THE APOPTOTIC RESPONSE OF HUMAN
UMBILICAL VEIN ENDOTHELIAL CELLS
TO OSCILLATING SHEAR STRESS

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A thesis submitted in partial fulfilment
of the requirements for the degree of

MASTER OF DENTAL SCIENCE (Paediatric Dentistry)

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August 2001
DECLARATION

This thesis describes the work done in the Department of Oral Pathology and Oral Medicine at Westmead Centre for Oral Health, the Faculty of Dentistry in the University of Sydney between February 1999 and June 2001. The research is entirely my own and has not been submitted in whole or in part for a degree at this or any other university. Nor to my best knowledge does it contain any material published or written by another person except as acknowledged in the text.

Signature

Sumant Gue
ACKNOWLEDGEMENTS

Completion of this thesis would not be possible without the advice and help of many individuals.

First of all, my greatest thanks go to my supervisor Dr. Hans Zoellner for his support and advice throughout the past three years. His guidance, dedication, encouragement, and technical help were a tremendous help and greatly appreciated. I must also make special mention and thank him for his enthusiasm during the difficult times, and his “dry sense of humour”, which always managed to put a smile on my face. I am also indebted for his help in the preparation of this thesis.

Thanks to Prof. D.M. Walker for allowing me to join the Department of Oral Pathology and Oral Medicine and for his help and advice with my research and other areas of pathology in Paediatrics. Thanks also to Dr. Angus Cameron and Associate Professor Richard Widmer for their help and support during the last three years.

I owe thanks to all my colleagues in the Department of Oral Pathology and Oral Medicine for their help and encouragement during the last three years. Special thanks go to Ms. Catherine Emmanuel, who has been incredibly supportive and patient during the past 18 months and whose encouragement has been second to
none. Thanks also go to Ms. Janice Matthews for her technical support, Dr. William Xu, Ms. Christine Donald and Dr. Munira Xaymardan for their help and expertise and Ms. Toni Chapman for assistance with FACS Analysis.

A special thanks to the staff of the Electron Microscopy Unit of Westmead Hospital, whose generosity with time and expertise was invaluable. My deepest thanks go to Dr. Ross Bodel, Ms. Levina Dear, Ms. Gail Versace, Ms. Carol Rebison and Ms. Mary Simonian.

Thanks also go to the New South Wales Branch of the Australian Dental Association for their funding towards research in Oral Pathology

Thanks to my friends, especially Dr. Erin Mahoney who has been exceptionally supportive and helpful during the last three years. Thanks also to my parents for their help and encouragement.

Lastly, and most importantly, my deepest and dearest thanks go to my wife, Mandy, who has been truly amazing during the last three years. Her support, encouragement and love have made this work possible.
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<td>Ang-1</td>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Angiopoietin-2</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis Activating Factor-1</td>
</tr>
<tr>
<td>API</td>
<td>Apoptosis Protection Index</td>
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<tr>
<td>BAEC</td>
<td>Bovine Aortic Endothelial Cell</td>
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<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Caspase Activated DNase</td>
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<tr>
<td>CDD</td>
<td>Caspase Dependent Deoxyribonuclease</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CM</td>
<td>Complete Medium</td>
</tr>
<tr>
<td>CSA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>DAB</td>
<td>Di-amino Tetrabenzohydrochloride</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
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<tr>
<td>ECGS</td>
<td>Endothelial Cell Growth Supplement</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Scanning</td>
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<tr>
<td>FADD</td>
<td>Fas-Associated Death Domain</td>
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<tr>
<td>Abbr.</td>
<td>Full Form</td>
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<tr>
<td>FCS</td>
<td>Iron Fortified Foetal Bovine Calf Serum</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
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<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
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<tr>
<td>ICE</td>
<td>Interleukin-β Converting Enzyme</td>
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<td>IL-1</td>
<td>Interleukin-1</td>
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<tr>
<td>IL-2</td>
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<td>IL-3</td>
<td>Interleukin-3</td>
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<td>INF-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>Int. β2</td>
<td>Integrin β2</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>c-Jun-N-terminal Kinase/Stress Activated Protein Kinase</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>Oxidized Low Density Lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M199</td>
<td>Medium 199</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP-ribose) Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed Cell Death</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>Prostaglandin I2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PLA</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear Leukocytes</td>
</tr>
<tr>
<td>RER</td>
<td>Rough Endoplasmic Reticulum</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal Deoxynucleotidyl Tranferase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA Buffer</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris Hydrochloride</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated Deoxy-Uridine Nick End Labeling</td>
</tr>
<tr>
<td>UEA-1</td>
<td><em>Ulex Europaeus</em> Lectin-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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SUMMARY

Programmed capillary regression is a frequent occurrence in growth and development. Despite the prevalence of this event, and the potential therapeutic value of an understanding of its mechanism, capillary regression is only understood at a rudimentary level. It is known that angiogenesis and vascular restructuring involve both proliferation and apoptosis of vascular endothelial cells (ECs). Excess cells are removed by apoptosis which is a highly regulated form of cell death frequently requiring protein synthesis. It is known that excess blood vessels are removed by apoptosis of ECs. However the full complement of signals and factors responsible for this have not been fully defined. It is thought that the factors which regulate EC apoptosis, are also responsible for microvascular remodelling, and in part contribute to atherosclerosis.

Earlier work suggested that chemical factors in blood regulate EC apoptosis, so that only those vessels with sufficient blood flow to supply the necessary anti-apoptotic factors survive.

The question arises as to how small blood vessels acquire their shapes, which seem usually to be minimally tortuous. This thesis investigates the possibility that oscillating flow as might be generated in excessively tortuous vessels may affect EC apoptosis.
Experiments were performed using human umbilical vein ECs (HUVEC), which were stimulated with oscillating shear stress generated using a rocking platform. One of three different media were used; serum (20%), human serum albumin (HSA) (4%) or medium 199 (M199) alone, suggested as representing "near proliferative", "structurally stable" or "degenerative" biological setting for ECs respectively.

Stimulation of HUVEC with oscillating shear stress produced morphological changes in the cells. Quantitation of cell death was achieved through direct cell counts and revealed that oscillating shear stress significantly reduced cell survival. DNA gel electrophoresis, transmission electron microscopy and FACS analysis indicated that oscillating shear stress induced cell death was due to apoptosis.

These data indicate a previously unreported pro-apoptotic effect of oscillating shear stress for ECs. This response may have possible relevance to capillary remodelling and atherosclerosis.
Chapter I. LITERATURE REVIEW

I.1 The Microvascular System

I.1.1. Introduction to the Microcirculation

The microvascular system is a complex network of minute blood vessels that are specifically adapted to optimise the efficiency of metabolic exchange between the tissues and circulating blood. No single cell within a tissue can be further from an exchange vessel of the microvasculature than the efficient range of the diffusion of the metabolites (Folkman, 1974a;b). There is enormous complexity of the microvascular network, which exists as a result of various physiological adaptations required in living organisms. Some tissues, especially those where metabolic demand may fluctuate considerably, such as skeletal muscles, contain many more exchange vessels within the microvascular network than are necessary to support basal function (Garbett and Gibbins, 1980). The flow of blood throughout the network is constantly modulated and directed by contractile sphincters within the precapillary arterioles such that the rate and direction of blood flow varies frequently. In response to increased tissue function and greater cellular demand, the average proportion of perfused vessels, volume and the rate of blood flow are appropriately elevated. In this way the control of blood flow within the microvascular network is subservient to local metabolic demand.

Literature Review. I. 1
Vascular ECs form the innermost lining of the cardiovascular system and play an essential role in the formation of blood vessels. ECs establish a monolayer to define a critical surface for metabolic exchange of nutrients, waste products, hormones, proteins, and cells (Cliff, 1976). In addition ECs mediate many physiological and pathological processes by expressing proteins and factors which function as vasodilators, vasoconstrictors, growth factors, growth inhibitors, adhesion molecules and chemotactants (Augustin, 1995; Azmi et al., 1984; Bombeli et al., 1997; 1999; Dimmeler et al., 1997a;b;c; 1998a;b; Oliver et al., 2000).

Many of the early studies of microvascular endothelial biology were performed by observing blood vessels in living animals. This was achieved using whole mounts of thin membranous tissues in-vivo (Chambers and Zweifach 1940; Colnheim 1889). Advancements from this technique involved using more elaborate tissue chamber techniques (Clark and Clark, 1932a;b). This approach provided a wealth of information regarding physical aspects of the behaviour of blood vessels, particularly with regard to changes occurring during acute inflammation. Histological studies were also performed to more clearly examine the phenomena seen in vivo (Majno et al., 1969). However, this approach did not permit the study of ECs in isolation. The advent of tissue culturing techniques, and particularly with the development of methods for the isolation and culture of ECs, has more recently confirmed and characterised in vitro observations that were made in vivo.
Research from the late 1980's and early 1990's reflected the focus of much of this work. The interaction of ECs with the coagulation and fibrinolytic pathways has been extensively studied, with a view to understanding atherogenesis and thrombosis (Rodgers, 1988). The vasoactive effect of EC products such as endothelin, endothelium-derived relaxing factor (NO), and at least one arachidonic acid derivative, have illustrated the complex interactions between ECs and the surrounding smooth muscle cells, indicating ECs have an important function in regulating blood flow through tissues (Furchgott and Vanhoutte 1989).

Of particular relevance to the work of this thesis are studies of the factors involved in formation of the microvascular system, and the pathogenesis of atherosclerosis. One of the factors investigated in the pathogenesis of atherosclerosis is the effect of hemodynamic forces upon ECs.

1.1.2 Introduction to the Formation of Blood Vessels

Tissue regeneration of wound repair, embryogenesis, and the cyclical proliferation of the endometrium in preparation for implantation of the fertilized egg, are strictly dependent on the rapid growth of new capillary blood vessels, a process termed "angiogenesis" (Folkman and Cotran 1976; Folkman 1985a;b; Folkman and Klagsbrun 1987). In adult vertebrates, capillary ECs divide relatively infrequently. Turnover rates for ECs are typically in the order of several months or years (Denekamp 1984; Engerman et al., 1967; Tannock and Hayashi, 1988).
1972). However, in response to the release of pro-angiogenic mediators, often sequestered in the ECM, ECs lining venules systematically degrade their basement membrane and proximal ECM, migrate directionally, divide, and organise into new functional capillaries, all within a matter of days. This dramatic amplification of the microvasculature is followed by remodelling of seemingly excess vessels which are removed.

As shown in Table I.1, angiogenesis is a feature of a number of physiological processes. In contrast, the aetiology and pathogenesis of a much larger and increasingly expanding number of pathological conditions (Table II.2) have been shown to be a consequence of an angiogenic response that is persistent either due to the overproduction of normal or aberrant forms of angiogenic mediators, or due to a relative deficiency in inhibitors of this process.

I.1.3 Development of Blood Vessels During Embryogenesis

Blood vessel formation occurs during embryonic development via two distinct mechanisms, vasculogenesis and angiogenesis. Vasculogenesis is defined as de novo formation of ECs from blood islands which differentiate from splanchnopleuric mesoderm very early in embryogenesis (Dieteren-Lievre and Pandanaud 1992; Gonzalez-Crussi 1971; Krah 1994; Pandanaud 1987; Poole and Coffin 1989; Risau et al., 1988 Risau 1991a;b;). In contrast angiogenesis is the sprouting and budding of ECs from pre-existing vessels, usually the post-capillary and small terminal venules of the microvasculature. It is a developmental process

*Literature Review. I. 4*
that occurs during embryogenesis and is down-regulated in the healthy adult. After initial differentiation of stem cells into ECs and their assembly into endothelial cell-lined channels (vasculogenesis), the embryogenic vasculature further develops via sprouting of new channels from pre-existing vessels (angiogenesis) (Carmeliet and Collen, 1997).

A further way in which new vessels may form is through inter-susception (Patan et al., 1993). In this form of angiogenesis, ECs project into the vascular lumen to reach the opposite side of the vessel and establish an area of contact. The subsequent perforation of this area by invading fibroblasts and pericytes produces a pillar-like structure, which with further remodelling defines new vascular channels. Progressive remodelling of these new vascular channels results in an expanded mature vascular network (Burri and Tarek, 1990; Caduff et al. 1986; Patan et al. 1993).

Vasculogenesis is initiated once the embryo attains a size in which simple diffusion is no longer able to satisfy the nutritional requirements of a rapidly expanding cell population of increasing morphological complexity. Within the course of two to three days, during the third week of development, the embryo is transformed from an avascular organism to one in which virtually every tissue is populated by blood vessels. Angiogenic amplification is the primary method in definitive vessel formation within the embryo and is the principle mechanism by
which new capillary blood vessels form in adult organisms (Folkman and Shing, 1992).

Finally, increased tortuosity and dilation of blood vessels also occurs in some chronic inflammatory lesions and the increased tissue vascularity resulting from these remodelling events has been described by some authors as a form of angiogenesis (Creamer and Barker, 1995; Jackson et al., 1997; Thurston et al., 1998; Zoellner and Hunter, 1991; 1994).

I.1.4 Angiogenesis in Adults

Much of the early understanding of angiogenesis in adults resulted from pioneering studies on neovascularization in wound healing (Clark, 1918, 1939; Sandison, 1931). Clark (1918, 1939) describes in detail the growth of solid cords of endothelium from the convex side of curved vessels, which continue to grow and anastomose with other endothelial cords or with pre-existing vessels. The new capillaries developed as solid cords of ECs that underwent canalisation rather than as patent tubes. Cliff (1963) expanded Clark’s work by using time-lapsed microscopy. As soon as the lumen of the new vessel and the rest of the vasculature were established, the pulsating vessels filled with red blood cells. The new vessels were unusually fragile and permeable, irregularly dilated and tortuous. Within 48 hours after the appearance of new capillaries, maturation began, and some of the apparently redundant vessels regressed.
These early studies were subsequently confirmed in many laboratories using other model systems, such as the chick chorioallantoic membrane and rodent cornea. Further research also lead to identification of mediator systems that participate in the angiogenic response (Folkman and Haudenschild, 1980).

1.1.5 The Angiogenic Cascade

The target vessels for angiogenic factors are the post capillary venules and small terminal venules. These small calibre vessels consist of flattened ECs that lie upon a basal lamina, surrounded by an interrupted layer of pericytes and smooth muscle cells, and are invested in an ECM. One of the steps in the angiogenic response involves the disruption of local contacts between adjacent ECs, pericytes, and smooth muscle cells (Ausprunk and Folkman, 1977; Phillips et al., 1991; Sims, 1986). The ECs at this stage are 'primed' for the subsequent steps of migration and proliferation. The activated ECs have reorganised cytoskeletal elements and have altered expression of cell surface adhesion molecules, such as integrins, members of the immunoglobulin supergene family, selectins, and components of ECM (Brooks, 1994a;b; Gamble et al., 1993; Ingber et al., 1991; Ingber and Folkman, 1988; 1989; Madri et al., 1988; Nguyen et al., 1993). These activated ECs generate proteolytic enzymes that enable them to degrade their ECM and migrate away from the parent vessel (Rifkin et al., 1982). This is associated with the formation of capillary buds and induces the release of growth factors which facilitate angiogenesis.
During migration, EC morphology changes from the usual tubular structure to a more flat and elongated structure, but returns to a more tubular structure once new capillaries are established (Folkman and Shing, 1992). Attachment of ECs to the ECM and the overall EC shape appear to be important in permitting growth and tube formation (Meredith et. al., 1993). In vitro studies have suggested if these interactions are not available, the cells are unable to form vessels, resulting in cell death (Pollman et al., 1999a). Both fibronectin and fibroblasts have been suggested to be important in the formation of new capillary networks in wound healing (Ingber, 1990; Villaschi and Nicosia, 1994). The degradation of the basement membrane and surrounding connective tissue by ECs in response to angiogenic stimuli allows further proliferation, migration and re-aligning of ECs into new capillaries (Kalebic et al., 1981; Kraling et al., 1999; Satake et al., 1998).

There is also induction of EC-derived cytokines and growth factors, which play an important role in the later stages of the angiogenic response (Baird and Ling, 1985; Bauer et al., 1992; Gerritsen and Bloor, 1993; Liaw and Schwartz, 1993; Sarma et al., 1992; Vlodavski et al., 1987). The expression of these angiogenic mediators may be important in the control of EC growth and for elongation, orientation, and maturation as they organise into functioning vessels. Vessel maturation occurs with the re-establishment of the basement membrane and lumen formation. Anastomosis occurs if developing buds intersect with other growing buds or pre-existing vessels and fuse to form intact capillary loops.
(Gamble et al., 1993). Once formed, the capillaries stabilise and persist for as long as the metabolic demands of the tissues necessitate their presence. They may continue to persist as capillaries or differentiate into mature venules or arterioles, however, often the capillaries are only temporary and regress. The signals responsible for capillary regression are only recently being identified. There is increasing evidence that families of mediators capable of down-regulating angiogenesis may function by initiating a program of events that leads to apoptotic death of ECs (Brooks, 1994a,b; Claesson-Welsh, 1998; Lang et al., 1994; Re, 1994). The relationship between ECs and apoptosis and both angiogenesis and microvascular remodelling will be discussed later (Chapter I.4.).

I.1.6 Regulation of Angiogenesis.

Angiogenic mediators can be broadly divided into two groups: stimulators (Table I.3) and inhibitors (Table I.4) (Di Pietro and Polverini, 1993; Folkman and Klagsbrun, 1987; Hanahan and Folkman, 1996; Klagsbrun and D’Amore, 1991; Moses and Langer, 1991; Pollman, 1999a,b; Polverini, 1989; Risau, 1995). The majority of stimulatory molecules are proteins, many of which are growth factors that induce ECs to divide, migrate directionally towards the inducing stimulus, and differentiate into tubular structures. Most are secreted by a variety of cells, including ECs themselves, in response to exogenous and endogenous stimuli and are produced locally to function in an autocrine and, or paracrine manner. These mediators can stimulate angiogenesis directly by interacting with receptors on the endothelial cell surface, or indirectly by attracting and activating accessory cells.

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While the mediators responsible for inducing new capillary growth have been the subject of extensive investigation (Folkman and Klagsburn, 1987; Klagsburn and Folkman, 1990; Klagsbrun and D’Amore, 1991), only recently has attention focused on the mechanisms and mediators responsible for the timely down-regulation of angiogenesis (Brock, 1990; Cjaesson-Welsh, 1998; Hanahan and Folkman, 1996; Klagsbrun and D’Amore, 1991; Lang et al., 1994; Moses and Langer, 1991; Pollman, 1999a;b; Polverini et. al., 1994; Risau, 1995). A common property of these inhibitors is that almost all of them can influence the ability of cells to produce, interact with, or degrade ECM (Canfield et al., 1986; Ingber and Folkman, 1988; 1989; Madri et al., 1988; Maragoudakis et al., 1988). Alteration in the organisation and composition of the ECM have been shown to have a profound effect on the growth and function of ECs and in determining whether ECs will differentiate and organise into three-dimensional capillary networks (Maragoudakis et al., 1988).

Of the many angiogenic factors described, only vascular endothelial cell growth factor (VEGF) seems specific for ECs alone, while most other factors have a wide range of functions and target cells (Flamme et al., 1995; Holash et al., 1999). These regulatory factors play an important role in embryonic
development. This is in contrasts with the adult vertebrates where the same mediators have a much more restricted role in angiogenesis and an entirely different complement of mediators comes into play (Desmouliere et al., 1997; Polverini et al., 1977; Polverini, 1989; Sunderrkotter et al., 1991;1994;). These mediators will be discussed later (Chapter I.4.3. and I.4.4.).

I.1.7 Angiogenesis in Wound Healing

Wound healing is a physiological process that is entirely dependent upon the ingrowth of new capillary blood vessels. This biological process involves the cooperative interaction of a variety of cell types and mediator systems. Normal tissue repair requires that the cells and mediators of the immune system, the connective tissue, and vascular endothelium coordinate to effect the repair process. Although numerous cells are involved, monocyte-derived macrophages appear to play a central role in the process by phagocytosing wound debris, becoming activated, and secreting diffusible cytokines (Leibovich and Wiseman, 1988). Three major phases in wound healing are defined as the inflammatory, proliferative and maturation phases respectively.

I.1.7.1 The Inflammatory Phase of Wound Healing

This phase in initiated after initial tissue damage occurs. During this phase there has been capillary disruption and resultant blood loss, which is controlled by vasoconstriction and activation of the coagulation cascade (Mutsaers et al., 1997). Tissue injury also leads to exposure of subendothelial matrix components, which
activate platelets, leading to platelet aggregation, fibrin formation and establishment of a haemostatic plug (Leibovich and Ross, 1975). This consists of platelets, fibrin and adhesive glycoproteins, such as fibronectin, thrombospondin and von Willebrand Factor (Bonnefoy and Legrand, 2000). Despite the low tensile strength of this plug, it serves as a provisional matrix that allows the migration of inflammatory cells into the wound (Mutsaers et al., 1997). These cells are activated by both the complement and kinin cascades, as well as cytokines released from platelets and other cells (Hunt et al., 1984).

Early events of wound healing are well illustrated in an experimental model using guinea pigs by Leibovich and Ross (1975). Polymorphonuclear leukocytes (PMN) act as the first line of defence against bacterial contamination, however, after two days these cells are gradually replaced by macrophages. Macrophages are the predominant cell type by day 5 post-injury, and are thought to be responsible for coordinating much of the growth and tissue remodelling that occurs during the subsequent stages of wound healing.

I.1.7.2 The Proliferative Phase of Wound Healing

During the proliferative phase, growing capillaries provide nutrient support for regenerating tissues. Granulation tissue develops from the connective tissue surrounding the wound. Its main components are fibroblasts, small blood vessels and inflammatory cells in the newly deposited ECM. Activated macrophages or their culture supernatants have been shown to induce new

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capillary growth in vitro and angiogenesis in vivo (Folkman and Klagsburn, 1987; Folkman and Shing, 1992; Hunt et al., 1984; Polervini et al., 1977; Sunderkotter et al., 1991; 1994; Thakral et. al., 1979). The angiogenic factors and cytokines released by macrophages include; interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β) and epithelial growth factor (EGF). Cytokines attract fibroblasts and ECs into the blood clot (Greenhalgh, 1998). Fibroblasts synthesise collagen, as well as glycosaminoglycans. Other matrix components, such as proteoglycans are distributed within the collagen meshwork so that there is a progressive increase in wound tensile strength.

Specific adhesion molecules and integrins play a central role in the adherence of cells to the matrix and the migration of cells into the tissues. The expression of integrins is regulated by growth factors and ECM proteins (Ruoslhti and Engvall, 1997). Fibronectin is also important in aiding the migration of cells into the provisional matrix by the upregulation of fibronectin binding integrins in fibroblasts (Xu and Clark, 1996)

Towards the end of the proliferative phase, the wound space is filled with granulation tissue containing large numbers of fibroblasts, myofibroblasts and blood vessels. The strength of the wound has also increased due to the deposition of collagen and ECM components.
I.1.7.3 The Maturation Phase of Wound Healing

In the maturation phase, the balance between deposition and degradation of collagen is shifted towards deposition (Greenhalgh, 1998). Type I collagen becomes predominant and this increases the strength of the wound as does remodelling and realigning of the collagen fibres (Latha et al., 1999; Mutsaer et al., 1997). There is a decrease in cellularity and vascularity of the wound, which together with increased collagen deposition results in the formation of scar tissue (Desmouliere et al., 1995; 1997). Many of the newly formed capillaries regress as tissue regeneration is complete. Recent evidence suggests that macrophages play a role in capillary regression through the production of inhibitors of EC growth (Besner and Klogsburn, 1991; Di Pietro and Polverini, 1993; Jaffe et al., 1987; Polverini, 1989).

Desmouliere et al., (1995) found that apoptosis was prominent during wound maturation and demonstrated that it is also the basis for the decrease of cellularity and vascularity during wound maturation. There is progressive maturation and remodelling of the scar tissue involving the degradation of wound collagen by proteinases and this process may occur over several years.

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I.2 Microvascular Remodelling

The microcirculation has tremendous structural plasticity (Pollman et al., 1999a;b). The determinants of capillary network architecture appear to be coupled to the metabolic demands of the tissues. During embryogenesis, segments of the circulation undergo involution in concert with changes in organogenesis (Flamme et al., 1995; Risau, 1995; Risau and Flamme, 1995). In the adult, physiological cyclical changes in organ structure, such as the mammary gland (Walker et al., 1989) and ovarian tissue (Augustin, 1995) are associated with dramatic changes in capillary network vascularity. These changes are presumed to also occur in tooth eruption and orthodontic tooth movement. Similarly, in pathological processes such as wound repair, there is a dynamic process of angiogenesis and capillary network regression (Desmouliere et al., 1987, 1995; Pollman et al., 1999a;b).

As mentioned earlier (I.1.2. – I.1.3), vasculogenesis, angiogenesis and microvascular remodelling are complex processes involving EC growth, migration, and differentiation (Hanahan and Folkman, 1996; Risau, 1995). Apoptosis appears to be important in this process, by mediating the removal of redundant excess vessels (Lang et al., 1994; Messon et al., 1996; Pollman et al., 1999a).

This has been well characterised in a number of biological settings including: involution of the mammary gland (Tatarczuch et al., 1997; Walker et
al., 1989), cyclic changes in ovarian tissue (Augustin, 1995), pressure atrophy of
the parotid gland (Walker and Gobe, 1987), and the maturation of scar tissue
(Desmouliere et al., 1995).

Remodelling of the vasculature also occurs in diseases, such as
hypertension and atherosclerosis, and is likely to be due to an altered balance
between EC survival, proliferation, and death (Gibbons and Dzau, 1994). Recent
reports have shown relatively high proportions of apoptotic cells, as measured by
terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling
(TUNEL) in both primary and restenotic atherosclerotic plaques (Isner et al.,
1995; Geng and Libby, 1995). Cho and Ballerman have demonstrated in the
neonatal lamb that apoptosis of both vascular smooth muscle and ECs is
responsible for regression of the intra-abdominal aorta and umbilical vessels (Cho
and Ballerman, 1995). However, in normal tissues, EC turnover is reportedly very
low (Desmouliere et al., 1995), which suggests that normal endothelium in a
steady state has mechanisms of maintaining cell number by promoting viability
independently of proliferation.

1.2.1 Microvascular Remodelling – Programmed Capillary Regression.

Programmed capillary regression is a frequent occurrence in development
(Meeson et al., 1996). Developmentally programmed capillary involution can be
associated with regression of complex tissues, such as the interdigital web (Hurle
et al., 1985). In regression of the interdigital web, surrounding mesenchymal and

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ectodermal cells undergo programmed death at the stage of development that capillaries regress (Hurle et al., 1985). Capillary involution has also been observed with differentiative events, including the appearance of avascular regions that arise prior to the condensation of cartilage in the developing digital plate (Feinberg and Noden, 1991). Further capillary regression has been observed in the hyaloid system and pupillary membrane of the mammalian eye (Lang et al., 1994).

There have been a number of models proposed to explain different examples of capillary regression at the cellular level. Induced corneal capillaries have offered an opportunity for analysis of the morphological events associated with regression and have indicated that blood stasis may be a requirement for the later stages of regression (Ausprunk et al., 1978). Vessel regression in the precartilaginous regions of the chick foot plate has been examined (Hallmann et al., 1987; Hanahan and Folkman, 1996; Wilson, 1986), and as yet the mechanism of capillary regression prior to cartilage formation remains unclear.

Electron microscopy in the study of morphological changes associated with the regression of hyaloid vessels has revealed the presence of macrophages (referred to as hyalocytes or vitreal cells) with a possible role in regression (Balazs et al., 1980; Jack, 1972; Latker and Kuwabara, 1981; Wang et al., 1990). It has been further noted that although macrophages are present, their numbers are low, while the cells lack phagosomes and thus may not play a major role in

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regression as previously thought (Latker and Kuwabara, 1981). More recent studies have provided evidence that regression of capillaries in the pupillary membrane may be macrophage dependent (Lang and Bishop, 1993). Furthermore, the pattern of programmed cell death during pupillary regression suggests a cellular mechanism to explain the regression process (Lang et al., 1994: Fig. I.1.a). This current model proposes that apoptosis of ECs early in the regression of individual capillary segments is macrophage dependent. These events are discerned histologically as isolated apoptotic ECs in otherwise normal segments and are referred to as initiating apoptosis (Fig. I.1.a). The model further proposes that initiating apoptosis results in the cessation of plasma flow when the capillary lumen becomes narrow (Fig. I.1.b). In turn, the model proposes that the lack of flow causes synchronous pattern of apoptosis that has previously been observed, perhaps due to deprivation of plasma survival factors (Lang et al., 1994).

This current model proposes that, directly or indirectly, macrophages drive all aspects of programmed capillary regression. This model implies that there is a link between macrophage-induced apoptosis and the flow stasis that is proposed to result in secondary apoptosis. Azmi and O'Shae (1984) have documented that where there is a mechanical block to blood flow, the consequence is apoptosis of the ECs within the involved capillaries.

Although all temporary or permanent flow restrictions are associated with EC apoptosis, the converse is not true. It has been shown that some EC apoptosis
occurs in capillaries that are still free flowing (Meeson et al., 1996). The level of EC apoptosis was found to be 5.9% of total cells. This figure compared to those capillaries undergoing the transition from flow to stasis showed some endothelial apoptotic cells that were not associated with the point of restriction (sporadic flow, 17%; oscillating flow, 31%) (Meeson et al., 1996).

Quantitative analysis of many capillary segments indicates that as the blood flow in a capillary is diminished, the percentage of cells undergoing apoptosis increases (Meeson et al., 1996). This suggests that, for the second phase of regression, the two phenomena are interrelated. Meeson et al., (1996) have proposed that one explanation for the relationship between death and flow is that the level of survival factor to which the ECs within a segment are being exposed is being reduced in proportion with reducing flow. The relationship between EC apoptosis and blood flow will be discussed later (Chapter I.5.3).

I.2.2. The Control of Vascular Turnover and The Role of Patency.

In stable tissue environments, blood vessels with minimal blood flow survive, maintaining a level of vessel patency within the vascular bed. This is thought to occur due to structural adaptations within the capillary network. These adaptations include (a) precapillary sphincters, which can almost entirely stop the capillary flow, (b) only a minority of sphincters are normally open in the tissues, and (c) hydrostatic pressures in unperfused capillaries are similar to pressures at their venous ends. It is thought that the action of the precapillary sphincters which

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open and close maintain a constant level of perfusion in the tissues and thus acts to maintain capillary survival.

I.3 Apoptosis

I.3.1 Introduction to Cell Death

The death of cells has historically been associated with injury and disease processes. The morphological features have been well described and most regarded the notion of loss of viability in relation to disease as a logical finale. There are now two recognised forms of cell death. Necrosis describes a process of cell death due to loss of homeostasis at the level of individual cells (Wyllie, 1980; 1981; Duvall and Wyllie, 1986). This is related to processes of disease, chemical and physical forces (Wyllie, 1980; 1981; 1987; Duvall and Wyllie, 1986). The second form of cell death has been termed apoptosis. The term apoptosis was derived from the Greek term used to describe falling off or dropping off of petals from flowers or leaves from trees. Kerr et al., (1972) first proposed the term to describe a process of cell death that was inherently programmed. Up to this point in time, necrosis had been the only term used to describe cell death. It was recognised that there were fundamental differences between apoptosis and necrosis. Subsequently apoptosis became a definition for physiological cell depletion of cells within healthy or diseased tissues.

In necrosis, cell injury damages critical structural or biochemical functions within cells, and this exceeds the adaptive limits so that irreversible degradation
of cellular systems occurs. Necrosis, therefore, pertains to circumstances beyond physiological limits. In this way, necrosis is seen in: complement – mediated cell lysis; inhibition of oxidative phosphorylation; interference with glycolysis or the Krebs cycle; exposure to a variety of toxins; lytic viral infections and severe physical embarrassment such as hyperthermia, hypoxia and changes to osmotic pressure (Duvall and Wylie, 1986; Wylie, 1980; 1981).

In necrosis there are irreversible changes in the nucleus and cytoplasm due to loss of homeostasis which result in membrane disruption and inability of the cell to regulate osmotic pressure (Duvall and Wylie, 1986; Lopez-Farre et al., 1998; Wylie, 1980; 1981). The cells, as well as the internal organelles swell and eventually rupture. The resultant liberation of cellular components into the intercellular spaces simulates a local inflammatory response, and may also damage surrounding cells (Gerschenson and Rotello, 1992; Kroemer et al., 1995). Also, DNA is degraded randomly during necrosis, producing a smear on DNA gel electrophoresis. Further, intercellular adhesion is lost at late stages of necrosis while this is an early event in apoptosis.

Apoptosis is an important process in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and also in responses to some toxins (Clarke and Clarke, 1995; Dewey et al., 1987). Inappropriate apoptosis has been implicated in many human diseases, including
neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, ischaemic damage, autoimmune disorders, and several forms of cancer (Folkman, 1976; Kerr et al., 1994; Kockx and Herman, 2000; Nicholson, 1996; Thompson, 1995).

I.3.2 The History of Apoptosis

A general misconception is the notion that naturally occurring cell death is a relatively new biological discovery. In reality, the first documented account of such a process appears to date back to 1842, when Carl Vogt described the death of notochordal and cartilaginous cells during development (Clarke and Clarke, 1995; Dewey et al., 1987). In 1885 the morphological changes were thoroughly described by Flemming and given the name 'chromatolysis', a term which was widely used for the next thirty years (Majno and Joris, 1995). At the turn of the 19th century interest in cell death had declined, and it was not until 1964 when the topic was resurrected by Lockshin and Williams who introduced the term 'programmed cell death' (PCD) to describe the developmentally regulated elimination of specific cells during the transformation of larvae from adult moths (Lockshin et al., 1964). The death of cells in the normal development of vertebrates and invertebrates was described by Glucksmann in 1951 and by Saunders in 1966 (Glucksmann, 1951, Saunders, 1966). Early evidence of PCD consisted of experiments by Saunders (1966), who demonstrated that embryonic chick tissue destined to die would die on schedule when explanted but could survive if transplanted elsewhere in the chick. Both Lockshin and Saunders

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emphasised what was otherwise obvious: if a cell died on a developmental schedule, a genetic control was implicit. This point was later further suggested by experiments by Lockshin (1964), in which cell death was prevented by application of inhibitors of mRNA or protein synthesis. Finally true genetic regulation of cell death was documented by the identification of genes targeting cells for death, effecting the death, or preventing it in the nematode (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994; Horvitz et al., 1994).

In 1965, Kerr described different types of liver cell death after portal vein branch ligation. Patches of confluent necrosis were seen early, but during the following weeks a different type of cell death was observed in scattered individual hepatocytes that had condensed nuclei, without any evidence for lysosomal rupture or inflammation (Kerr, 1965). In 1971, the same author described the ultrastructure of what he called "shrinkage necrosis". The nuclear mass or masses were found to be membrane enclosed bodies containing pieces of condensed chromatin and well-preserved organelles (Kerr, 1971). Finally, the word 'apoptosis' was coined in 1972 by Kerr et al., (1972) to describe the common morphological changes that characterise the process of cellular self destruction (Kerr et al., 1972; Kerr and Searle, 1972).

Historically the term “apoptosis” meant simply to describe the morphological changes associated with most instances of cellular self destruction, but not necessarily requiring new gene expression. As is now known, not all PCD
occurs by apoptosis, and not all instances of apoptosis require new gene expression. For instance, the cytotoxic T-Lymphocyte killing of target cells induces an apoptotic morphology, but does not require new gene expression and, therefore, does not qualify as PCD (Martz and Howell, 1989). In contrast, de novo gene expression is required for PCD of intersegmental muscle cells during hormone-induced metamorphosis of the tobacco hawkmoth, but these cells do not show the typical morphological signs of apoptosis possibly due to their syncytial nature (Schwartz and Osborne, 1993). Over the past years, the two terms have lost their specific meanings and now any process that results in the morphological changes associated with apoptosis is also referred to as PCD, regardless of the trigger or absence/presence of new gene expression.

1.3.3 Phases and Time Course of Apoptosis

Under physiological conditions only a small proportion of cells are apoptotic, while the process is complete within hours so that only a small number of apoptotic cells can be seen at a given time point. Studies with cytosolic extracts of apoptotic cells have revealed distinct biochemical phases of apoptosis (Lazebnik et al., 1993; Solary et al., 1993). The first stage, known as the ‘initiation phase’, involves pro-apoptotic stimuli which trigger the central molecular machinery of apoptosis. In the second, ‘effector phase’, the molecular executioners become fully activated. This is followed by the third, or ‘degradation phase’, in which the hallmarks of apoptosis become morphologically evident. It is thought that the asynchronous nature of apoptotic death in cell populations is due
to the highly variable duration of the initiation phase (Saraste and Pulkki, 2000). *In vivo*, the duration of an apoptotic cell death has been estimated to be between 6 and 24 hours, although this varies with cell type (Gavrieli et al., 1992; Kerr et al., 1972). As a result of the short time scale of apoptosis, only few cells undergoing apoptosis are present at a single point and the quantitative significance of apoptosis may be underestimated.

1.3.4 Morphological Changes of Apoptosis.

Apoptosis as defined by Kerr et al., (1972, 1994) was first based on a distinct sequence of morphological features observed by transmission electron microscopy. During development, this natural form of death is responsible for the deletion of unwanted cells in a manner that does not disturb healthy surrounding tissue and fails to elicit an immune response. In contrast to the swelling of a necrotic cell that leads to eventual membrane rupture and dissolution of organelles, an apoptotic cell rapidly and inconspicuously condenses into varying sized membrane-bound bodies or vesicles, in which the cytoplasmic organelles appear to be intact and most of the apoptotic bodies have condensed nuclear fragments.

Characteristically, apoptosis affects single cells rather than groups of cells (Kerr et al., 1972; Searle et al., 1975). The onset of apoptosis is characterised by shrinkage of the cell and the nucleus, as well as condensation of the nuclear chromatin into sharply delineated masses that become marginated against the
nuclear membrane. Destruction of the nucleolus is followed by the appearance of dark granules in its place. Later on, the nucleus progressively condenses and breaks up. The cell detaches from the surrounding tissue and its outline becomes convoluted and forms extensions. The term “budding” has been coined for a process whereby the extensions separate to produce plasma membrane bound cellular particles. Wyllie et al., (1981) described blistering of the nuclear membrane with condensed chromatin in crescent shaped masses around the periphery. Cytoplasmic shrinkage leads to dense packing of structurally intact organelles and loss of contact with neighbouring cells, concomitant with the loss of specialised surface elements such as microvilli and cell-cell junctions. The nuclear outline often becomes convoluted prior to dissolution of the nucleus into discrete fragments that scatter throughout the cell. Time-lapse studies have revealed that these changes are accompanied by extraordinary surface convolutions that separate and seal off from each other to produce membrane-bound apoptotic bodies (Wyllie et al., 1981). These bodies contain various cellular elements ranging from intact organelles and nuclear fragments to cytoplasm alone. The fine structures, including membranes and mitochondria, are well preserved inside these bodies. In vivo the apoptotic bodies are rapidly phagocytosed into neighbouring cells, including macrophages and parenchymal cells. Apoptotic bodies can be easily recognised inside these cells, but eventually they become degraded. In vitro, phagocytosis by surrounding cells is often incomplete so that progressive loss of homeostasis by apoptotic particles results in so called “secondary necrosis” of apoptotic cells (Kerr et al., 1972; 1994).
Proteolytic cleavage of a set of key proteins by activated caspase proteases plays a role in apoptosis (Table I.5) (Martin and Green, 1995). Although, the exact mechanism explaining how the degradation of these proteins results in apoptotic morphology remains unknown, many target proteins of caspases participate in the formation and regulation of the membrane-associated cortical microfilaments cytoskeleton, which is an important determinant of cell shape (Brancolini et al., 1995; 1997; Brown et al., 1997; Kayalar et al., 1996; Kothakota et al., 1997; Martin et al., 1995; Mashima et al., 1997; Rudel et al., 1997; Vagags et al., 1996; Wang et al., 1998). Over expression of the caspase cleaved forms of Gas2 or gelsolin result in dramatic changes in cell shape, resembling apoptosis (Brancolini et al., 1995; Kothakota et al., 1997). Other proteases, such as calpains (Brown et al., 1997; Wang et al., 1998), have been implicated in signalling of the apoptotic changes in the cytoskeleton. Caspases are currently implicated as the executioners in apoptosis and will discussed further in section 1.3.7.3.

Two protein kinases at the cell-to-cell and cell-to-matrix attachment sites are also targets of caspases. Their cleavage by caspases results in further enhancement of the pro-apoptotic signalling and possibly detachment of the cell from the surrounding tissue (Cardone et al., 1997; Wen et al., 1997). Disassembly of the nuclear protein laminin, the supporting structure of the nuclear envelope, is also an essential feature of nuclear breakdown in apoptosis (Lazebnik et al., 1993). This process depends on caspase-mediated degradation of nuclear laminins.
A and B (Orth et al., 1996; Takashi et al., 1996). Other proteins cleaved include those involved with the regulation of chromatin structure or interactions between chromatin and nuclear matrix proteins, such as nuclear mitosis associated protein.

1.3.5 Biochemical Changes in Apoptosis

A widely accepted biochemical hallmark of apoptosis is internucleosomal degradation of DNA by endogeneous DNases. This has the effect of fragmentating the genome into 180-200 base pairs oligomers, resulting in a DNA 'ladder' as seen by DNA gel electrophoresis (Wyllie, 1987). This internucleosomal DNA cleavage is mediated by a Ca²⁺-dependent endonuclease (Arends et al., 1990; Kroemer et al., 1995; Majno and Joris, 1995), and occurs in two steps, with the appearance of large (50 and 300kb) fragments preceding the production of the smaller internucleosomal fragments (Brown et al., 1993). Vanderbilt et al. (1982) demonstrated that the endonuclease preferentially attacks transcriptionally active DNA. Importantly, the internucleosomal cleavage characteristic of apoptosis is not seen in necrosis where DNA fragmentation is random and presents as a smear in DNA gel electrophoresis.

The characteristic DNA fragments were originally thought to be produced by double stranded cleavages as a result of frequent nicks of both strands of DNA in the internucleosomal region (Peitsch, 1993). More recent work suggests that these breaks are more specific, producing double-stranded DNA fragments that are either blunt ended or have 3' single base overhangs with ligatable 5' ends,
features consistent with the activity of pancreatic DNase I (Didenko and Hornsby, 1996). Although some studies have suggested that the morphological changes associated with apoptosis occur as a result of DNA laddering (Tounekti et al., 1995), others have shown that morphological cell death can occur in the complete absence of such internucleosomal fragmentation (Cohen, 1997; Cohen et al., 1992; Lockshin, 1981; Lockshin et al., 1991; Oberhammer, 1992), so that internucleosomal cleavage may not be a universal apoptotic event. Although the role of DNA degradation in apoptosis remains unclear, it is present in most apoptotic cells and is a useful biochemical marker for this process.

A variety of caspase substrates are involved with the regulation of DNA structure, repair and activity (Nicholson and Thornberry, 1997). The DNase enzymes responsible for the fragmentation during apoptosis include DNA fragmentation factor (DFF40) (Liu et al., 1998), and caspase activated DNase (CAD) (Enari et al., 1998; Sakahira et al., 1998). These enzymes are selectively activated upon cleavage by caspase 3 (Liu et al., 1997; 1998), or by other members of the caspase family. Many other cellular proteins are also degraded in apoptotic cells including poly-ADP-ribose polymerase (PARP), so that PARP cleavage has been used as a marker for apoptosis.

Cytoplasmic acidification has been suggested as essential for apoptosis, perhaps activating pH-dependent enzymes (Meisenholder et al., 1996). Concomitant with this are changes in the plasma membrane. Phosphatidylserine,
which is normally restricted to the inner leaflet of the bilayer in healthy cells, becomes expressed on the extracellular surface of apoptotic cells and thus can be detected through the binding of Annexin V. (Fadok et al., 1992; Lincz, 1998).

I.3.6 Identification of Apoptosis

A variety of laboratory tools are available to detect apoptosis. Light microscopy reveals both cellular and nuclear fragmentation, both of which can be displayed in greater detail using transmission electron microscopy (TEM). This technique also allows visualisation of the maintained integrity of organelles. EC apoptosis involves the formation of canalicular structures seemingly unique to apoptosis in endothelium, therefore allowing presence of ‘canalicular fragmentation’ to be used as a marker for EC apoptosis in TEM (Zoellner et al., 1996a,b; 1999).

Internucleosomal DNA fragmentation can be detected as a laddered pattern after electrophoresis of isolated DNA (Wyllie, 1980). With the TUNEL assay, cells containing DNA strands breaks become visible in light microscopic analysis (Gavrieli et al., 1992). Fluorescence activated cell scanning analysis (FACS) can be used to quantify the amount of DNA in cells and apoptotic particles, identifying “sub-diploid” apoptotic populations (Darzynkiewicz et al., 1992).
Caspase activation can be detected by a variety of methods (Bedner et al., 2000; Durrieu et al., 1998), but recent reports demonstrating caspase independent pathways of apoptosis (Slowik et al., 1997) imply limitations for this approach to verifying apoptosis.

1.3.7 Mechanism of Apoptosis

1.3.7.1 Caenorhabditis elegans: A Model System

Pioneering studies by Horvitz et al., (1994) on the development of the nematode Caenorhabditis elegans have established both an important role for apoptosis in nematode development as well as the identity of some genes involved. Of the 1090 somatic cells produced during the genesis of this hermaphrodite worm, exactly 131 are destined to die (Driscoll, 1992). The pattern of cell death has been well described, with the same cells always dying at defined stages of development. Over 14 genes in C elegans are known to be operative in this genetic pathway of apoptosis, and mutations which affect the regulation of the death program have been mapped to three principle C elegans genes: ced-3, ced-4, and ced-9.

Mammalian homologues of C elegans death genes have been identified, indicating a high level of evolutionary conservation for these genes (Yuan et al., 1993) and include: Bcl-2 related proteins (Hengartner and Horvitz, 1994; Vaux et al., 1992) and Apaf-1 (Zou et al., 1997). The ced-9 protein has sequence homology with mammalian Bcl-2, which acts to prevent cell death in mammals.
(Hengartner and Horvitz, 1994; Vaux et al., 1992). The ced-3 protein is similar in sequence to mammalian interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993).

Apoptosis can be initiated either by specific pro-apoptotic signals or through deprivation of specific survival signals. The main stages of the apoptotic pathway appear to be: the activation of the cell; loss of mitochondrial membrane potential and release of cytochrome C from mitochondria; activation of the caspase cascade; cell shrinkage, DNA fragmentation, and fragmentation into apoptotic bodies (Lopez-Farre et al., 1998).

I3.7.2 The Bcl-2 Family of Proteins

B cell lymphoma/leukaemia gene 2, better known as Bcl-2, was originally identified as a consequence of its involvement in human B cell follicular lymphoma. Vaux et al., (1988) were the first to show that over-expression of Bcl-2 could prolong cell survival. Pre-B cells that were normally dependent on exogenous interleukin-3 (IL-3) for their survival in vitro could avoid death in the absence of IL-3 if they had expressed the Bcl-2 protein (Vaux et al., 1988). Since then, Bcl-2 has been found to block cell death induced by a variety of methods and in many different cell types (Table I.6).

Numerous proteins have been identified that share one or more of the four conserved regions within Bcl-2 known as the Bcl-2 homology domains: BH1,
BH2, BH3 and BH4 (Ellis and Horwitz, 1986). However, not all of these proteins protect against cell death; some are pro-apoptotic, while others may perform either function as dictated by cellular circumstances. Regulation of apoptosis by this family of proteins has been shown to occur by protein-protein interactions, as well as through phosphorylation (Lincz, 1998). Most members of the Bcl-2 group are located on intracellular membranes, particularly on the outer mitochondrial membrane, nuclear envelope and the endoplasmic reticulum (Reed, 1998). Dimerisation of Bcl-2 family members is thought to determine the fate of the cell. One of the main pro-apoptotic homologues, Bax, can form heterodimers with both Bcl-2 and Bcl-XL, as well as homodimers with itself. It is believed that the ratio of heterodimers to homodimers within the cell determines its fate; at least half of the Bax proteins must be heterodimers with Bcl-2 for the cell to be protected from apoptosis, while if the number of Bax homodimers exceeds the number of Bax-Bcl-2 heterodimers the cell will undergo apoptosis (Lincz, 1998). Consistent with this, it has been found that in certain mammalian cells, expression of Bax alone is enough to trigger apoptosis, which can be inhibited by co-expression of Bcl-2 (Hunter and Parslow, 1996). Furthermore, mutation of specific residues within the BH1 and BH2 domains of Bcl-2 results in proteins that can no longer form complexes with Bax, and are likewise unable to suppress apoptosis (Yin et al., 1994). Bcl-2, which was the first member of this group to be identified, is thought to prevent the release of cytochrome C from mitochondria or prevent Apaf-1 function (Reed, 1998). Most roles of the Bcl-2 family in the regulation of apoptosis are only now being defined.
I.3.7.3 Caspases in Apoptosis

Caspases are cysteine proteinases, which are synthesised as inactive proenzymes requiring cleavage at specific aspartate residues to become active. These active complexes are composed of two heterodimers, which contribute to a tetramer containing two catalytic sites (Nunez et al., 1998). The active enzymes then activate further caspases in a proteinase cascade amplifying the apoptotic signal and accelerating apoptosis. Caspases therefore may display autocatalytic activity and can also activate further caspases as part of a caspase cascade.

Mid 1980's research has proposed a role for a number of different proteinases, calpains and proteasomes. Early attention has focused on ICE, due partly to the enormous progress made by Horvitz et al., (1994) in understanding programmed cell death in the nematode. Of the three principle genes mapped, ced-3 protein bears marked sequence similarity to, and identity with, mammalian caspases. This finding, together with the observation that over-expression of caspases induces apoptosis, suggests that caspases may play a key role in the induction of apoptosis (Miura et al., 1993; Yaun et al., 1993). Further evidence supporting a critical role for caspase proteinases in apoptosis is the ability of specific protease inhibitors, including cowpox viral serpin Crma and baculovirus p53, to inhibit apoptosis (Clem et al., 1991; Miura et al., 1993).

Since the recognition of the similarity between ced-3 and ICE in 1993, a further ten related caspases have been identified (Lincz, 1998). By degrading
specific cellular components, these proteinases are thought to be responsible for many of the morphological changes typical of apoptosis (Nunez et al., 1998)

Phylogenetic analysis of caspases reveals that there are three subfamilies: an ICE subfamily, comprising caspases-1,-4, and -5, a CED-3/CPP32 subfamily, comprising caspases -3, -6, -7, -8, -9 and -10, and an ICH-1 (where ICH is Ice and ced-3 homologue) /Nedd2 subfamily (Cohen, 1997).

There are two pathways whereby caspases may be activated. Firstly, caspases may be activated through the release of cytochrome C from the mitochondria in response to stimuli such as growth factor withdrawal or chemotherapeutic drugs. Cytochrome C activates Apaf-1, which then activates caspase-9. The signal is amplified through the subsequent cleavage of caspases – 3, -6, and -7, which then target the cellular substrates (Nunez et al., 1998). In the second pathway, caspases are activated through receptors such as Fas, which contain “death domains” (Nunez et al., 1998). The activated caspases then target substrates throughout the cell.

Caspase substrates can be broadly categorised as apoptosis regulators or structural proteins. They may act to cleave and activate further caspases, activating apoptosis regulators and these amplify the apoptotic signal. Alternatively, such cleavage may activate quite separate pathways.
The cleavage of structural proteins by caspases results in the characteristic morphological changes of apoptosis. For example, nuclear lamins, which are major cytoskeleton components of the nucleus, are cleaved by caspase –6, while actin is also cleaved by caspases during apoptosis (Nunez et al., 1998). The caspase activated deoxyribonuclease (CAD), is normally bound to its inhibitor ICAD in the cytosol but is activated by caspase –3 mediated cleavage of ICAD. Active CAD is then translocated to the nucleus and initiates DNA degradation (Dragovich et al., 1998)

I.3.7.4 Apaf-1

A novel human protein designated Apoptosis activating factor-1 (Apaf-1) has been identified which shows homology to ced-3 at its N-terminus followed by a ced-4- like region and a COOH-terminus consisting of multiple WD-40 repeats believed to mediate protein-protein interactions (Zou et al., 1997). It was identified as one of the three cytosolic components (Apaf-1, Apaf-2, Apaf-3) required for activation of caspase –3 in the presence of exogeneous dATP (Liu et al., 1996). Apaf-2 was identified as the electron transport protein cytochrome C (Liu et. al., 1996), while Apaf-3 is yet to be cloned. Cytochrome C normally resides in the mitochondrial inner-membrane space, and has been found to be an important component of cellular apoptotic machinery, suggesting that the mitochondria contribute to apoptosis by releasing the protein into the cytoplasm (Liu et al., 1996).
Apoptotic pathways may be divided into Ca\textsuperscript{2+} dependent and independent mechanisms. In the Ca\textsuperscript{2+} dependent pathway, Ca\textsuperscript{2+} is released from intracellular stores in response to apoptotic stimuli, activating caspases and resulting in apoptosis (Lincz, 1998). Ca\textsuperscript{2+} has also been implicated in activation of the endonuclease responsible for DNA fragmentation (Arends et al., 1990). In one Ca\textsuperscript{2+} independent pathway, caspases are activated by a Fas dependent mechanism. The Fas ligand binds to the Fas receptor, which transduces the signal through further molecules known as Fas-associated death domain (FADD), which is subsequently activated. FADD then activates caspases 8 and 10, which then activate caspases 3, 6, and 7 (Nunez et al., 1998). In both pathways, caspases then target cellular substrates ultimately causing apoptosis.

L3.8 Factors Regulating Apoptosis

General apoptotic signals for cells include: loss of communication with the surrounding environment, withdrawal of growth factors, viral infections, UV light, ionising radiation, hydrogen peroxide, high glucose, ischaemia, chemotherapeutic agents, and some cytokines, such as TNF-\textalpha (Desmouliere et al., 1997; Harada-Shiba et al., 1998; Robaye et al., 1991). Loss of EC anchorage also induces apoptosis, suggesting a role for integrins (Meredith et al., 1993). Apoptosis also occurs in response to low levels of irradiation (Gerscheneson and Rotellom 1992) or oxidized low density lipoproteins (Oxidized LDL) (Harada-Shiba et al., 1998). Specific growth factors, such as insulin-like growth factor (Greenhalgh, 1998), fibroblast growth factor (Araki et al., 1990a;b; 1993), serum
albumin (Zoellner et al., 1996a; 1999) and laminar shear stress (Dimmeler et al., 1996; 1998b; 1999), have been shown to inhibit apoptosis in endothelium.

I.4 Endothelial Cell Apoptosis

I.4.1 Introduction to Endothelial Cell Apoptosis

The inflammatory cytokine TNF-α induced EC apoptosis (Robaye et al., 1991). Many other factors have also been shown to be either pro-apoptotic or anti-apoptotic for ECs. Apoptosis in ECs share many morphological and biochemical similarities with that in other cells, as described in sections 1.3.d. and 1.3.e. One difference, however, is that EC apoptosis involves the formation of canalicular structures which are seemingly unique to apoptosis in endothelium, and this has been proposed as minimizing microembolic potential of the cells (Zoellner et al., 1996a;b; 1999).

I.4.2 Anti-Apoptotic Signals for Endothelium

I.4.2.1 Matrix and Intercellular Adhesion

Several factors have been identified which inhibit EC apoptosis. The ECM plays an important role in the regulation of cell growth, differentiation, and behaviour (Daniels and Solursh, 1991). ECM-cell interactions are mediated to a large extent by integrins, a family of more than 20 different αβ heterodimer proteins. Matrix and intercellular adhesion have been shown to inhibit EC apoptosis (Re et al., 1994; Xaymardan, 2001). This seems biologically important, as ECs not confined to an endothelial monolayer or detached from the vessel wall

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are by definition, non-functional, and therefore would be expected to become apoptotic. These observations have been further supported by subsequent observations in vitro, where ECs failing to form intercellular contact, in addition to matrix interactions, were found to undergo apoptosis (Frisch and Francis, 1994; Pollman et al., 1999a;b).

1.4.2.2 Anti-Apoptotic Chemical Factors

Several growth factors inhibit EC apoptosis including fibroblast growth factor (FGF) (Araki et al., 1990a), vascular endothelial growth factor (VEGF) (Spyridopoulos et al., 1997), endothelin-1 and angiopoietin-1 (Holash et al., 1999; Kim et al., 2000). FGF inhibits EC apoptosis in serum deprived EC (Araki et al., 1990a;b). The potent protein kinase C activators, PMA and TPA, have also been shown to inhibit EC apoptosis and this is implicated in FGF mediated protection (Araki et al., 1990b, Hase et al., 1994). Estrogen-receptor-mediated inhibition of EC apoptosis has also been observed (Spyridopoulos et al., 1997). Also, estradiol (E2) treatment results in a dose-dependent, receptor mediated inhibition of TNF-α-induced EC apoptosis (Spyridopoulos et al 1997).

Serum has been established as important for the survival of ECs (Arika et al., 1990b; Zoellner et al., 1996b), and both FGF and serum albumin have been identified as specific anti-apoptotic factors in serum. However, serum albumin is the only known plasma protein with anti-apoptotic activity, as FGF is not normally present in plasma. Serum albumin strongly inhibits human EC apoptosis.
in serum free conditions (Zoellner et al., 1996b). This activity is identical for both native bovine serum albumin (BSA) and human serum albumin (HSA) from several sources and maximal anti-apoptotic activity is observed at physiological concentrations (4%) of the protein. The anti-apoptotic activity of albumin is not due to serum contaminants of native albumin preparations, as recombinant human serum albumin from yeast, also displays identical anti-apoptotic activity. Further, the anti-apoptotic activity is not due to a non-specific protein effect, as neither ovalbumin, nor denatured albumin display any anti-apoptotic activity under similar conditions (Zoellner et al., 1996b). These observations have been confirmed for microvascular ECs in both human and rat tissue explants, importantly, no other cell type displays apoptosis or necrosis in these tissues (Zoellner et al., 1999). From these observations, it has been suggested that albumin may be an anti-apoptotic factor in plasma signalling vascular perfusion to ECs, and in this way contributes to vascular remodelling (Zoellner et al., 1996b; 1999).

Several experimental and clinical studies suggest that cyclosporin A (CSA), which is commonly used to suppress allograft rejection, reduces hyperlipidemia-induced atherosclerosis (Drew and Tipping, 1995), and ameliorates transplant atherosclerosis (Anderson et al., 1994; Valentine et al., 1994). In addition, CSA has been shown to stabilise mitochondrial transmembrane potential and thereby inhibit apoptosis induced by different stimuli (Zanzami et al., 1996). The mechanism underlying the protective effect of
CSA appears to involve stabilisation of the mitochondria, thereby preventing release of mitochondrial protein cytochrome C into the cytosol (Walter et al., 1998). Cytosolic cytochrome C has been shown to be necessary to activate the apoptosome complex leading to caspase activation and apoptosis induction. EC apoptosis and its possible relationship to atherosclerosis will be discussed later.

1.4.3 Pro-Apoptotic Signals for Endothelium

Several potent pro-apoptotic stimuli for ECs have been identified. These include; hemorrhagic snake venom (Araki et al., 1993), sulfur mustard gas, (Dabrowska et al., 1996), hydrogen peroxide (De Bono and Yang, 1995), oxidized LDL (Dimmeler et al., 1997a), and IFN-γ (Maier et al., 1995). Bacterial lipopolysaccharide (LPS) has also been found to stimulate EC apoptosis in certain circumstances (Choi et al., 1998; Haimovitz-Freidman and Cordon-Cardo, 1997)

1.4.3.1 Ionising Radiation

Ionising radiation exposure has also been associated as a pro-apoptotic factor of ECs, specifically ECs of the microvascular network (Langley et al., 1997). The magnitude of this apoptotic response depends on two factors. Firstly, apoptosis is dependent upon the duration of FGF withdrawal. Secondly, ionising radiation induced apoptosis of ECs occurs in a dose-dependent manner (2-10Gy).
1.4.3.2 Angiopoietin II

Angiopoietin II (Ang II) has been shown to have pro-apoptotic activity for ECs. This pro-apoptotic activity is dose-dependent, and is associated with activation of caspase 3, a central down-stream effector of the caspase cascade executing the cell death program. Importantly, the effect of NO, is to completely abrogate Ang-II-induced apoptosis by interfering with activation of the caspase cascade (Dimmeler et al., 1997c).

1.4.3.3 Nitric Oxide

NO is synthesized by the endothelium through enzymatic activity of the endothelial isoform of NO synthase. The role of NO in EC apoptosis is still unclear. When ECs are cultured in subconfluent conditions, inhibition of NO synthesis results in increased EC proliferation and growth. However, inhibition of NO in confluent cultures of ECs induces apoptosis (Lopez-Farre et al., 1998). Other studies have shown that low levels of NO inhibit TNF-α induced apoptosis through a cGMP dependent pathway, while higher levels of NO increase EC apoptosis through a cGMP independent pathway (Shen et al., 1992). Furthermore, it has been suggested that NO can block the induction of EC apoptosis and protect activated EC in vitro (DeMeester et al., 1998). It seems likely that one role of NO in endothelium is to maintain the confluent monolayer, rather than inhibit subconfluent EC growth (Lopez-Farre et al., 1998). Reports supporting a pro-apoptotic action are challenged by those observing an anti-apoptotic action. The
lack of a consistent effect of NO on apoptosis may be due to differences in experimental conditions and models.

I.4.3.4 Tumour Necrosis Factor-α

TNF-α is a cytokine synthesised by a variety of cells including macrophages (Hase et al., 1999) and fibroblasts (Natoli et al., 1998), and induces both pro-coagulant and pro-inflammatory responses in ECs. TNF-α exerts its effect by binding to two receptors, both of which are expressed by ECs. The pro-apoptotic activity of TNF-α for ECs was reported by both Frater-Schroder et al., (1987) and Robaye et al. (1991). Subsequent research has shown that protein synthesis inhibitors, such as protein synthesis inhibitor, cycloheximide (CHX) and RNA synthesis inhibitor actinomycin D, potentiate the apoptotic response (Polunovsky et al., 1994). TNF-α induced apoptosis of ECs is suggested to be dependent on pathways involving sphingolipid turnover. Sphingomyelinases are activated by TNF-α and produce ceramide, which has apoptotic activity, but TNF-α also activates sphingosine kinase which catalyses the formation of sphingosine-1-phosphate, a protective factor for ECs (Jones et al., 1989). Thus, the balance of the two factors, ceramide and sphingosine-1-phosphate, may determine the fate of the cells exposed to TNF-α (Xia et al., 1999).
I.4.3.5 Transforming Growth Factor -β

TGF-β is a cytokine synthesised by a wide variety of cells including platelets, ECs, monocytes, and lymphocytes. TGF-β is an important cytokine in inflammation and wound healing (Blobe et al., 2000). Synthesis of TGF-β is also increased in response to inflammatory stimuli such as TNF-α and IL-1. The growth inhibitory properties of TGF-β for ECs have been well documented (Frater-Schroder et al., 1986; Heimark et al., 1986; Muller et al., 1987), however, TGF-β was first shown to be apoptotic for ECs in vitro by Tsukada et al. (1995). They demonstrated that the pro-apoptotic activity of TGF-β was coupled to the down-regulation of Bcl-2, an anti-apoptotic protein. Choi and Ballerman (1995) found EC apoptosis and TGF-β to be important in capillary morphogenesis. TGF-β induced apoptosis of ECs was shown to involve the down-regulation of p21, which is an inhibitor of G1 cyclin/cyclin dependent kinase contributing to the regulation of the cell cycle, specifically the passage of cells from G1 to M phase (Pollman et al., 1999b).
I.5 Endothelium and Blood Flow

I.5.1 Haemodynamic Forces of Blood Flow

The pulsatile flow of blood through the branched tubular network of the mammalian circulatory system generates forces that act on blood vessels to modulate changes in their intrinsic structure and function (Nerem et al., 1981; Resnick and Gimbrone, 1995). These hemodynamic forces include hydrostatic pressures, cyclic strains, and frictional wall stresses. They constitute a specific category of biophysical stimuli and can elicit important biological responses in the cells that compose the blood vessel wall. The luminal surface of the blood vessel and its endothelial surface are constantly exposed to hemodynamic shear stresses (Davies, 1995; Fung, 1997). The magnitude of the shear can be estimated in most of the vasculature by Poiseuille’s law (Fung, 1997), which states that shear stress is proportional to blood flow viscosity, and inversely proportional to the third power of the internal radius (Kamiya et al., 1980; 1984; LaBarbera, 1990). Measurements using different modalities have shown that shear stress ranges from 1 to 6 dyne/cm² in the venous system and between 10 and 70 dyne/cm² in the arterial vascular network. In numerous experiments, shear stress has been shown to actively influence vessel wall remodelling (Kamiya et al., 1980; Langille, 1984; Langille and O’Donnell, 1986). Specifically, chronic increases in blood flow, and consequently shear stress, such as seen in the radial artery of a dialysis patient proximal to their arteriovenous fistula (Girerd et al., 1996), or in feeder arteries supplying cerebral arteriovenous malformations (Rossitti and Svendsen, 1998), lead to the expansion of the luminal radius such that mean shear
stress is returned to its baseline level (Girerd et al., 1996; Langille and O’Donnell, 1986). Conversely, decreased shear stress resulting from lower flow or blood viscosity induces a decrease in the internal vessel radius (Langille and O’Donnell, 1986). The net effect of these endothelial-mediated compensatory responses is maintenance of mean arterial hemodynamic shear stress magnitude at approximately 10 to 70 dyne / cm². This shear stress stabilising process is dependent on intact endothelial function and is abolished by prior selective destruction of the endothelial monolayer (Davies et al., 1986, 1993).

1.5.2 In Vitro Fluid Mechanical Model Systems

To explore the hypothesis that fluid mechanical forces could act as direct stimuli modifying vascular endothelial cell structure and function, several centres undertook research in the early 1980s. This led to the development of in vitro model systems to simulate the in vivo biomechanical environment of the vascular endothelium (Bussolari et al., 1982; Davies et al., 1984; Dewey et al., 1981; 1984; 1987; Eskin et al., 1984; Grabowski et al., 1985; Levesque and Nerem, 1985). This experimental strategy has permitted deliberate control of selected parameters (e.g., amplitude, duration, and spectral properties of the applied force), as well as more precise measurements of biological response at the cellular level.

For flow simulation studies, two basic systems have been designed. The first is a relatively simple parallel-plate flow chamber, whereby well-developed laminar shear stress flow patterns are generated by a pump device, and used to
stimulate confluent endothelial monolayers grown on transparent cover-slides. In this system, wall shear stress, the tractive force imparted by the movement of the viscous fluid over the luminal endothelial surface, is a linear function of the volume flow rate through the channel. Using a closed sterile system with periodic replenishment of medium, cells can be maintained under defined flow for several days. Coupled with a phase-contrast microscope or microfluorimeter, this system permits visualisation of shear stress-induced changes in cell morphology or live-time functional analyses (Dull and Davies, 1991; Mo et al., 1991; Shen et al., 1992).

The second system is a modified cone-plate viscometer (Bussolari et al., 1982), in which shear stresses are produced in a layer of fluid contained between a stationary base plate and a rotating cone. By adjusting cone angle, medium viscosity, and cone reduction speed, a broad dynamic range of shear forces (typically in the range of 1 to 50 dyne / cm²) can be generated, in both laminar and turbulent shear stress flow patterns (Sdougos et al., 1984). Confluent endothelial monolayers grown on small cover-slides and mounted at different positions in the base plate allow multiple samplings of the flow field. Alternatively, a single, large plastic plate insert can be substituted, thus enabling scale-up of sample size for molecular biological analysis. Unsteady (oscillatory) laminar shear stress flow can be generated by tipping the cone with respect to the axis of rotation. In addition, small, defined areas of disturbed flow can be created on each cover-slide by the addition of a barrier to the primary flow in the form of

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a step. The latter system can generate a separate flow region downstream with complex secondary flows and large spatial variations in the shear gradient (Sdougos et al., 1984), thus mimicking the disturbed laminar flow patterns that occur in vivo near arterial bifurcations (Asakura and Karino, 1990).

1.5.3 Endothelial Responses to Defined Flow Stimuli In Vitro

The role of fluid shear stress in promoting changes to ECs is uncertain. Early research suggested that both high and low shear stresses were implicated. High shear stress has been linked to alignment of ECs (Langille and Adamson, 1981), cell loss (Fry, 1969), increased arterial permeability (Krueger et al., 1971; Thibault and Fry, 1980), and enhanced biosynthetic capabilities (DeForrest and Hollis, 1980).

One of the first consistent observations made using the in vitro mechanical model systems, was the morphological observation that uni-directional laminar shear stresses applied to cultured large vessel EC monolayers induce a time- and force-dependent change in cell shape and alignment that is reversible upon cessation of flow (Barbee et al., 1994; Dewey et al., 1981; Levesque and Nerem, 1985; Remuzzi et al., 1984; 1986). This shear-induced shape change was accompanied by reorganisation of the cytoskeleton (Franke et al., 1984). Accordingly the flow-rate and flow direction over the cell layer caused changes in the cell shape and intracellular microfilament alignment, which was further accompanied by changes in cell proliferation and migration.

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In addition to these morphological changes, cultured ECs exposed to shear stress have altered gene expression. Briefly, shear stress regulates gene expression of various proteins including: NO synthase (Tsao et al., 1996), endothelin-1, TGF-β (Diamond et al., 1989; 1990; Ohno et al., 1995), fibrinolytic activators (Kawai et al., 1996; Wechezak et al., 1985), bFGF (Hsieh et al., 1991; 1992; 1993; Malek et al., 1993; Malek and Izumo, 1992; 1994), platelet-derived growth factor (Resnick et al., 1993; Sampath et al., 1995), intercellular adhesion molecule-1 (Nagel et al., 1993), integrins, vascular cellular adhesion molecule-1 and monocyte chemoattractant protein-1, prostaglandins (Bhagyalakshmi and Frangos, 1989), coagulation factors, the proto-oncogenes c-fos, c-jun and antioxidant enzymes superoxide dismutase (Chien et al., 1996; Urbich et al., 2000). Shear stress also activates shear-sensitive K+ current in ECs (Olensen et al., 1988). Other shear stress-dependent modulation has been reported for various endothelial cell functions (Lansman, 1988; Resnick and Gimbrone, 1995; Takada et al., 1994; Ziegler and Nerem, 1994).

Pioneering research by Davies et al. (1986) demonstrated that EC turnover in vitro is considerably more sensitive to relatively low shear stresses in turbulent flow than to much higher shear stresses applied in laminar flow, implying EC susceptibility to the flow characteristics rather than the magnitude of shear stress alone. Importantly for this thesis, it was noted by Caplan and Schwartz (1973) that enhanced EC turnover could be mediated by apoptosis activation. Forces acting on the endothelium in both types of flow have the same tractive
hemodynamic component, shear stress, but in turbulent flow both the duration of the shear stress applied to the cell and its direction are fluctuating. In contrast, the shear signal received by the cell subjected to laminar flow is unidirectional and non-fluctuating. The actual mechanisms initiating increased EC turnover in turbulent flow conditions is unknown, but maybe related to loss of contact inhibition of growth, or possibly turbulent shear stress flow stimulating other mechanisms that initiate entry to the cell cycle. These observations of increased cell turnover under turbulent shear stress flow, were also reported by Langille et al., (1986) in regions of disturbed flow in vivo. Furthermore, cultured ECs in a disturbed laminar shear stress flow field exhibited a complex spatial pattern with maximal perturbation in the flow reattachment region where the most extreme fluctuations in wall shear stress had occurred (DePaola et al., 1992).

More recent research has concentrated on the application of unidirectional laminar shear stress to confluent endothelial monolayers, which has been shown to suppress cell replication and exert an anti-apoptotic influence on ECs (Cho et al., 1997; Dimmeler et al., 1996; Kaiser et al., 1997; Malek and Izumo, 1994). This is in contrast with the observation that static conditions lead to the onset of EC apoptosis (Cho et al., 1997; Dimmeler et al., 1996; Kaiser et al., 1997), and the previous observations that turbulent shear stress stimulated increased EC turnover (Davies et al., 1986; Langille et al., 1986). The EC apoptosis after external stimulation was described as a significant increase above a background level (Polunovsky et al., 1994): i.e., 0.5-3% apoptosis in untreated cultures versus
up to 52% in TNF-α treated cultures. The evidence of a “basal level” of EC apoptosis has been established in static cultures of EC and this is further supported by studies monitoring the proliferative status of the confluent EC monolayer. EC in static cultures retain a residual population of proliferating cells, whereas fluid-mechanical stimulation results in a truly quiescent monolayer (Kaiser et al., 1997). Furthermore, hemodynamic forces suppress EC apoptosis, and thus indirectly sustain the proliferative resting state of the ECs (Cho et al., 1997; Dimmeler et al., 1996; Kaiser et al., 1997). Conversely, conventional static culture conditions lacking any hemodynamic forces exhibit significantly higher levels of EC apoptosis. An alternative to the term; “basal level” of EC apoptosis has been suggested by Kaiser et al., (1997): “cryptic apoptosis”. In cultures of human umbilical vein endothelial cell (HUVEC) or bovine aortic endothelial cell (BAEC) the frequency of cryptic apoptosis ranges between 1-10% increasing with the passage number of the culture and the time span of post-confluent culturing. Further studies have demonstrated laminar shear stress abrogates apoptosis of EC induced by TNF-α, as well as by serum withdrawal (Dimmeler et al., 1996). Research by Bartling et al. (2000) supported the earlier studies of unidirectional laminar shear stress (Dimmerler et al., 1996; Kaiser et al., 1997), however in contrast they found low shear stress could not reduce the susceptibility of human EC to undergo apoptotic cell death, which may represent static culture conditions analysed in previous studies. Their results were also in agreement with previous studies showing an anti-apoptotic effect of high shear stress after serum deprivation (Bartling et al., 2000; Dimmeler et al., 1996, Kaiser et al., 1997).
These results suggest that hemodynamic forces, specifically laminar shear stress significantly contribute to EC integrity by inhibition of apoptosis.

Many of these flow-induced responses resemble classic receptor-mediated, second-messenger coupled events. Studies of adenosine nucleotide-induced endothelial responses have pointed to a role for flow-related alterations in the mass transport of agonists in the vicinity of cell-surface receptors (Dull and Davies, 1991; Mo et al., 1991). There are several examples of biological responses mediated by direct mechanical stimulation of ECs (Ando et al., 1993a;b; Davies and Tripathi, 1993), which can be grouped broadly into three temporal categories: immediate (seconds to minutes), intermediate (minutes to hours), and delayed (hours to days). The earliest detectable events, such as ion movement and activation of polyphosphoinositide metabolism, presumably are very proximal manifestations of the biomechanical transduction process and appear causally linked to other immediate metabolic effects, such as changes in nitric oxide production. At the other end of the temporal spectrum, delayed responses in cultured ECs such as cell shape change, cytoskeletal reorganisation, and ECM remodelling represent more long-term cellular adaptations. The immediate category consists of primary biological responses that are detectable at the cellular or molecular level, and include membrane deformation, changes in cell cycle kinetics, cellular changes associated with EC apoptosis, and the up- and down- regulation of gene products. Many of the latter represent key components in the pathophysiological effector systems located in the vascular endothelium.
that modulate hemostasis and thrombosis, vascular tone, cell growth, and inflammatory and immune reactions.

Also laminar shear stress diminishes EC apoptosis induced by several pro-atherosclerotic factors, such as; oxidized LDL (Dimmeler et al., 1997a), reactive oxygen species (Hermann et al., 1997), or Ang-II (Dimmerler et al., 1997c). Laminar shear stress from flowing blood is considered an anti-atherosclerotic stimulus (Glasgov et al., 1988).

The anti-apoptotic effect of laminar shear stress for ECs is seemingly mediated by flow-dependent expression of apoptosis-regulating genes. These genes encode potentially pro-apoptotic (Fas, Bak, Bax) and anti-apoptotic (Bcl-xl, Bcl-2, Mcl-1) factors. Alternate splicing of the primary Fas transcript can generate anti-apoptotic isoforms of this mainly pro-apoptotic protein. The antagonistic Fas isoform FasExo6Del lacking the transmembrane domain can be secreted as a soluble protein and exerts its anti-apoptotic effect by competing with Fas for the Fas ligand. In contrast, anti-apoptotic members of the Bcl-2 family are intracellular proteins localised on the outer surface of the mitochondria and other membrane structures. Both Bcl-xL and Bcl-2 prevent the apoptosis associated disruption of the mitochondrial transmembrane potential, mitochondrial cytochrome C release and consequent cytochrome C-mediated activation of caspase 9 (Kharbanda et al., 1997; Kluck et al., 1997; Reed, 1996).
In addition, Mcl-1 mediates an analogous anti-apoptosis activity (Wang and Studzinski, 1997).

Bartling et al., (2000) also observed that high shear stress reduced EC apoptosis, and this was accompanied by elevated expression of the anti-apoptotic FasExo6Del, Bcl-2 and Bcl-xL proteins, while the mRNA level of apoptosis-inducing Fas receptor was decreased. The Fas receptor and its ligand FasL are expressed in human ECs (Sata et al., 2000). Although EC in vitro are usually resistant to apoptosis via Fas receptor (Richardson et al., 1994; Sata et al., 2000), pro-atherosclerotic factors, including oxidized LDL (Sata and Walsh, 1998), and hydrogen peroxide (Suhara et al., 1998) can sensitize ECs to Fas-mediated apoptosis. Furthermore, concurrent Fas over-expression and apoptosis is observed by immunohistochemistry, mainly in ECs of arterial tissue with atherosclerosis (Dong et al., 1996). These data indicate a role of the Fas receptor in EC apoptosis and a contribution to atherosclerosis. In contrast to Fas, elevated expression of apoptosis-inhibiting Bcl-XL by high shear stress might be involved in the control of viability of ECs even without the additional apoptosis-sensitising and pro-atherosclerotic factors such as TNF-α (Ackerman et al., 1999). In addition, the anti-apoptotic Mcl-1 expression is augmented by high laminar shear stress, while the anti-apoptotic Bcl-2 remains unchanged. The finding that high shear stress exerts an anti-apoptotic effect for ECs is further supported by the observation that the anti-apoptotic induction of FasExo6Del/Fas and Bcl-xL are only found under high shear stress conditions (Dimmeler et al., 1998a). These data suggest an

*Literature Review. I. 54*
increased sensitivity by ECs to pro-apoptotic factors in areas of reduced laminar shear stress or turbulent shear stress flow.

Shear stress modulates the levels of NO (Marletta, 1989; Nishida et al., 1992; Noris et al., 1995; Urbich et al. 2000). Laminar shear stress flow increases NO synthase gene expression and subsequent release of NO (Buga et al., 1991; Hutcheson et al., 1991; Noris et al., 1995; Rubanyi et al., 1986; 1993). This is a dose-dependent relationship between laminar shear and NO synthesis by cultured ECs, while turbulent shear stress has no reported effect on the NO-synthetic pathway (Noris et al., 1995). Low levels of NO have been shown to inhibit apoptosis in human B cells (Genaro et al., 1995; Mannick et al., 1994). In contrast, high levels of NO induce apoptosis in macrophages (Messmer et al., 1994). It is suggested that high concentrations of NO lead to direct DNA damage or cause other cytotoxic effects (Nathan, 1992), whereas low levels of NO inhibit apoptosis via post-transitional modification of ICE (Dimmeler et al., 1997). The constitutively expressed EC NO synthase produces low levels of NO (Nathan, 1992; Noris et al 1995; Uematsu et al., 1995) suggesting that EC NO production in response to laminar shear stress may render ECs resistant to other potent triggers of apoptosis.

In addition, the enhanced anti-oxidative capacity of ECs stimulated by shear stress seems to contribute to the anti-apoptotic response (Hermann et al., 1997). This seems mediated by shear stress-induced expression of Cu/Zn
superoxide dismutase (Dimmeler et al., 1998a) and interference with the glutathione redox system (Hermann et al., 1997).

The question arises as to the effect pulsatile and oscillatory shear stress may have upon EC apoptosis, and this thesis attempts to answer this issue.

I.6. The Possible Relationship of Shear Stress with the Pathogenesis of Atherosclerosis

Numerous studies suggest that normal functioning of the endothelium is critical in limiting the development of atherosclerosis, as illustrated by the correlation between risk factors for atherosclerosis and endothelial dysfunction (Ross, 1993). Impairment of endothelial cell-mediated processes and subsequent injury of the vascular endothelium has been postulated as a critical event in the pathogenesis of atherosclerosis (Dimmeler et al., 1998b; Friedman, 1990; Kockx and Herman, 2000; Malek et. al., 1999; Vanhoutte, 1989;). Importantly, ECs in lesion-prone regions, where atherosclerotic plaques preferentially develop are characterised by increased EC turnover rates, suggesting a mechanistic link between EC turnover and susceptibility to atherosclerotic plaque development (Caplan and Schwartz, 1973; Oliver et al., 2000). This enhanced turnover is most likely secondary to an increased level of apoptosis (Dimmeler et al., 1998). Furthermore, classical risk factors for atherosclerosis such as high glucose concentrations (Du et al., 1998), oxidized LDL (Dimmerler et al., 1997a), and
increased oxidative stress stimulate EC apoptosis (Dimmeler et al., 1997a;c; Lizard et al., 1997).

The focal nature of atherosclerotic lesion development in areas with turbulent, oscillatory, or low blood flow, such as bifurcations has suggested that one of the most potent endogenous anti-atherosclerotic factors is high laminar blood flow (Caro et al., 1971; Nerem, 1992; Triscot et. al., 2000). Normal levels of laminar shear stress have been shown to completely prevent apoptosis induced by various stimuli (Cho et al., 1997; Dimmeler et al., 1996; Kaiser et al., 1997). Moreover, a lack of hemodynamic force has been shown to trigger apoptosis of ECs (Dimmeler et al., 1996; Kaiser et al., 1997). Taken together, these data suggest a mechanistic link between low shear stress and/or turbulent shear stress, EC apoptosis, and susceptibility to atherosclerotic plaque development (Dimmeler et al., 1998b). EC death by apoptosis may also participate in plaque disruption and thrombosis. Exposure of the subendothelium to blood flow promotes platelet aggregation and vasospasm, and it has been recently observed that apoptotic ECs exhibit marked pro-coagulant activities and become pro-adhesive, even for non-activated platelets (Bombeli et al., 1997; 1999), although other work suggests maintained anti-aggregating (Xu et al., 2001) and pro-fibrinolytic (Zoellner et al., 1999) activities during EC apoptosis.

Triscot et al., (2000) have recently provided the first in vivo evidence that blood flow directly influences EC survival or apoptosis in human atherosclerosis.
Luminal EC apoptosis was observed in 60% of plaques examined (Triscot et al., 2000). It was found that in these plaques, there was a 7-fold increase in EC apoptosis in downstream parts of the plaques, where low shear stress prevails (Ku et al., 1985; Zarins et al., 1983), or compared with the upstream regions. Data suggests that low shear stress and, more importantly, oscillatory flow and flow reversal are permissive or even causative in the pathogenic process of atherosclerosis (Davies, 1995; Topper et al., 1996).

It has been postulated that shear stress modulates atherogenesis via its effects on endothelial-mediated alterations in coagulation, leukocyte and monocyte migration, smooth muscle growth, lipoprotein uptake and metabolism, and EC survival (Traub and Berk, 1998). A model for signal transduction in ECs in response to shear stress has also been proposed, including possible mechanotransducers (integrins, caveolae, ion channels, and G proteins), intermediate signalling molecules (c-Src, ras, Raf, protein kinase C), mitogen activated protein kinases (ERK1/2, JNK, p38, BMK-1), and effector molecules (nitric oxide) (Traub and Berk, 1998).
### Table I.1

**Physiological Angiogenesis**

- Ovulation
- Development of the corpus luteum
- Embryogenesis
- Lactating breast
- Immune response
- Orthodontic tooth movement
- Physiological tooth eruption

* Adapted from Polverini, 1995*
### Table L2

**Angiogenesis and Vasoproliferative-Dependent Diseases**

<table>
<thead>
<tr>
<th>Neoplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid tumours</td>
</tr>
<tr>
<td><strong>Vascular Malformations and Cardiovascular Disorders</strong></td>
</tr>
<tr>
<td>- Angiofibroma</td>
</tr>
<tr>
<td>- Hemangiomatosis</td>
</tr>
<tr>
<td>- Arteriovenous malformations</td>
</tr>
<tr>
<td>- Atherosclerosis</td>
</tr>
<tr>
<td>- Restenosis/ reperfusion injury</td>
</tr>
<tr>
<td>- Vascular adhesions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Syndromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Dyschondroplasia with vascular hamartomas (Maffucci’s syndrome)</td>
</tr>
<tr>
<td>- Hereditary hemorrhagic telangiectasia (rendu-Osler-Weber Syndrome)</td>
</tr>
<tr>
<td>- Van Hipple Lindau Syndrome</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ocular Disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Corneal graft neovascularisation</td>
</tr>
<tr>
<td>- Diabetic retinopathy</td>
</tr>
<tr>
<td>- Neovascular glaucoma</td>
</tr>
<tr>
<td>- Retrolental fibroplasia</td>
</tr>
<tr>
<td>- Trachoma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chronic Inflammatory Diseases and Wound Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Diabetes</td>
</tr>
<tr>
<td>- Granulations; burns</td>
</tr>
<tr>
<td>- Haemophiliac joints</td>
</tr>
<tr>
<td>- Hypertrophic scars</td>
</tr>
<tr>
<td>- Non-healing fractures</td>
</tr>
<tr>
<td>- Osteoradionecrosis</td>
</tr>
<tr>
<td>- Psoriasis</td>
</tr>
<tr>
<td>- Pyogenic granuloma</td>
</tr>
<tr>
<td>- Rapidly progressing adult and juvenile periodontitis</td>
</tr>
<tr>
<td>- Rheumatoid arthritis</td>
</tr>
<tr>
<td>- Systemic sclerosis</td>
</tr>
<tr>
<td>- Venus stasis ulcers</td>
</tr>
<tr>
<td>- Gingivitis</td>
</tr>
<tr>
<td>- Periodontitis</td>
</tr>
<tr>
<td>- Dental periapical granulomas</td>
</tr>
<tr>
<td>- Wound healing</td>
</tr>
<tr>
<td>- Healing fractures</td>
</tr>
</tbody>
</table>

* Adapted from Moses and Langer, 1991

*Literature Review. I. 60*
Table I.3

Pro-angiogenic Cytokines and Mediators of Angiogenesis

<table>
<thead>
<tr>
<th>Growth Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Acidic fibroblast growth factor (aFGF)</td>
</tr>
<tr>
<td>- Basic fibroblast growth factor (bFGF)</td>
</tr>
<tr>
<td>- Epidermal growth factor</td>
</tr>
<tr>
<td>- Interleukin 1 (IL-1)</td>
</tr>
<tr>
<td>- Interleukin 2 (IL-2)</td>
</tr>
<tr>
<td>- Scatter factor/ Hepatocyte growth factor</td>
</tr>
<tr>
<td>- Transforming growth factor alpha</td>
</tr>
<tr>
<td>- Transforming growth factor beta (TGF-β)</td>
</tr>
<tr>
<td>- Tumour necrosis factor alpha (TNF-α)</td>
</tr>
<tr>
<td>- Vascular endothelial growth factor (VEGF)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbohydrates and lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 12α-hydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>- Hyaluronan fragments</td>
</tr>
<tr>
<td>- Lactic acid</td>
</tr>
<tr>
<td>- Monobutyrin</td>
</tr>
<tr>
<td>- Prostaglandin E1 and E2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other Proteins and Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Angiogenin</td>
</tr>
<tr>
<td>- Angiopoietin II (Ang-II)</td>
</tr>
<tr>
<td>- Ceruloplasm</td>
</tr>
<tr>
<td>- Fibrin</td>
</tr>
<tr>
<td>- Human angiogenic factor</td>
</tr>
<tr>
<td>- Interleukin 8 (IL-8)</td>
</tr>
<tr>
<td>- Plasminogen activator</td>
</tr>
<tr>
<td>- Polyamines</td>
</tr>
<tr>
<td>- Substance P</td>
</tr>
<tr>
<td>- Urokinase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Adenosine</td>
</tr>
<tr>
<td>- Angiotropin</td>
</tr>
<tr>
<td>- Copper</td>
</tr>
<tr>
<td>- Heparin</td>
</tr>
<tr>
<td>- Nicatinamide</td>
</tr>
<tr>
<td>- ESAF</td>
</tr>
</tbody>
</table>

* Adapted from Polverini, 1995

* Literature Review. I. 61
### Table I.4

**Endogenous Inhibitors of Angiogenesis**

- Angiostatic steroids
- Angiostatin
- Eosinophilic major basic protein
- High molecular weight hyaluronan
- Interferon γ (INF-γ)
- Interferon γ-inducible protein 10
- Interferon α - 2 α and 2β
- Interleukin 1, 4 and 10
- Laminin and fibronectin peptides
- Nitric oxide (NO)
- Placental RNAse (angiogenin) inhibitor
- Platelet factor IV
- Prostaglandin synthetase inhibitor
- Somatostatin
- Substance P
- Thrombospondin 1
- Tissue inhibitors of metalloproteinases
- Retinoids
- Vitamin A
- Vitreous fluids

* Adapted from Polverini, 1995*
Table I.5

**Structural Proteins Processed by Caspases Associated With Apoptotic Morphology**

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Function/location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Microfilament forming protein with various localisation and functions, i.e. regulation of cell shape in the cortical cytoskeleton</td>
</tr>
<tr>
<td>Spectrin/fodrin</td>
<td>Actin cross-linking protein in cortical cytoskeleton</td>
</tr>
<tr>
<td>Beta-catenin</td>
<td>Intracellular attachment protein in cell-to-cell junction sites</td>
</tr>
<tr>
<td>Gelsolin</td>
<td>Microfilament fragmentation protein</td>
</tr>
<tr>
<td>Gas2</td>
<td>Microfilament organising protein</td>
</tr>
<tr>
<td>PAK2</td>
<td>Protein kinase involved in regulation of cytoskeleton</td>
</tr>
<tr>
<td>MEKK-1</td>
<td>Regulate cell survival and morphology at cell-matrix and cell-cell contacts</td>
</tr>
<tr>
<td>FAK</td>
<td>Regulate cell adhesion at cell-matrix and cell-cell contact sites</td>
</tr>
<tr>
<td>Keratins 18 and 19</td>
<td>Intermediate filament protein in keratinocytes</td>
</tr>
<tr>
<td>Rabaptin 5</td>
<td>Membrane protein that regulates intracellular vesicle traffic</td>
</tr>
<tr>
<td>Lamin A and B</td>
<td>Intermediate filament that forms the nuclear lamina</td>
</tr>
<tr>
<td>NuMa</td>
<td>Mediator of nuclear chromatin-matrix protein interactions</td>
</tr>
</tbody>
</table>

* Adapted from Saraste and Pulkki, 2000
<table>
<thead>
<tr>
<th>Bcl-2 blocks apoptosis caused by:</th>
<th>Bcl-2 does not block apoptosis caused by:</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Growth factor derivatives</td>
<td>- Negative selection in thymocytes</td>
</tr>
<tr>
<td>- Ionizing radiation</td>
<td>- CTL-mediated killing</td>
</tr>
<tr>
<td>- Calcium ionophore</td>
<td>- IgM-induced death of B cells</td>
</tr>
<tr>
<td>- Phorbol ester</td>
<td>- fas APO-1 ligation</td>
</tr>
<tr>
<td>- Methotrexate</td>
<td>- Hydrogen peroxide</td>
</tr>
<tr>
<td>- Anti-metabolites vincristine</td>
<td>- Activation-induced apoptosis T-cell</td>
</tr>
<tr>
<td>- Glucocorticoids</td>
<td></td>
</tr>
<tr>
<td>- Sodium azide</td>
<td></td>
</tr>
<tr>
<td>- Etoposide</td>
<td></td>
</tr>
<tr>
<td>- Oxygen depletion (hypoxia)</td>
<td></td>
</tr>
<tr>
<td>- Chemotherapeutic drugs</td>
<td></td>
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<tr>
<td>- Viral infection</td>
<td></td>
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<tr>
<td>- Fas ligation</td>
<td></td>
</tr>
<tr>
<td>- Tumour necrosis factor-α</td>
<td></td>
</tr>
<tr>
<td>- c-myc</td>
<td></td>
</tr>
<tr>
<td>- p53</td>
<td></td>
</tr>
<tr>
<td>- R-Ras</td>
<td></td>
</tr>
<tr>
<td>- Loss of adhesion</td>
<td></td>
</tr>
</tbody>
</table>

* Adapted from Saraste and Pulkki, 2000
Figure 1.1 A model for developmentally programmed capillary regression. Current evidence suggests that programmed capillary regression may occur through a two step mechanism. The model suggests that the first apoptosis of vascular ECs are dependent upon the macrophage (Lang and Bishop, 1993). This event is termed initiating apoptosis (A). Meeson et al., (1996) proposed that macrophage-mediated vascular EC death ultimately results in lumen restriction and a block to plasma flow within a capillary segment. Red arrows indicate flow and the red bar, a block to flow (B). Meeson et al., (1996) suggests that vascular ECs die subsequent with a synchronous pattern because they are denied survival factors present in plasma. This is referred to as secondary apoptosis (C). The large blue arrows indicate the likely chemotactic response of macrophages to apoptotic cells. From Meeson et al. 1996.
Chapter II. MATERIALS AND METHODS

II.1 Materials

II.1.1 Cell Culture Materials

Type 1A collagenase, Hanks Balanced Salt Solution (HBSS), and gelatin were purchased from Sigma (St. Louis, USA). JRH Biosciences (Lenexa, USA) supplied Medium 199 (M199), iron fortified foetal bovine calf serum (FCS) and trypsin/ Ethylene Diamine Tetra-acetic Acid (EDTA). Heparin was obtained from Pharmacia and Upjohn (Perth, WA, Australia). Penicillin/streptomycin solution was used in all cultures and obtained from CSL Biosciences (Parkville, Australia) while amphoteracin B was supplied by ICN Biomedicals (Ohio, USA). Endothelial Cell Growth Supplement (ECGS) was extracted from bovine hypothalamus using a method modified from that described by Maciag et al., (1984). Phosphate Buffered Saline (PBS) tablets were obtained from Oxoid (Hampshire, England) while Dimethylsulfoxide (DMSO) was from Ajax Chemicals (Auburn, Australia). 25 cm², 75cm², and 225cm² cell culture flasks were obtained from Costar (Cambridge, MA, USA). Disposable plastic centrifuge tubes were purchased from Iwaki, Scitech Division (Chiba, Japan). Thermax tissue culture coverslips on which HUVEC were grown were brought from Nunc Inc. (Naperville, Denmark). Disposable membrane filters were supplied by Sartorius, Minisart (Gottingen, Germany).
II.1.2 Histological and Immunological Reagents

*Ulex europaeus* lectin-1 (UEA-1), rabbit anti-UEA-1 antiserum, BSA, diamino-benzo-tetrahydrochloride (DAB) and haematoxylin were obtained from Sigma (St. Louis, USA). Normal goat serum (NGS) was obtained from Hunter Antisera (Jesmond, NSW, Australia). Hydrogen peroxide was supplied by Laboratory Supply (Milperra, NSW, Australia). Goat poly-clonal antibodies to PECAM-1, VE-CAD, Intergrin chain β2 (Int. β2), con-43 Donkey anti-Goat Biotin conjugate, and Donkey-anti-Goat FITC conjugate were purchased from Santa Cruz Biotechnology (CA, USA). Goat-anti-rabbit Origan Green and Streptavidin Texas Red conjugates were obtained from molecular probes (Eugene, USA). Triton X-100 was obtained from BDH Laboratory Supplies (Poole, England) while Dako (Carpinteria, California, USA) supplied both the rabbit anti factor VIII anti-serum and the peroxidase labelled goat anti-rabbit antibody. Streptavidin peroxidase conjugate was purchased from BioSource (Camberwell, VIC, Australia). Mediglass microscope slides were purchased from Lomb Scientific (Sydney, Australia) while Aquamount, aqueous mounting medium, used for coverslipping Termanox coverslips, was obtained from BDH Laboratory Supplies (Poole, England).
II.1.3 Materials for DNA Gel Electrophoresis

RNAse A and Proteinase K were supplied by Roche Molecular Biochemicals (Nutley, New Jersey, USA), while glycerol was purchased from BDH Laboratory supplies (Poole, England). Tris, Tris-HCl, Tris-borate, EDTA, sodium acetate, ethidium bromide and bromophenol blue were purchased from Sigma (St. Louis, USA). Ajax Chemicals (Auburn, NSW, Australia) supplied phenol, absolute ethanol, sodium chloride and potassium chloride. Molecular biology grade agarose, electrophoresis units, Minisub DNA Cell, Wide Minisub Cell and DNA Sub Cell, as well as a Model 200/2.0 Power Supply power pack were purchased from BioRad (Hercules, California, USA).

II.1.4 Materials for Histology and Transmission Electron Microscopy

Cacodylate buffer and uranyl acetate were purchased from Taab Laboratories Equipment Inc. (Aldermaston, Berkshire, UK). Reynolds’s lead citrate was prepared by stoichiometric reaction of lead nitrate supplied by Fluka (Buchs, Switzerland) and Trisodium Citrate supplied by Ajax Chemicals (Auburn, NSW, Australia). Ajax Chemicals (Auburn, NSW, Australia) also supplied Absolute Ethanol. Osmium Tetroxide, Glutaraldehyde, Spur’s Epoxy Embedding resin, slot grids and 300 mesh copper grids were purchased from ProSciTech (Thuringowa, QLD, Australia). Acetone was purchased from BDH Laboratory Supplies (Poole, England). Methylene Blue was obtained from Medos Co. Pty. Ltd. (Lidcombe, NSW). DiffQuick Giemsa stain was purchased from Laboratories Aid (Narrabeen,
Australia). Kodak electron microscope film, type 4489, was used to record results in electron microscope studies.

II.1.5 Materials for Fluorescence Activated Cell Scanning Analysis

Propidium iodide was supplied by Sigma (St. Louis, USA) while sodium citrate was obtained from Ajax Chemicals (Auburn, NSW, Australia). Triton X-100 was obtained from BDH Laboratory Supplies (Poole, England). Falcon FACS tubes were from Becton Dickinson Labware (New Jersey, USA).

II.1.6 Stimuli

IISA was purchased from Calbiochem (Alexandria, USA). BSA was purchased from Sigma (St. Louis, USA). Oscillating shear stress generation is explained in II.1.7.

II.1.7 Materials For Shear Stress Experiments

Experiments were performed with a simple laboratory rocking platform. Triplicate 25 cm² tissue culture flasks were placed on the rocking platform for a period of 24 hours in a humidifier CO2 incubator. The laboratory rocking platform produces oscillating shear stress. Figure II.1 illustrates the action of the rocking platform on EC cultures.
II.2 Methods

II.2.1 Isolation of Human Umbilical Vein Endothelial Cells

HUVEC were isolated from umbilical cords obtained from normal vaginal deliveries from the maternity ward of Westmead Hospital with ethical approval from Westmead Hospital Human Research Ethics Committee (HREC 95/6/4.14). HUVEC were isolated using a method modified from that described by Jaffé et al., (1973).

Briefly, cords were collected from Westmead Hospital Delivery Ward, stripped of as much blood as possible and stored at 4°C in sterile containers with HBSS, for subsequent isolation of HUVEC.

Umbilical cords were washed with normal saline before cannulation of veins at one end. The veins were then perfused with up to 10 ml of HBSS to remove remaining blood before perfusion with 8 ml and incubation with 2 ml for 20 minutes of 0.1% collagenase solution in HBSS and clamping of the remaining free end. Cords were then incubated at room temperature for 30 minutes before incision of the clamped end and collection of the collagenase solution containing endothelial cells into 2 ml of FCS in 50 ml centrifuge tubes. Remaining loosely adherent HUVEC were collected by further perfusion with up to 30 ml of HBSS and pooling with the initial collagenase perfusate. HUVEC were pelleted by centrifugation for 10 minutes at 1500 rpm before discarding the supernatant and re-suspension of cells into 5 ml of complete culture medium consisting of M199.
with FCS (20%), ECGS (50 µg/ml), Heparin (30 U/ml), Penicillin (100 U/ml), Streptomycin (100 µg/ml) and Amphotericin B (2.5 µg/ml). HUVEC were then plated into 25 cm² tissue culture flasks and incubated at 37°C under CO2 (5%) in a humidified incubator. All tissue culture surfaces were pre-coated with gelatin (0.1%) in PBS. Fresh culture medium was supplied on the day following initial isolation and on every third to fourth day thereafter.

II.2.2 Culture of Human Umbilical Vein Endothelial Cells

Confluent cells were passaged at a split ratio of 1:3 into 75 cm² and 225 cm² flasks as convenient. Briefly, cells were split by first washing the monolayers with M199 and then treating with trypsin (0.25%) in HBSS with EDTA (0.02%) (trypsin/EDTA). The culture medium was initially removed, adherent cells were washed with M199 before application of 5 to 15 ml of trypsin/EDTA, depending on the size of the tissue culture flask. Cells were agitated and then placed in the humidified incubator for 5 minutes to allow for adequate detachment. HUVEC readily detached using this procedure. This process was confirmed by phase microscopy. Once detached HUVEC were collected into centrifuge flasks containing 5 ml of FCS to neutralise trypsin activity. HUVEC were then pelleted by centrifugation at 1000 rpm and 4°C for 5 minutes. After pelleting, cells were resuspended in an appropriate volume of complete culture medium and seeded for further culture. All culture surfaces for HUVEC were treated with gelatin (0.1%) in PBS. Cells were stored frozen in liquid nitrogen with DMSO. HUVEC were cultured to a maximum of fifth passage. All experiments were performed with

*Materials and Methods, II. 6*
cells from fourth and fifth passage. The identity of HUVEC was confirmed on the basis of their characteristic cobblestone morphology, staining for Factor VIII associated antigen (Jaffe et al., 1973) and the binding of *Ulex Europeaus* lectin (Holthofer et al., 1982) as performed by Dr. M. Xaymardan and Dr. H. Zoellner of this laboratory, and described elsewhere in II.1.4.

II.2.3 Storage of Human Umbilical Vein Endothelial Cells in Liquid Nitrogen

HUVEC from 75 cm² or 225 cm² flasks were released using trypsin/EDTA as described in Section 2.2.b. Cell pellets were then resuspended in complete medium with DMSO (10%) in M199. Confluent cells from 75 cm² of culture surface were resuspended in 1 ml of this cryopreserving solution. Cell suspensions were transferred to cryovials for gradual freezing. This was achieved by firstly storing cryovials in a foam container at −40°C overnight before transferring to liquid nitrogen for long-term storage. When needed, vials were removed from liquid nitrogen and rapidly thawed by gentle agitation in a 37°C water bath. These cells were then resuspended in 10 ml of complete medium and pelleted by centrifugation at 1000 rpm, 4°C for 5 minutes. The resulting pellets were then resuspended in 15 ml of complete medium and cultured in gelatin coated 75 cm² flasks.
II.2.4 Immuno and Lectin Fluorescence Staining of Human Umbilical Vein Endothelial Cells

Cells were cultured on gelatin-coated tissue cover-slips, and fixed with formaldehyde (1%) in PBS for 10 minutes before washing with PBS and blocking of endogeneous peroxidase activity and non-specific protein binding sites with H$_2$O$_2$ (1%) and FCS (10%) in PBS for 20 minutes at room temperature. UEA-1 and Factor VIII antigen were detected by lectin and immuno-histochemistry as described elsewhere (Zoellner et al., 1996a; 1999).

II.2.5 Culture Conditions for Human Umbilical Vein Endothelial Cells During Experiments

Araki et al., (1990a; b), Meredith et al., (1993) and Zoellner et al., (1996a) reported that serum deprivation activates apoptosis in HUVEC. HUVEC from 4th to 5th passage were harvested and seeded at confluence in triplicate 25cm$^2$ tissue culture flasks in a volume of 5 ml of either: a) M199 with FCS (20%); b) M199 with HSA (4%); and c) M199. In all media Penicillin (100 U/ml), Streptomycin (100 µg/ml) and Amphotericin B (2.5 µg/ml) were added. It is important to note that both ECGS and Heparin were excluded from these media, as these agents are used to promote proliferation of cultured endothelium, which was not desired for these experiments. Cells were cultured for 24 hours at 37°C under CO2 (5%) in a humidified incubator.
Briefly, monolayers of HUVEC were washed with 5 ml of M199, taking care that the monolayers did not dry out during this procedure. This was followed by the addition of 5 ml of the appropriate medium: M199 alone or supplemented with either HSA (4%) or FCS (20%). The cells were then incubated for 24 hours at 37°C under CO2 (5%) in a humidified incubator.

Earlier work demonstrated that HUVEC detach early during apoptosis while levels of cell proliferation are negligible in these culture conditions. From this, the number of surviving adherent cells provides an accurate, although indirect measure of apoptosis in HUVEC cultures (Zoellner et al., 1996b, 1999). In all experiments, the remaining surviving adherent cells were quantified by adherent cell counts. This was performed by washing each set of triplicate tissue culture flasks with 5 ml of M199, before release of adherent cells with 5 ml of trypsin/EDTA. The released cells were then counted using a haemocytometer and cell counter. An apoptosis protection index (API) was calculated for each triplicate set of data in which the apoptosis protection index represented the percentage of adherent surviving HUVEC. An apoptotic protection index ranging from 0 to 100 was defined. A value of 100 was assigned to the number of adherent cells remaining after treatment with FCS (20%) so that the effect of other agents upon EC survival is expressed as a percentage of protection by serum. This method gave good reproducibility both within triplicates and between experiments, permitting the application of Student t-tests to the data. This apoptosis index has been used in earlier studies in this laboratory (Zoellner et al.,

Materials and Methods, II. 9
1996b; 1999) and permits ready comparison of data performed on different days collected from different donors.

II.2.6 Exposure of Cultured Human Umbilical Vein Endothelial Cells to Oscillating Shear Stress

HUVEC were cultured in the same way as described in II.2.5 Cells were cultured for 24 hours at 37°C under CO2 (5%) in a humidifier incubator and were either exposed to combined pulsatile and oscillatory shear stress by incubating on a simple laboratory rocking platform, tipping 6° from horizontal in both directions at a rate of 15 cycles per minute, or alternatively cultured in static conditions on an adjacent shelf. The remaining adherent surviving HUVEC were quantified by cell counts as described in II.2.5. Confirmation of apoptosis in detached cell populations was achieved using phase contrast microscopy, TEM, DNA gel electrophoresis and FACS analysis.

II.2.7 DNA Gel Electrophoresis

DNA fragmentation was observed using a method modified from that described by Smith et al., (1989). HUVEC were cultured in 225cm² tissue culture flasks as described in II.2.5 and II.2.6. It has been previously reported that detached HUVEC are apoptotic in these culture conditions (Robaye et al., 1991; Tsukada et al., 1995; Zoellner et al., 1996b). The detached, non-adherent, floating cells were collected by washing with M199 (15 ml), while the attached, adherent and presumably non-apoptotic cells were released by trypsin/EDTA, as described

*Materials and Methods, II. 10*
in II.2.2. The non-adherent and adherent HUVEC were then pelleted separately by centrifugation at 2000 rpm at 4°C for 5 minutes. Pellets were then resuspended in 1 ml of tris-buffered saline (TBS) (25 mM, pH 8.0) in eppendorf tubes, followed by further centrifugation at 1500 rpm at 4°C for 10 minutes. The resulting pellets were resuspended in 500 μl of lysis buffer comprising Tris (10 mM), EDTA (1 mM), SDS (0.5%) and RNase A (0.1 mg/ml). Preparations were then incubated for 1 hour at 37°C before adding proteinase K to a final concentration of 0.5 mg/ml and further incubation for 3 hours at 55°C. The solution was made up to 500 μl with Tris-EDTA (TE) buffer, consisting of Tris-HCL (10 mM) with EDTA (1 mM) at pH 8.0 and subjected to three rounds of extraction with Tris-buffered phenol. DNA in the aqueous phase was precipitated by the addition of 50 μl sodium acetate (3 M) followed by two volumes of ice cold absolute ethanol (1 ml). The solution was then mixed by inversion before storage at -40°C for at least 30 minutes. Prior to electrophoresis, the solution was centrifuged at 13000 rpm for 10 to 15 minutes to pellet precipitated DNA. The pellet was then washed with ice cold ethanol (70%) and recentrifuged before removal of the supernatant and allowing the pellet to dry at room temperature. The dried pellet was resuspended in 15 μl of TE buffer before adding 5 μl of a loading buffer, consisting of bromophenol blue (0.25%) and glycerol (30%) in water. DNA was then subjected to electrophoresis using agarose gel (2%) with ethidium bromide (0.5%) at 70 V for 30 to 45 minutes and electrophoretic motility assessed by visualisation with UV light. Results were recorded photographically using a DS34 Polaroid Direct Screen Instant Camera and Polaroid 667 Black and White film.
II.2.8 Transmission Electron Microscopy

HUVEC stimulated in 225 cm² tissue culture flasks (II.2.5 and II.2.6) were collected. Briefly detached, non-adherent, floating cells were collected by washing with M199 (15 ml), while the attached, adherent and presumably non-apoptotic cells were released by trypsin/EDTA, as described in II.2.2 The non-adherent and adherent HUVEC were then pelleted separately by centrifugation at 2000 rpm at 4°C for 5 minutes. Cell pellets were resuspended in 1 ml of glutaraldehyde (0.25%) in PBS for 15 minutes at 4°C followed by centrifugation at 2000 rpm at 4°C for 5 minutes. The resulting pellets were fixed with glutaraldehyde (2.5%) in PBS for 1 hour at 4°C. After this, the pellets were washed three times with PBS and stored in PBS at 4°C. Pellets were then post-fixed with osmium tetroxide (2%) in cacodylate buffer (0.1 M, pH 7.4) for 3 hours, washed with water and further treated with uranyl acetate (2%) for 1 hour. Specimens were dehydrated with 10 minute immersions in graded alcohol prepared with aqueous NaCl (1%) with consecutive concentrations of ethanol of: 50%, 75%, 100% and 100%. Ethanol was then removed by two further 10 minute immersions in dry acetone. Samples were infiltrated with an acetone/resin mixture (1:1) for 1 hour followed by three exposures to resin at 70°C for 10 minutes each. Fresh resin was used for polymerisation at 70°C for 10 hours. The resulting blocks were cut from the eppendorf tubes and semi-thin sections prepared using a Reichert Ultracut E microtome (Vienna, Austria). The semi-thin sections were stained with methylene blue (1%) for 2 minutes each. Ultra-thin sections were collected on either slot or 300 mesh copper grids and briefly stained

Materials and Methods, II. 12
with uranyl acetate (2%) for 15 minutes and washed with ultrapure water for at least 15 seconds before further staining with Reynold’s lead citrate for 4 minutes and again washing with ultrapure water. Grids and slots were examined using Phillips 400 and Phillips CM 120 BioTWIN, transmission electron microscopes (Eindhoven, Netherlands) at 100kV.

II.2.9 Fluorescence Activated Cell Scanning Analysis

HUVEC were cultured in 25 cm² tissue culture flasks as described in II.2.5 and II.2.6. Non-adherent cells were collected by washing with M199, while the attached cells were released using trypsin/EDTA as described in II.2.2. The non-adherent and adherent cell populations were combined and pelleted by centrifugation at 1500 rpm at 4°C for 8 minutes. Cell pellets were resuspended in HBSS (1 ml) and then added dropwise to 5 ml of ice cold ethanol (70%), while gently tapping, ensuring adequate mixing of the cells. The ethanol mixture was then stored at -20°C until required. 24 hours prior to FACS analysis, 3 ml of the ethanol suspensions were transferred to FACS tubes and centrifuged at 2000 rpm at 4°C for 5 minutes, while the remaining 3 ml aliquots were stored at -20°C for further analysis if required. Cell pellets were allowed to dry at room temperature and then resuspended in 400 μl of HBSS containing propidium iodide (50 μg/ml), RNase (0.1 mg/ml), sodium citrate (0.1% w/v) and Triton X-100 (0.1%). Cell suspensions were then incubated at room temperature in the dark for 24 hours before analysis with a Becton Dickinson FACScan Device using Cell Quest
version 3.2.1 fl software. Data was analysed using Windows multiple Document Interface (WinMDI) Flow Cytometry Application, Version 22.8
Figure II.1 Diagrammatic Representation of Rocking Platform. Illustrating culture flasks on rocking platform and the generation of oscillating shear stress through movements of the rocking platform, as shown in figures II.1.A, II.1.B, and II.1.C. The maximum inclination angle of 6° is shown, with a maximum of 15 cycles per minute.
Chapter III. RESULTS

III.1 Oscillating Shear Stress Resulted in Denudation of Culture Surface on the Basis of Phase Contrast Microscopy

Figure III.1 illustrates the typical appearance of cells cultured in the three separate media exposed to either oscillating shear stress or static cultures, not exposed to any form of shear stress used in this study using phase-contrast microscopy. Phase-bright cells indicative of EC fragmentation and apoptosis, loss of EC adhesion, denudation of the culture surface and decreased levels of cellular confluence can be observed.

Static EC cultures in FCS (20%) retained their cobblestoned morphology. Occasional phase-bright apoptotic cells were seen, indicative of a baseline rate of apoptosis in this medium. Increased numbers of phase-bright cells were observed in ECs cultured in HSA (4%). Although showing a similar morphology, there were areas of denudation in the culture surface and subsequently a noticeable reduction in confluence in these cultures which was not observed in cultures with FCS (20%). EC cultures in serum free conditions (M199) exhibited the highest rate of apoptosis, which was characterised by high numbers of phase-bright cells, early loss of EC adhesion and the exposure of denuded areas in the culture.

Phase contrast microscopy of EC cultures exposed to oscillating shear stress revealed greater numbers of phase-bright cells undergoing fragmentation

Results, III.1
typical of EC apoptosis. By 4 hours of exposure to oscillating shear stress, EC fragmentation could be observed. This was subsequently followed by loss of EC adhesion and the resultant denudation of cell culture surface and decreased cellular confluence. These changes were noted throughout the entire period of the experiments. Although these observations were seen in static cultures, greater levels were observed in all cultures exposed to oscillating shear stress in all experiments performed.

As with ECs from static cultures, cells cultured in FCS (20%) exposed to oscillating shear stress partly retained their typical cobblestoned morphology. Increased numbers of phase-bright cells were observed, indicative of higher levels of EC apoptosis. This further resulted in small areas of denudation in the culture surface.

ECs cultured in HSA (4%) exposed to oscillating shear exhibited increased levels of phase-bright cells, in comparison with the control culture and those cells cultured in FCS (20%). The increased level of EC apoptosis resulted in larger areas of denudation in the culture surface with reduced confluence.

The highest numbers of phase-bright cells were observed in serum free cultures (M199 alone) exposed to oscillating shear stress. These cultures also exhibited the largest areas of culture surface denudation and the lowest levels of

*Results, III.2*
cell confluence, indicating the exposure to oscillating shear stress in M199 alone resulted in the greatest levels of EC apoptosis.

III.2 Quantitation of Cells in Static and Oscillating Shear Stress Cultures Revealed Reduced Cell Survival

Quantitation of surviving cells in all experiments of oscillating shear stress cultures are shown in Figure III.2. Experiments were performed 18 times using triplicate flasks and 6 donors, with all cells at 5th passage.

Loss of EC adhesion was found to be an early event in EC apoptosis and was exploited to quantitate apoptosis. In static serum free conditions (M199 alone), many cells started to fragment into small apoptotic bodies and detach. Cells undergoing such fragmentation rapidly lost adhesion to the surface. EC fragmentation continued after loss of adhesion. It is important to note that few cells with this apoptotic morphology could be found in EC cultures before serum deprivation.

Quantitation of surviving ECs in static cultures confirmed previous observations by phase contrast microscopy. EC cultured in FCS (20%) exhibited the lowest levels of detached cells, while EC cultured in HSA (4%), exhibited slightly higher levels of detached cells. Furthermore, it was observed that EC cultured in M199 alone, exhibited the lowest level of cell survival, and thus the

Results, III.3
greatest level of apoptosis in static cultures. These findings were consistent in all 18 triplicate experiments performed.

The additional stimulus of oscillating shear stress significantly increased apoptosis in all three media used (FCS (20%) p<0.01; HSA (4%) p<0.001; M199 alone p<0.005). These observations were consistent in all 18 triplicate experiments. The data demonstrated a significantly greater loss of EC adhesion, and thus decreased cell survival in all media stimulated by oscillating shear stress in comparison with static cultures. Morphologically, ECs started to fragment into small apoptotic bodies and detach earlier in comparison to static cultures. This was observed to begin within 4 hours of exposure to shear stress. EC fragmentation continued after the loss of adhesion to the surface monolayer.

III.3 The Basis of Reduced Survival of Endothelium in Oscillating Shear Stress was Apoptosis

III.3.1 Transmission Electron Microscopy Revealed Typical Apoptotic Features in Detached Endothelial Cells

Ultrastructural features of adherent and detached cells were to be identical in all culture conditions studied. TEM revealed no signs of significant cellular toxicity or necrosis.

Figures III.7 and III.8 show the typical ultrastructural appearance of adherent ECs exhibiting no apoptotic characteristics in all media used, both

Results, III.4
exposed to oscillating shear stress and the static group. There were no signs of cellular or organelar swelling, ribosomes remained studded across the rough endoplasmic reticulum (RER) and chromatin appeared loose and vesicular, consistent with the absence of cytotoxicity. It is interesting to note that occasional lipid vacuoles were observed. This was suggestive of some degree of stress in the attached cultured cells exposed to oscillating shear stress. This observation was not present in the adherent population from cultures not exposed to oscillating shear stress. There was also minimal surface blebbing, but this was attributed to the processing for TEM. Adherent cells were also larger compared with detached cells.

Figures III.3. - III.6 and III.9 show the ultrastuctural features typical of EC apoptosis in detached cells in all media used, both exposed to oscillating shear stress and in the static group. Typical features included: the formation of small apoptotic particles with electron dense cytoplasmic contents; condensation and fragmentation of nuclear material into small spherical bodies, and canalicular fragmentation. Intracellular organelles such as mitochondria, endoplasmic reticulum, ribosomes, and Weibel Palade bodies were preserved and intact in detached cells. There was a tendency for canalicular fragmentation to displace cytoplasmic residues to the periphery of apoptotic particles. These changes indicated that loss of adherence of EC was a result of apoptosis.

Results, III.5
III.3.2 DNA Gel Electrophoresis was Consistent with Apoptosis of Detached Cell Populations in Endothelial Cultures Exposed to Oscillating Shear Stress

Figure III.10 shows DNA gel electrophoresis of detached ECs in cultures exposed to oscillating shear stress. A 180 BP ladder typical of apoptotic internucleosomal DNA fragmentation was seen. Internucleosomal DNA fragmentation was seen in all samples. This characteristic DNA "ladder" was visible in DNA gels of nuclear material from cultured EC not exposed to shear stress and stimulated in serum free media. This confirmed the detached population of ECs as apoptotic. Adherent cells did not show any of the characteristic features of apoptosis. This was consistent with the expectation that detached ECs were apoptotic.

III.3.3 A Sub-diploid Population Typical of Apoptosis was Present in Populations Exposed to Oscillating Shear Stress

Figure III.11 shows typical results of FACS Analysis for the DNA content in cultured ECs in all media, both exposed to oscillating shear stress and those in static conditions. FACS analysis indicated that the cell populations in the S, G0-G1 and G2-M phases progressively decreased after the cells were subjected to oscillating shear stress, while the sub-diploid apoptotic peak increased in all EC populations subjected to oscillating shear stress. FACS analysis was repeated four times using the same conditions, each time confirming EC apoptosis. This technique was used to confirm EC apoptosis, but although quantitation of the sub-diploid peak was possible, the occurrence of secondary necrosis and

Results, III.6
fragmentation made quantitation of apoptosis by this method inaccurate and this was not used to quantitate EC apoptosis. These data indicate that detached EC exposed to oscillating shear stress were apoptotic.

III.4 A Flow Cell Apparatus For Quantitative Analysis of Shear Stress was Designed and Manufactured.

III.4.1 Materials for flow apparatus chamber

A parallel plate laminar flow chamber modified from that described by Levesquw and Nerem in 1985 was designed jointly by the Department of Oral Pathology and Oral Medicine, University of Sydney, and the Department of Mechanical Engineering, University of Sydney. The chamber flow apparatus was designed after experiments with the laboratory rocking platform, in order to quantitate the level and type of laminar shear stress and the effect on EC apoptosis. The apparatus was constructed from Perspex to hold two separate single tissue culture slides (Nunc). The apparatus was constructed by Ralua Engineering, Dee Wee, NSW, Australia. The rubber gasket used in the flow chamber was purchased from Amaando, Silverwater, NSW, Australia.

III.4.2 Design and Manufacture of Flow Chamber Apparatus

Figures III.12 - III.15 illustrate the design of the flow chamber apparatus. The apparatus was constructed using two rectangular Perspex blocks. The main Perspex block (A) was constructed in three phases in order to achieve the specified design. It measured 200.0 mm in length, with a width of 100.0 mm and

Results, III.7
depth of 20.0 mm. The apparatus contained two inlet and outlet flow paths constructed of stainless steel tubing, each with an internal diameter of 5.0 mm, and length 70.0 mm. The inlet and outlet tubing was inserted into the Perspex block to a depth of 30.0 mm, at an angle of 9°. From the inlet tubing the internal aspect of the flow chamber was machined to create two flow channels measuring a length of 50.0 mm, with a width of 16.0 mm and depth of 1.0 mm. These flow channels were designed to establish uniform laminar shear stress over the cultured cells with uniform flow rates. This upper Perspex block was designed to sit upon a perspex base into which an area had been machined to a depth of 1.0 mm, with a length 46.0 mm and width of 16.0 mm forming the floor of the flow chamber.

Tissue culture cover slides with monolayers of HUVEC were placed into this recess for stimulation. Medium passing across the HUVEC exited flow chambers into exit flow channels and metal tubing to return to the culture pump. The rectangular border recess housed rubber gasket, and also stabilised tissue culture slides with HUVEC. Figures III.15 illustrate the design of the apparatus and medium flow through the apparatus. The assembled apparatus was held together with six stainless steel bolts evenly distributed.

The fluid mechanical properties of similar channel flow devices have been described at both theoretical and experimental levels (White, 1974). In such a channel and for a Newtonian fluid, the velocity profile has the simple parabolic form:

Results, III.8
\[ u = \frac{1}{2} \mu \times \frac{dp}{dx} \left( \frac{h^2}{4} - y^2 \right) \]  

(1)

where \( y \) is measured in the vertical dimension from the centreline both up and down, and the position of the upper and lower surfaces is at \( y = \pm \frac{h}{2} \), respectively, where the velocity is zero. The maximum velocity is at the centreline, i.e., \( y = 0 \), and there

\[ u_{\text{max}} = - \frac{1}{8} \mu \times \frac{dp}{dx} \times h^2 \]  

(2)

Here \( \mu \) is the fluid viscosity and \( \frac{dp}{dx} \) is the pressure gradient along the channel. The mean velocity, \( \bar{u} \), averaged over the height of the channel, is given by the equation

\[ \bar{U} = \frac{2}{3} u_{\text{max}} = - \frac{1}{12} \mu \times \frac{dp}{dx} \times h^2 \]  

(3)

and the flow rate, \( Q \), thus may be expressed as

\[ Q = h \times (b) \bar{u} = - \frac{1}{12} \mu \times \frac{dp}{dx} \times h^3 (b) \]  

(4)

where \( b \) is the width or span of the channel. The pressure drop, \( \Delta p \), divided by the length of the channel, \( L \), is the pressure gradient, \( \frac{dp}{dx} \). Thus, as may be seen, knowing \( \Delta p \), the viscosity of the perfusing fluid, \( \mu \), and the geometry of the channel, i.e., \( h, b, \) and \( L \), then the mean velocity, \( \bar{u} \), and the flow rate, \( Q \), can be determined.

For such a flow, the wall shear stress, \( \tau_w \), to which a test specimen of cultured cells will be exposed is defined mathematically as

\[ \tau_w = \mu S_w \]  

(5)

where \( S_w \) is the wall shear rate and given by the equation

\[ S_w = - \frac{\partial u}{\partial y} \]  

(6)

Equation (6) may be evaluated using equation (1) with the result that

Results, III.9
\[ S_w = -\frac{h}{2\mu} \times \frac{dp}{dx} \quad (7) \]

and
\[ \tau_w = -\frac{h}{2} \times \frac{dp}{dx} \quad (8) \]

thus, knowing the pressure gradient, \( \frac{dp}{dx} \), which can be determined experimentally from a measurement of the pressure drop, \( \Delta p \), over the length of the channel, \( L \), one can determine the wall shear stress, \( \tau_w \).

It is also important that the flow conditions to which HUVEC are exposed are in the physiological range. To ensure this, it is necessary that the flow be laminar and of a two-dimensional, fully developed character. The parameter of interest in this is the Reynolds number, \( \text{Re} \), where \( v \) is the kinematic viscosity, i.e., \( \mu = \nu \rho \), where \( \rho \) is the density of the perfusing fluid.

\[ \text{Re} = \frac{u(h)}{v} \quad (9) \]

Combining equations (3), (8), and (9), the wall shear stress can then be expressed as
\[ \tau_w = 6\nu \rho h^2 \times \text{Re} \quad (10) \]

Taking the perfusing fluid to be normal culture media and assuming its viscosity and density are that of water, then equation (10) is a relationship between the wall shear stress, the Reynolds number of the channel flow, and the channel height, where \( \mu = \) fluid viscosity.

For laminar flow to be created, the Reynolds number, \( \text{Re} \), must be less than 2000. For the flow to be two-dimensional in character, the channel width or length must be much greater than the channel height (or depth). Finally, for the velocity profile to be fully developed, then the inlet length for the flow (70.0 mm) is less compared to the total length of the channel (148.0 mm).

Results, III.10
The chamber flow apparatus was positioned in a continuous flow loop. The flow loop consisted of an elevated reservoir and a roller pump to return the outflow from the collecting reservoir back to the feeding reservoir. There were a further four 100.0 ml glass containers within the continuous loop which served to establish constant flow from the peristaltic pump by allowing air in the sealed bottles to dampen pulses. The flow loop had a volume of 200.0 ml and was filled with water for testing.

The chamber flow apparatus will be modified so that a syringe attached to a Scotch Yoke is connected to a motor drawing medium in the reverse direction to that of the otherwise normal flow by compression of the silicone tubing.

Prior to experimentation, the flow chamber may be sterilised using plasma sterilisation. Unfortunately, time restraints of this project precluded actual use of the apparatus after manufacture.

III.4.3 Proposed Exposure of Cultured HUVEC to Laminar, Oscillating and Pulsatile Shear Stress Using Chamber Flow Apparatus

HUVEC were cultured in the same way as described in II.2.5, with the difference that cells were cultured on tissue culturing cover slides (Nunc) for insertion into the flow chamber apparatus. A culture medium consisting of M199 with antibiotics and HSA (4%) buffered with HEPEC was used in these experiments, while cells were stimulated for up to 24 hr. Tissue culture slides

Results, III.11
were fixed with formalin (10%) in PBS before staining with haematoxylin. Changes in cell shape and number were recorded by light microscopy and manual counts of cells in five randomly identified fields as defined using a microscope graticule.

Unfortunately, due to time constraints it was not possible to proceed with experiments using cultured cells and the designed chamber flow apparatus.
Figure III.1 Phase Contrast Photomicrograph of Human Umbilical Vein Endothelial Cells Cultured in Foetal Calf Serum (20%), Human Serum Albumin (4%) and M199 Alone with or without Exposure to Oscillating Shear Stress for 24 hrs. Cells cultured in FCS (20%) retained confluence with a cobblestone morphology as did cells cultured in HSA (4%), while cells cultured in the absence of serum were significantly less confluent. Floating above the layer of adherent cells, were detached phase-bright cells with morphology typical of apoptotic endothelium. Exposure of cells to shear stress resulted in increased levels of endothelial apoptosis. After 24 hr of exposure to shear stress, significantly more floating cells were visible in all cultures exposed to shear stress. Phase contrast photomicrographs are taken at 0 hrs and 24 hrs.

(Bar = 100μm.)
Figure III.1 Phase Contrast Photomicrograph of Human Umbilical Vein Endothelial Cells Cultured in Foetal Calf Serum (20%), Human Serum Albumin (4%) and M199 Alone with or without Exposure to Oscillating Shear Stress for 24 hrs. Cells cultured in FCS (20%) retained confluence with a cobblestone morphology as did cells cultured in HSA (4%), while cells cultured in the absence of serum were significantly less confluent. Floating above the layer of adherent cells, were detached phase-bright cells with morphology typical of apoptotic endothelium. Exposure of cells to shear stress resulted in increased levels of endothelial apoptosis. After 24 hr of exposure to shear stress, significantly more floating cells were visible in all cultures exposed to shear stress. Phase contrast photomicrographs are taken at 0 hrs and 24 hrs.

(Bar = 100μm.)
Figure III.2 Histogram Illustrating the Effect of Oscillating Shear Stress on Human Umbilical Vein Endothelial Cell Survival. Histogram showing the effect of oscillating shear stress on HUVEC survival in FCS (20%), HSA (4%) and M199 alone after 24 hr compared to HUVEC cultured in static conditions. Results are obtained from 18 experiments using triplicate cultures. There was a decreased level of EC survival in cultures exposed to oscillating shear stress.
Figure III.3 Transmission Electron Micrograph of Detached Human Umbilical Vein Endothelial Cells Exposed to Shear Stress Cultured in M199 for 24 hr.

Cells were clearly apoptotic (arrows) with condensed nuclear material (stars) and evidence of canalicular fragmentation (C), while the electron density of the cytoplasm was increased relative to that of non-apoptotic adherent cells (Figures III.7 and III.8). These cells were also significantly smaller than non-apoptotic cells. Loosely adherent debris was noted of approximately the same size as apoptotic cells and this was interpreted to be secondary necrosis of apoptotic particles (SN). At low magnification, various forms of apoptotic cells are illustrated. All detached ECs displayed the typical features of EC apoptosis.

(Bar = 5 μm)
Figure III.4 Transmission Electron Micrograph of Detached Human Umbilical Vein Endothelial Cells in Figure III.3 Cell (i) at High Magnification.

At higher magnification the finer detail of RER (large arrowheads), mitochondria (m) and canalicular fragmentation (C) are clearly seen. The appearance of detached HUVEC in transmission electron microscopy were identical, regardless of the medium used.

(Bar = 2 μm)
Figure III.5 Transmission Electron Micrograph of Detached Human Umbilical Vein Endothelial Cells in Figure III.3 Cell (ii) at Low (A) and High (B) Magnification. At higher magnification the apoptotic EC appears to be breaking up into smaller apoptotic bodies, and then possibly undergoing secondary necrosis. Fragmentation of the cell appears to have originated from canalicular fragmentation (C). The cell was also significantly smaller and had more electron dense cytoplasm than non-apoptotic cells. Condensation of nuclear material was not as clear as on previous TEMs and it is thought that this cell had passed the typical and classic apoptotic stage. Despite the obvious cellular fragmentation, organelles including mitochondria (m), RER (large arrowheads) and Weibel Palade bodies (WP) are well preserved and intact. The organellar structures of mitochondria (m) and Weibel Palade bodies (WP) are seen more clearly at higher magnification (B), as was evidence of canalicular fragmentation (C). These observations are consistent with apoptotic, but not necrotic death of HUVEC.

(Bar for A = 2 µm, Bar for B = 500 nm)
Figure III.6 Transmission Electron Micrograph of Detached Human Umbilical Vein Endothelial Cells Exposed to Shear Stress Cultured in M199 for 24 hr.

This cell has all the features of classic cellular apoptosis, including: significant reduction in cell size in comparison with non-apoptotic cells; increased cytoplasmic electron density; nuclear condensation and fragmentation (stars); and maintained intracellular organelles, including mitochondria (m) and RER (large arrowheads) structures.

(Bar = 1μm)
Figure III.7 Transmission Electron Micrograph of Adherent Human Umbilical Vein Endothelial Cells Exposed to Shear Stress Cultured in M199 for 24 hr after Release from the Culture Surface with Trypsin/EDTA. The adherent non-apoptotic cells were significantly larger in size compared to detached apoptotic cells. Large vesicular nuclei (N) were present while the cytoplasm was electron lucent and plentiful. There is evidence of organellar structures which are more clearly seen in Figure III.8 at higher magnification. These features were typical of non-apoptotic endothelium. (Bar = 10 μm).
Figure III.8 Transmission Electron Micrograph of Adherent Human Umbilical 
Vein Endothelial Cells in Figure III.7 at Low (A) and High (B) Magnification.
At higher magnification large vesicular nuclei (N) is present while the cytoplasm 
was electron lucent and plentiful. Golgi apparatus (g) and lipid vacuoles (L) can 
be identified in (A), while at higher magnification (B), mitochondria (m) and RER 
(large arrowheads) structures can be seen. The presence of lipid vacuoles is the 
only clear sign of stress in HUVEC. These cells and their features were typical of 
all adherent non-apoptotic endothelium in all cultures.
(Bar for A = 2 μm, Bar for B = 1 μm)
Figure III.9 Transmission Electron Micrograph of Detached Human Umbilical Vein Endothelial Cells not Exposed to any Shear Stress Cultured in M199 for 24 hr at Low (A) and High (B) Magnification. At lower magnification (A) condensed nuclear material (star), canalicular fragmentation (C), mitochondria (m) and RER (large arrowheads) can be seen. At higher magnification (B) mitochondria (m), Weibel Palade bodies and RER (large arrowheads) structures can be identified and seen to be well preserved. These features are consistent with EC apoptosis.

(Bar for A = 2 μm, Bar for B = 1 μm)
Figure III.10 Photograph of Ethidium Bromide Agarose stained DNA Separated by Agarose Electrophoresis Following Isolation from Human Umbilical Vein Endothelial Cells after 12 hrs of Exposure to Oscillating Shear Stress cultured in M199 and HSA (4%). The size of molecular mass markers is indicated. Internucleosomal fragmentation of DNA is evidenced by the 180 BP ladder typical of apoptosis.
Figure III.11 Fluorescence Activated Cell Scanning Analysis of Human Umbilical Vein Endothelial Cells Exposed to Oscillating Shear Stress and Static Cultures. HUVEC cultured in FCS (20%); HSA (4%) and M199 alone. FACS Analysis for the DNA content in HUVEC indicated that the cell populations in the S, G0-G1 and G2-M phases progressively decreased after the cells were subjected to oscillating shear stress, while the apoptotic peak increased in all HUVEC populations subjected to oscillating shear stress as indicated by the percentage of apoptotic cells.
Number of Events

FCS (20%) Static
28.4%

FCS (20%) Rocking
36.1%

HSA (4%) Static
54.3%

HSA (4%) Rocking
67.9%

M199 Alone Static
60.3%

M199 Alone Rocking
63.4%

DNA CONTENT (PI Fluorescence)
Figure III.15 Schematic Representation of Assembled Chamber Flow Apparatus. Superior view of Chamber Flow Apparatus, showing HUVEC cover slide and microscope viewing area in green as anticipated in a flow cell with medium in the apparatus.