colleagues (1996), have recently characterized the changes which occur early in wounded porcine endothelial cells and note morphological changes as early as 30 minutes post-wounding through 16 hours when a majority of cells along the wound edge exhibited a perpendicular microfilament orientation conducive to migration into the wound. Further cytoskeletal analysis of harvested cells at earlier times following wounding is necessary. One plausible reason to account for the relatively decreased
capacity of branch-harvested cells to migrate may be a delay in growth factor-influenced cytoskeletal reorganization. A number of growth factors released at various times during wound healing such as platelet derived growth factor (Bonin, 1994), epidermal growth factor (D’Amore, 1992; D’Amore, 1993), the interleukins (Ku, 1992), and basic fibroblast growth factor (Edelman et al, 1992; Bikfalvi et al, 1995) play as yet undetermined roles in the stimulation/inhibition of cell proliferation and migration. Basic fibroblast growth factor has been suggested to play a role in effecting the transition of central actin microfilaments in cells from a parallel to a perpendicular orientation (unpublished observations). Cation-dependent protein phosphorylation and downstream activation of protein kinase C has also been shown to result in the enhancement of cytoskeletal reorganization (Camussi et al, 1995).

As mentioned previously, differences in the rates of wound closure observed between cells from branch regions and cells from unbranched regions may also reflect differences in proliferation. While wounding does induce the division of endothelial cells lining the wound edge (Ryan et al, 1982), growth curve data suggests that such differences were not significant between the cells studied. However, it is arguable whether the initial plating of cells for growth curve measurements is a proliferative stimulus comparable to that effected in cells at the leading edge by mechanical wounding. Studies conducted by Pepper and colleagues (1989) in bovine microvascular cells, have shown that intercellular communication in mechanically wounded cultures is significantly greater at 24 hours compared to that observed in sparse and preconfluent cell monolayers. Although it was also determined that increased junctional communication did not correlate with BME cell proliferation, the
possibility remains, that in our model, such wounding may affect the proliferation of endothelial cells from unbranched areas of the aorta to a greater extent than cells from the intercostal branch region.
CHAPTER 4

General Discussion

4.1 General Discussion

Organ culture studies to date have identified the fact that there exist different reactions to tissue injury in endothelial cells from different regions of the porcine thoracic aorta. Such reactions are likely due to tissue remodeling in response to the removal of flow stimuli. However, the fact that a single injurious stimulus has the potential to affect endothelial cells over relatively short distances to such disparate extents implies that certain populations of endothelial cells in the porcine thoracic aorta may be more prone to injury and subsequent dysfunction.

Attempts at harvesting endothelial cells from the regions of the thoracic aorta under study have largely been successful inasmuch as they have proven that the cells harvested are indeed endothelial in nature. Whether they retain the same characteristics in tissue culture that they maintain in organ culture remains to be determined. The simple fact that harvested cells no longer differ in terms of proliferation as measured by growth curve analysis serves to further the notion that cells are changing upon their isolation from the vessel wall. Furthermore, factors that may influence one model may do so in a reduced capacity in another. For instance, organ culture studies showed that the wall opposite the flow divider had a lesser capacity to proliferate and therefore to “repair” in response to organ culture injury. This was reaffirmed in tissue culture experiments however, the observation that cells from the flow divider also showed a decreased capacity for repair suggests that this region is far too complex to be characterized on the basis of one parameter.
Additionally, the release of growth factors such as basic fibroblast growth factor from injured endothelial cells may play a role in wound healing in tissue culture. It is highly unlikely however that it exerts any appreciable influence over the differences observed in organ culture. As such, interpretation of organ and tissue culture data should be analyzed with caution and in their respective contexts.

Whereas previous work in our laboratory has focused on the mechanisms of neointimal development, the emphasis of this work has been the comparison of the cellular response to non-specific injury, related to the removal of the arterial wall from the circulation, between different areas of the thoracic aorta. From these studies I hoped to discover changes that were constitutive in cells from different locations within the vessel that reflected differences in gene expression, ultimately inferring differences in cell phenotype. However, there is always the possibility that cells in culture lose constitutive functions rapidly while taking on new functions. In this respect, one limitation of the in vitro aortic organ culture model is that it is an artificial environment, one without flow or access to the host of immunological and physiological responses that would accompany any form of trauma to the vessel wall in vivo. Components of these systems may be added back to investigate both specific and synergistic actions; however, once removed from the host and placed into culture, each piece of tissue is effectively isolated, able only to react and interact with the components that comprise the tissue and nutritive media itself. Thus, findings in organ culture must be validated in in vivo conditions whenever possible.
4.2 Future Studies

The data presented herein is provocative in that it suggests heterogeneity of form and function within intimal cells of the vessel wall within microenvironments relatively close to one another. Questions regarding the mechanism whereby unbranched and control cells migrate faster than branch-harvested cells may be answered by analyzing cytoskeletal structure and organization at time points up to 16 hours.

If indeed different cell phenotypes in specific areas of the aorta coexist and remain in short term culture, then it is important to determine the relative proportion of such cells in harvested cultures. An indirect means of addressing this issue involves the cloning of individual cells from established cell cultures. Preliminary work in this area has in fact yielded intriguing results. Specific clones established through limiting dilution techniques were observed to differ in terms of cell morphology once confluence was reached but not before, some resembling the elongated cells observed at the leading edge of wounded endothelial cultures at 24 hours. All clones grew as islands similar to endothelial cells and methods used previously to identify endothelial characteristics in cell scrapes verified that cloned cells were endothelial. These results suggest the possibility that the relative proportions of specific types of cells in specific areas may influence the response of a particular region of the vessel to tissue injury. Further work in this area may include the measurement of wound closure and growth curve characteristics between clones from different regions of the vessel as well as clones established from the same region. Subtractive hybridization might then be employed to determine differences in mRNA expression between different clones, the
products of which may be responsible for differences in morphological and/or functional characteristics between clone populations.

The experiments outlined above are the first step towards gaining a firm mechanistic understanding of the molecules which govern the injury-response characteristics of individual cells. As new molecular techniques are developed and the efforts of science persist, the complexities of the body and its abilities to heal itself will gradually become unraveled. Studies as have begun herein may ultimately help to provide insight into the response of cells to injury at branch points as it relates to neointimal formation, the development of atherosclerosis-prone areas, and subsequent plaque growth in humans.
References


Bjorkerud S, Bjorkerud B: Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. Am J Path 1996, 149: 367-380


Caplan B, Schwartz C: Increased endothelial cell turnover in areas of in vivo Evans Blue uptake in the pig aorta. Atherosclerosis 1973, 17: 401-417


Coltera MD, Gown AM: PCNA/cyclin expression and BrdU uptake define different sub-populations in different cell lines. J Histochem Cytochem 1991, 39:23-30


D'Amore PA, Smith SR: Growth factor effects on cells of the vascular wall: a survey. Growth Factors 1993, 8: 61-75


Giachelli C, Bae N, Lombardi D, Majesky M, Schwartz S: Molecular cloning and characterization of 2B7, a rat mRNA which distinguishes smooth muscle cell phenotypes in vitro and is identical to osteopontin. Biochem and Biophys Res 1991, 177: 867-873


Gotlieb AI: The endothelial cytoskeleton: organization in normal and regenerating endothelium. Tox Path. 1990, 18: 603-617


Graf H: Endothelial control of cell migration and proliferation. Eur heart J. 1993, 14: 183-186


Kumar I, West D, Ager A: Heterogeneity in endothelial cells from large vessels and microvessels. Differentiation 1987, 36: 57-70


Langille L: Chronic effects of blood flow on the artery wall. Physical Forces and the Mammalian Cell 1993, Ch. 8: 249-274


Langille L, O'Donnell F: Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. Science 1986, 231: 405-407


Leak R: The cell cycle and regulation of cancer cell growth. Annals New York Acad Sci USA, 252-262


Ley K: Molecular mechanisms of leukocyte rolling and adhesion to the microvascular endothelium. Eur Heart J 1993, 14: 68-73


Reidy M: Neointimal Proliferation: The role of basic FGF on vascular smooth muscle cell proliferation. Throm and Haemo 1993, 70: 172-176


Schwenke D, Carew T: Quantification in vivo of increased LDL content and rate of LDL degradation in normal rabbit aorta occurring at sites susceptible to early atherosclerotic lesions. Circ Res 1988, 62: 699-710


Segal S: Cell-cell communication and blood flow control. Hypertension 1994, 23 (6) Pt. 2: 1115-1120


Shirotani M, Yui Y, Kawai C: Restenosis after coronary angioplasty: pathogenesis of neointimal thickening initiated by endothelial loss. Endothelium 1993, 1: 5-22


Stary HC: Macrophages, macrophage foam cells, and eccentric thickening in the coronary arteries of young children. Atherosclerosis 1987, 64: 91-108


Stewart R, Kashour T, Marsden P: Vascular endothelial platelet endothelial cell adhesion molecule-1 (PECAM-1) expression is decreased by TNF-α and IFN-γ. J Immunology 1996, 156: 1221-1228


Vaux D: Towards an understanding of the molecular mechanisms of physiological cell death. Proc Natl Acad Sci USA 1993, 90: 786-789

Vaux D, haeker G, Strasser A: An evolutionary perspective on apoptosis, cell 1994, 76: 777-781


Wright H: Mitosis patterns in aortic endothelium. Atherosclerosis 1972, 15: 93-100


