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COMPARISON OF STRESSED HUMAN ENDOTHELIAL CELLS DERIVED FROM DIFFERENT VASCULAR BEDS

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COMPARISON OF STRESSED HUMAN ENDOTHELIAL CELLS
DERIVED FROM DIFFERENT VASCULAR BEDS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Laurie B. Joseph

The Ohio State University
1986

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ABSTRACT

COMPARISON OF STRESSED HUMAN ENDOTHELIAL CELLS
DERIVED FROM DIFFERENT VASCULAR BEDS

By

Laurie B. Joseph, Ph.D.
The Ohio State University, 1986

Dr. Howard A.I. Newman, Adviser
Dr. Ralph E. Stephens, Adviser

Vascular disease is the leading cause of morbidity and mortality in the United States. Most research into the underlying mechanisms of these diseases has involved whole animal systems. Explanation of these mechanisms has benefited from the development of more simplified experimental models. To date most research in vitro has involved endothelial cells other than human. I have developed a model system in which human endothelial cells from umbilical vein (HUVE), saphenous vein (SVE) and aorta (HAE) can be compared. I have hypothesized that since certain
diseases appear in specific vascular beds, that cells from different vascular beds have a fixed (heritable) and differential response to stress. The effects of 2 types of stress physical and chemical were investigated. Cellular response to stress was measured morphologically (wound healing) and biochemically (cyclic nucleotide and prostaglandin production). Vascular response was measured as prostacyclin production. The time required for these cells to repopulate an artificially induced wound varied according to their bed of origin (HAE > SVE > HUVE). Since, these data suggested that endothelial cells derived from various vascular beds were different, cellular prostacyclin production was investigated. Endothelial cells are the major producers of prostacyclin (PGI₂). Under non-stimulated conditions arterial-derived endothelial cells produced significantly more PGI₂ than the venous-derived endothelial cells (HAE = 1900, SVE = 376, HUVE = 217 picograms per 10⁶ cells). HAE and HUVE responded to A 23187 by an increased production of PGI₂, while SVE did not. Although, there was an increase in PGF₂α after the addition of A 23187, there were no significant differences between the cell types. Addition of the ionophore did not effect the cAMP and cGMP production of the cells, the amount of cyclic nucleotide produced ranged between 2 and 6 picomoles per 10⁶ cells. In conclusion: 1). Endothelial cells from different vascular beds respond differentially to physical and chemical stress. 2). Endothelial cells elicit 2 types of response: vascular and cellular. The cellular response was the same for PGF₂α, cAMP and cGMP.
for all 3 cell types. The vascular response or ability to produce prostacyclin was dependent on the site of vascular cell origin. 3). Development of an in vitro model for the study of vascular diseases must not assume that all endothelial cells are the same.
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Abbreviations

HAE, human arterial-derived endothelial cells; HUVE, human umbilical vein-derived endothelial cells; SVE, saphenous vein-derived endothelial cells; FBS, fetal bovine serum; ECGF, endothelial cell growth factor; cAMP, cyclic 3'5'-adenosine monophosphate; cGMP, cyclic 3'5'-guanosine monophosphate; PG, prostaglandin; PGI₂, prostacyclin; 6-keto PGF₁α, 6-keto Prostaglandin F₁α; prostaglandin F₂α, PGF₂α; prostaglandin E₂, PGE₂; Prostaglandin H₂, PGH₂; Prostaglandin G₂, PGG₂; M-199, medium 199; cpm, counts per minute; ³H TdR, tritiated thymidine; RIA, radioimmunoassay; HPLC; high performance liquid chromatography; TCA, trichloroacetic acid; LSC, liquid scintillation counting; μCi, micro Curies; ml, milliliters.
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Chapter 1
Introduction

1.1 Endothelial Cells - A Historic Perspective

The structure and function of cells lining both the lymphatics and vascular tree have been of interest since the development of the microscope. The eminent German pathologist Virchow, of the mid 19th century developed the concept of the maintenance and integrity of the vessel wall and described how inflammation could be responsible for endothelial injury\(^1\). By the beginning of the twentieth century there was evidence that foreign bodies could induce injury to the endothelium. Subsequent intimal damage resulted in removal of endothelial cells, thus inducing smooth muscle cell proliferation\(^2,3,4,5\). In the mid-1920’s researchers became interested in the interaction of blood products with endothelial cells and noted that changes in the environment will effect the endothelial cell integrity. Also, in the 1920’s
the first attempt to propagate endothelial cells was made\textsuperscript{1}, but not until the early 1970's was there renewed interest in the culturing of human endothelial cells\textsuperscript{6,7}. Today, cell biologists are able to study the complex vascular system by dissecting it into various components using \textit{in vitro} techniques pioneered by the early pathologists.

1.2 Recent Advances in Endothelial Cell Studies

1.2.1 Development of Culture Techniques

Human derived-endothelial cells were first routinely isolated by Gimbrone\textsuperscript{6} using collagenase infusion of the umbilical vein. This technique has proven to be highly reproducible from laboratory to laboratory. In the past 10 years numerous laboratories have made modifications to Gimbrone's original technique for isolating human endothelial cells from adrenal gland\textsuperscript{8}, foreskin\textsuperscript{9,10}, renal glomeruli\textsuperscript{11}, iliac artery\textsuperscript{12}, pulmonary artery\textsuperscript{13}, and saphenous vein\textsuperscript{14}. In the laboratory of Dr. Ralph Stephens, I have successfully isolated human endothelial cells from umbilical vein, saphenous vein, and aorta. Umbilical vein and saphenous vein-derived endothelial cells were isolated using a modification of Gimbrone's original technique. Aortic endothelial cells were released from the media by scraping.
Since the discovery of fibroblast growth factor, which stimulates bovine aortic endothelial cells\textsuperscript{15,16}, research has focused on the entire family of insulin-like growth factors ability to stimulate human cell growth\textsuperscript{15}. Endothelial cell growth factor (ECGF), which is a member of the same family of polypeptide mitogens as FGF\textsuperscript{17}, was identified and characterized by Maciag\textsuperscript{18}. ECGF has enabled researchers not only to isolate these cells but also maintain and propagate human derived endothelial cell cultures to population doubling levels exceeding 40. Heparin stabilizes ECGF\textsuperscript{17} thereby decreasing the amount of ECGF required in the medium\textsuperscript{19}. The combination of ECGF and heparin enhances the longevity of adult human endothelial cells in culture\textsuperscript{20}. The use of ECGF allows scientists: 1) the study the same population of cells as they age in culture, and 2) grow large numbers of cells necessary for biochemical and molecular biological studies.

1.2.1.1 Endothelium

Endothelial cells are uniquely situated. They have 3 types of surfaces: 1) non-thrombogenic, 2) cohesive, and 3) adhesive. The non-thrombogenic surface faces the lumenal portion of the vessel. The adhesive surface allows the cells to bind firmly to the intimal elastica and the cohesive surface allows for cell-cell connection. The location of the endothelial cell reflects the cohesiveness of the endothelial cell - endothelial cell junction from very tight gap junctions in the cerebrovascular system to very loose junctions in the renal system.
In this project the endothelial cells have been divided into 3 types according to their location within the body. Endothelial cells were derived from the aorta, the umbilical vein and the saphenous vein. Most research to date involving human endothelial cells has made use of the abundance of umbilical cords and the ease of harvesting cells from a large non-branching vessel. Umbilical cords are an excellent source of endothelium because of the ease of access to healthy donors. Saphenous vein endothelial cells (SVE) have been harvested from adults undergoing various types of vascular surgery (peripheral and coronary bypass)\textsuperscript{14} and arterial endothelium (HAE) was derived from the aortae of cadaver organ donors.

1.2.2 Endothelial Cell Biology and Vascular Disease

1.2.2.1 Inflammation

Changes in permeability are among the numerous endothelial responses to acute inflammation. Endothelial cells elicit a variety of chemical mediators upon stimulation by vasoactive amines\textsuperscript{21}. Vasodilation and vasoconstriction is modified by the cells ability to produce prostaglandins\textsuperscript{22} which play a causal role in the modification of vasoconstriction and vasodilation of the underlying media\textsuperscript{4,3}. They are produced during inflammation and cause edema and deviation from normal local temperature (skin)\textsuperscript{22}. The production of the PG can be inhibited by such anti-inflammatory agents as aspirin and Indomethacin. PGs specifically PGE\textsubscript{2} and PGI\textsubscript{2} are inducers of increased
blood flow$^{23,24}$. PGE$_2$ and PGI$_2$ can interact synergistically with other mediators of inflammation, complement, to promote increased vascular permeability$^{25}$. The endothelial cell response to bradykinin elicits an increase in PGI$_2$ production, which can affect the production of cAMP by platelets and decreases the formation of platelet aggregation$^{26,27}$. As part of inflammation there are increases in the vasoactive amines which elevates intracellular calcium, and initiates the metabolism of arachidonic acid. The PGI$_2$ will then activate adenylate cyclase catalyzed production of cAMP which in turn modulates the PGI$_2$ concentration$^{27}$.

Another important mediator of vessel function is the production of platelet derived growth factor for regulation of smooth muscle cell growth. Platelet derived growth factor is produced by platelets during clotting and by damaged endothelial cells$^{28,29}$.

1.2.2.2 Atherosclerosis

Endothelium is the major barrier to the formation of atherosclerotic lesions$^{30}$. Endothelial cells are in intimate contact with blood and provide a non-thrombogenic surface$^7$. Acting as a selectively permeable barrier, endothelium influences the behavior of the other cells in the vessel wall and the circulating blood$^{30}$. It was thought until recently$^{31}$ that the endothelium needed to be removed for the formation of the atherosclerotic lesion$^{3,4,29,2}$. 
Repair of the endothelium is directly related to the damage incurred by the underlying smooth muscle cells\textsuperscript{31}. Recently, interest has been focused on spontaneous injury to the endothelium resulting in increased permeability of the vessel at cell junctions\textsuperscript{31,29}. Researchers are trying to understand the factors which cause changes in the endothelial cells before the formation of the atherosclerotic lesion\textsuperscript{29}. Changes in endothelial permeability is thought to be the initial event in the formation of atherosclerotic lesions\textsuperscript{29}. The alterations in the overlying endothelium could lead to the ingress of lipoproteins and increased lipid deposition in the media, or even frank necrosis of endothelial cells, increased endothelial cell turnover, and platelet interaction with the media at sites of endothelial injury\textsuperscript{3,4,32}. The platelet-endothelial cell interaction at the site of injury would allow interaction of mitogens, such as platelet-derived growth factor, with smooth muscle cells. Damaged and regenerating endothelial cells release a platelet derived growth factor-like substance, which enhances smooth muscle growth and endocytosis\textsuperscript{28,29}. Gerrard and White developed a model to describe the potential relationships of the interaction of prostaglandin and thromboxane with the development of atherosclerosis\textsuperscript{33}. Briefly, injury to the endothelium and platelet activation will cause activation of the phospholipases. Formation of PG and thromboxane will result in: 1) return to normal through healing and repair, 2) local bleeding diathesis, 3) increased production of pro-aggregatory prostaglandins and thromboxanes. Result
number 3 will lead to increased platelet activation, endothelial injury and thrombus formation with the release of smooth muscle mitogens. Thrombus organization and smooth muscle cell proliferation can occur. Then ultimately lipid infiltration of the vessel wall due to direct entry of the lipids or by macrophage presentation of lipids$^{33}$.

1.2.3 Wound Healing

Recent developments in in vivo vascular research suggests that increased permeability between the endothelial cells may lead to the formation of thrombi or atherosclerotic lesions$^{31}$. Earlier work focused on the assumption that the intima must be removed for the development of vascular lesions$^{31}$ this was accomplished using balloon and nylon catheters to denude portions of the aorta$^{31}$. The major sites of physical damage within arteries are downstream from the arterial branch points. Arterial endothelial cells have the highest rate of turnover at these sites$^{29}$. The study of mechanical wound healing has been shown to be a good model of cellular injury.

Damage to the endothelium of the major arterial vessels is considered to be one of the initial events in the formation of atherosclerotic lesions. Endothelial injury has been postulated to be an early event which occurs in the continuum of pathogenic events leading to the formation of the atherosclerotic lesion. Wound healing of confluent human umbilical vein-derived endothelial (HUVE) cell cultures has proven to be a good in
vitro model for this process in vivo. Endothelial cells must achieve stationary confluence before wounding; since sparsely plated cells do not response as a tissue. Therefore, there must be damage introduced separating contact-inhibited cells for "healing" to occur.

Repair of the in vitro wound is separated into 2 distinct stages: 1) cellular migration, and 2) proliferation. Any alteration in the cells' ability to function properly in either of these stages could interfere with the restoration of the normal structure and function of the endothelium.

Wound healing experiments of bovine aorta, and HUVE have shown that cells respond to damage by increased proliferation at the wound edges within approximately 12 hours of denuding. Both cellular proliferation and migration into the wound were shown to be highly correlated using chemical inhibitors of cellular proliferation and/or migration. Cytochalasin B which affects microfilament formation, decreases cellular movement and also has a profound effect on incorporation. Other chemicals such as puromycin, dibutyryl cAMP and theophylline also affect the movement of endothelial cells. Sholley, et al. 1977, using 1500 rads of x-irradiation, which prevents cellular division, showed that endothelial cells will migrate into the wound without the necessity of DNA replication. More recently, Thorgeirsson et al. 1979 repeated and confirmed the work of Sholley. They noted that by placing a layer of agarose on top of the wounded endothelium
the cells moved as a sheet into the denuded area. The cells grew out in a concentric arc from the monolayer at the wound edge.

1.3 Biochemistry of Human Endothelial Cells

1.3.1 Phospholipids

The cell or plasma membrane is composed of a phospholipid bilayer with accessory proteins and carbohydrates. This membrane is a semi-permeable barrier between the cell and the surrounding environment. Its integrity is of the utmost importance for maintaining the cell's status quo. Perturbations will induce modifications in the cellular machinery and, if the perturbations are extreme, cell death. Minor modifications of the phospholipid composition could cause loss of receptors or loss cellular response to specific messengers.

Phospholipids are synthesized either from phosphatidate (phosphatidylinositol) or 1,2-diacylglycerol (phosphatidylcholine and phosphatidylethanolamine). Interconversion between PE and PC species is a simple stepwise methylation of the PE to PC.

Phosphatidylserine can be formed directly from phosphatidylethanolamine by reacting with serine. Phosphatidylserine and
phosphatidylethanolamine are found on the inside of the cell membrane\textsuperscript{40}. Upon methylation PE becomes PC and is transferred to the outside (flip-flop theory)\textsuperscript{40}. PC is involved with information transfer into the cell\textsuperscript{41}. Cardiolipin is found in the mitochondria\textsuperscript{42}, and is formed by the condensation of CDP-diacylglycerol with phosphatidylglycerol\textsuperscript{42}. Phosphatidylinositol is, also, formed from CDP-diacylglycerol. The inositol is added by CDP-diacylglycerol inositol transferase with the release of CMP\textsuperscript{42}. Phosphatidylinositol has recently been associated with receptor activation and the transfer of information into the cell through the activation of protein kinase C\textsuperscript{42}.

Some phospholipids are the stores for arachidonic acid. Arachidonic acid is not normally free in the cell; it is esterified to the SN\textsubscript{2} position primarily of PI, PC and PE.

1.3.2 Arachidonic Acid Metabolism

Arachidonic acid is a 20- carbon polyunsaturated fatty acid (5,8,11,14-eicosatetraenoic acid) which can be incorporated directly from exogenous dietary arachidonic acid or through the conversion of linoleic acid. It is present in membrane lipids mainly in the esterified form. The arachidonate is released by either phospholipase A\textsubscript{2} or phospholipase C from phosphatidylcholine and phosphatidylinositol, respectively\textsuperscript{39}. The enzymatic
release can be triggered by chemical or physical stimuli at the cell surface\textsuperscript{43,44}, which cause an increase in intracellular calcium. Both of these phospholipases are calcium dependent\textsuperscript{45}. The arachidonate is metabolized by either of 2 enzyme cascade systems, cyclooxygenase or lipoxygenase. Prostaglandins, thromboxanes and prostacyclin are formed by the metabolism of arachidonate along the cyclooxygenase pathway and the hydroperoxy metabolites are formed along the lipoxygenase path\textsuperscript{46}.

1.3.2.1 Cyclooxygenase

Cyclooxygenase metabolites are potent vasodilators, vasoconstrictors, inhibitors of platelet aggregation, and platelet aggregators\textsuperscript{46}. Endothelial cells are the major producer of prostacyclin, which is important in maintaining blood equilibrium\textsuperscript{47}. Free arachidonic acid is enzymatically converted to PGG\textsubscript{2} and then PGH\textsubscript{2}, prostaglandin endoperoxides\textsuperscript{46}. These endoperoxides can than be converted to either the thromboxane series or to the rest of the prostaglandin series. Important to this work is the conversion of the PGH\textsubscript{2} to PGI\textsubscript{2} and to PGE\textsubscript{2} then PGF\textsubscript{2\alpha}. PGI\textsubscript{2} is relatively unstable and will convert non-enzymatically to 6-keto PGF\textsubscript{1\alpha}. PGE\textsubscript{2} will enzymatically convert via 9-keto reductase to PGF\textsubscript{2\alpha}. PGF\textsubscript{2\alpha}, also, can be converted non-enzymatically directly from the prostaglandin endoperoxide PGH\textsubscript{2}\textsuperscript{46}.

Production of prostacyclin [PGI\textsubscript{2}] is a necessary element in the maintenance of normal blood flow\textsuperscript{3,29,30}. PGI\textsubscript{2} regulates platelet aggregation.
and vascular smooth muscle cells. Moncada and Vane discovered a unique enzyme which converts prostaglandin endoperoxides to prostacyclin. The enzyme was found to be primarily located in the vasculature. Upon examination of this reductase it was found to be localized in the endothelium. Endothelial cells are the major producer of prostacyclin. Prostacyclin not only is important for its anti-platelet aggregating ability but also for vasodilation. Arterial endothelial cells derived from both human and animal sources, in vitro, consistently produce higher levels of PGI₂ than endothelial cells from venous beds. Human umbilical vein-derived endothelial cells under stressed conditions can produce more PGI₂ than arteries. But, this is thought to be due to the nature of the umbilical vein being a special case. SVE and HUVE production of PGI₂ was consistently lower than that of HAE during prolonged subculturing [Figure 17]. HUVE produced PGI₂ up to and including passage 47.

PGF₂α, another metabolite of arachidonic acid, is formed either directly non-enzymatically from PGH₂ or from PGE₂ via 9-keto reductase. PGE₂ and PGF₂α have been implicated as being important in cellular division. PGF₂α causes changes in the cAMP profiles. Addition of pharmacological levels of PGF₂α and physiological levels of PGE₂ increased the clonal growth of human diploid fibroblasts 110% and 160%, respectively. Elevation of both PGE₂ and PGF₂α cause vasoconstriction. PGE₂ and PGF₂α have been found to be elevated in pre- and postpartum women and individuals having...
diabetic retinopathy\textsuperscript{55}. Implying that PGE\textsubscript{2} and PGF\textsubscript{2\alpha} are important in cell proliferation.

Endothelial cells produce prostacyclin (PG\textsubscript{I\_2}) as their major arachidonic acid metabolite with lesser quantities of PGE\textsubscript{2} and PGF\textsubscript{2\alpha}\textsuperscript{56}. The production of prostacyclin is important in maintaining vascular equilibrium by preventing platelet aggregation and adherence to the endothelium. \textit{In vitro}, prostacyclin can be released by simple agitation upon changing of the culture medium\textsuperscript{57}. Endothelial cells derived from various vascular beds have been shown to produce PG\textsubscript{I\_2} in various amounts\textsuperscript{49}. Venous and arterial endothelial cells both produce PG\textsubscript{I\_2} upon application of various agonists; i.e., histamine, bradykinin, A\textsubscript{23}187\textsuperscript{49,27,58,59}.

1.3.3 Production of Cyclic Nucleotides

Cyclic nucleotides can be used as a measure of the cell's function. The cyclic nucleotides cAMP and cGMP (cyclic 3'5'adenosine monophosphate and cyclic 3'5'-guanosine monophosphate, respectively) are important mediators of cell growth\textsuperscript{60}. cAMP concentrations reflect the stage of cell growth and the concentration of cAMP is directly correlated with the confluence of the monolayer\textsuperscript{61,62}. Confluence or contact inhibition is inversely correlated with cGMP concentration\textsuperscript{60} in human\textsuperscript{61} and mouse (3T3) fibroblasts\textsuperscript{63}, human epithelial cells\textsuperscript{61}, and lymphocytes\textsuperscript{64,60}. Changes in cyclic nucleotide ratios
can be encountered with the addition of membrane-damaging agents, such as psoralens and ultra-violet light\textsuperscript{61}. Histamine, bradykinin and A\textsubscript{23}187 can also induce membrane perturbations and cause changes in the cyclic nucleotides\textsuperscript{52,62,53,27}.

1.3.3.1 Agonists

Agonists are compounds which are capable of combining with receptors to initiate "drug" actions, they possess affinity and intrinsic activity\textsuperscript{65}. Natural agonists such as histamine, thrombin and bradykinin disrupt the lipid bilayer\textsuperscript{59,66}. Membrane changes can be monitored by increased prostacyclin production from endothelial cells, \textit{in vitro}\textsuperscript{59}. Hong et al. showed that histamine and A\textsubscript{23}187 had similar effects on the production of phosphatidic acid, 1,2-diacylglycerol, lysophospholipids and the production of arachidonic acid metabolites\textsuperscript{59}. A\textsubscript{23}178, a partially aromatic polyether ionophore, specifically allows movement of divalent calcium by the formation of large channels in the phospholipid membrane\textsuperscript{67}. This calcium ionophore can be considered a wounding agent, causing changes in cellular metabolic functions\textsuperscript{62}. The influx of calcium activates phospholipase A\textsubscript{2} and C which release arachidonic acid from the SN\textsubscript{2} position of phosphatidylcholine and phosphatidylinositol, respectively. The arachidonate is then metabolized by either the cyclooxygenase or lipoxygenase pathway. A\textsubscript{23}187, also, inhibits cAMP regulation of calcium transport\textsuperscript{62,68}, thus effectively blocking cAMP regulation of cellular division\textsuperscript{62}. 
1.3.4 Calcium Mobilization and Its Effects on Cyclic Nucleotide and Prostaglandin Production

A 23187, like thrombin, histamine and bradykinin, causes direct or indirect activation of phospholipase A$_2$ and C$^{50}$, which mobilizes calcium. This interaction leads to an enhanced influx of calcium with the release of arachidonic acid and the subsequent metabolism of free arachidonate by either the cyclooxygenase or lipoxygenase pathway$^{69}$. Bareis et al.$^{69}$ found that bradykinin, which is released during clotting, effectively produced increased phospholipid methylation, calcium influx, release of arachidonic acid, prostaglandin formation and cellular accumulation of cAMP in human fibroblasts. Adams Brotherton and Hoak$^{27}$ investigated the effects of thrombin and A 23187 on endothelial cell production of cAMP. They found, like Bareis et al., that calcium influx plays an important role in PG biosynthesis, activation of phospholipases. When A 23187 was added to platelets there was Agonists are compounds which are capable of combining with receptors to initiate "drug" actions, they possess affinity and intrinsic activity$^{65}$. Natural agonists such as histamine, thrombin and bradykinin disrupt the lipid bilayer$^{59,66}$. Membrane changes can be monitored by increased prostacyclin production from endothelial cells, in vitro$^{59}$. Hong et al. showed that histamine and A 23187 had similar effects on the production of phosphatidic acid, 1,2-diacylglycerol, lysophospholipids and the production of arachidonic acid metabolites$^{59}$. A 23178, a partially aromatic polyether
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like Bareis et al., that calcium influx plays an important role in PG biosynthesis. When A 23187 was added to platelets there was an increase in intracellular calcium causing an activation of phospholipases with a sudden release of arachidonic acid.

Cyclic nucleotide concentrations are directly proportional to the available calcium. Cyclic AMP is decreased under high calcium conditions due to the activation of phosphodiesterase. Increased calcium concentrations favor the formation of cGMP. In platelets under normal conditions free cytoplasmic calcium levels are maintained by cAMP. Calcium efflux inhibits the production of PG. Adams Brotherton and Hoak support the contentions that: 1) PGI₂ biosynthesis is stimulated by calcium in HUVE, and 2) increased cAMP inhibits PGI₂ production. These findings agree with earlier work of Schafer et al. that PGI₂ stimulates an increase in cAMP and that PGI₂ acting through cAMP might affect its own production in intact cells. The response to PGI₂ was not seen in cell lysates. Catecholamines stimulated a four fold increase of PGI₂ in intact cells. PGI₂ formed by endothelial cells can elevate cAMP in platelets and endothelial cells. The cAMP subsequently causes attenuation of the PGI₂. These data suggest that endogenous PGI₂ may regulate PGI₂ biosynthesis by regulating adenylate cyclase. The cAMP inhibition may be to limited to phospholipase. Berridge suggested that PGI₂ production by vascular endothelium could be under bidirectional control of cAMP and calcium.
Adams Brotherton and Hoak\textsuperscript{27} and Hopkins et al.\textsuperscript{71} found that HUVE produce elevated amounts of phosphodiesterase which modulates PG\textsubscript{I\(_2\)} induced increases in cellular cAMP. To detect elevations of cAMP induced by PGs the cells were treated with a phosphodiesterase inhibitor, suggesting that normal intact endothelium has a low steady-state level of cAMP. Adams Brotherton and Hoak\textsuperscript{27} found that cAMP phosphodiesterase may regulate a negative feedback mechanism involving the activation of adenylate cyclase by PG\textsubscript{I\(_2\)}. This model\textsuperscript{27,69} implies that adenylate cyclase is stimulated by PG formation. Ager\textsuperscript{52} found that neither porcine aortic endothelial nor smooth muscle cells had an elevated production of cAMP when vasoactive amines were added to the cells maybe related to elevated phosphodiesterase.

Guanylyl cyclase is activated by an increase in intracellular calcium\textsuperscript{64,60}. The introduction of A 23187 causes an influx of divalent calcium from the medium into the cell or an efflux from mitochondria regulating both cGMP and cAMP\textsuperscript{62}. Increased calcium concentrations stimulate the formation of cGMP and the degradation of cAMP. Studies of cAMP and cGMP in umbilical arteries showed that cAMP was involved in smooth muscle relaxation and cGMP was involved in contraction\textsuperscript{72}. Bradykinin and histamine caused a 2 fold elevation in cGMP without an increase in cAMP in the whole artery\textsuperscript{72}. An agent which causes vasodilation, PG\textsubscript{I\(_2\)}, causes the accumulation of cAMP intracellularly whereas agents which cause contraction, bradykinin and histamine, cause an accumulation of cGMP.
The bidirectional model of Berridge\textsuperscript{64} seems appropriate for endothelial cells. He stated that stimulant 1 activates the calcium signal which causes contraction, secretion, metabolism and cell division. Stimulant 2 activates cAMP which lowers intracellular calcium causing relaxation and cessation of cell division.

1.4 Statement of Hypothesis

Atherosclerosis remains the leading source of human morbidity and mortality in the United States. Physical or chemical modification to the intima allows for the development of disease. Diseases of the arterial and venous system manifest themselves differently. Atheromas mainly occur in the arterial tree. Are these differences due to a fixed (heritable) predisposition of the endothelium or are these differences due to the cell’s environment? To test whether isolated endothelial cells had the same response \textit{in vitro}, I decided to investigate the effects of known modifiers of cellular integrity on endothelial cells derived from large vessels of both the venous and arterial tree. This was accomplished by studying the effects of chemically and physically induced stress. Cellular response to stress was measured morphologically (wound healing) and biochemically (cyclic nucleotide and prostaglandin production). Investigating the effects of various agents on isolated cells allows the determination as to whether endothelial cells from
different vascular beds are functionally the same. If indeed these cells are the same then the development of vascular diseases is only due to their environment and not due to tissue specific differences. Therefore, comparison of the response of isolated human endothelial cells from different beds to the same environment over several passages should help in the understanding of the development of vascular diseases.
2.1 Culture and Characterization of Endothelial Cells

2.1.1 Endothelial Cell Culture Techniques

2.1.1.1 Saphenous Vein [SVE]

At the time of coronary bypass surgery before the individual was systemically heparinized, the saphenous vein was removed and a portion was allocated for development of adult SVE cultures\textsuperscript{14}. Autologous arterial blood (20 ml) was removed and mixed with preservative-free porcine Na-heparin (5 mg) (Gibco) and chilled. The vein can be stored at 4°C for up to 10 hours before processing. After all the side branches were tied, the vein was canulated and flushed with cold phosphate buffered saline (PBS, see Appendix B: Formularies) to remove residual blood. Type II collagenase
(Cooper Biomedical) in cold PBS (1 mg/ml) was flushed through the vein. The vein was closed at one end with a bulldog clamp and then distended with collagenase. After incubation at room temperature for 25 minutes, the vein was drained into a sterile 15 ml polystyrene centrifuge tube (Corning) and flushed 3 times with M-199 (Gibco, see Appendix B: Formularies) plus 10% fetal bovine serum (FBS, HyClone). The cells and effluent were centrifuged at 900 x g in a Beckman model GLC table top centrifuge for 10 minutes at 4°C. The supernatant was aspirated and the cells plated on a human fibronectin (see Section 2.1.7, Fibronectin Preparation, gift of Dr. Mitchell) coated (10ug/cm sq.) tissue culture dish (Corning) containing M-199 supplemented with 20% FBS, 150ug/ml endothelial cell growth factor (ECGF)18 (see Section 2.1.6, Endothelial Cell Growth Factor), 90ug/ml Na-heparin19, plus 150U/ml amphotericin-B and 100U/ml penicillin and 100U/ml streptomycin with 20mM HEPES (Sigma) buffer (growth medium). The cultures were incubated in a Forma (model number 3315) triple gas microprocessor controlled incubator set at 5% CO2/95% air, 37°C, humidified environment. Six (6) hours later the medium was removed, cells were rinsed with room temperature PBS to remove the red blood cells and other debris, and replaced with fresh growth medium. Growth medium was changed every 48 hours; for the first few days the cultures were rinsed with incomplete M-199 to remove any residual debris. The cultures were usually passaged 2 weeks after inoculation (Figure 1a). After the second passage,
Figure 1: Confluent Cultures of Human Derived Endothelial Cells
Figure 1a  Confluent monolayer of saphenous vein-derived endothelial cells. Original magnification of 100x.

Figure 1b  Confluent monolayer of umbilical vein-derived endothelial cells. Original magnification of 100x.

Figure 1c  Confluent monolayer of arterial-derived endothelial cells. Original magnification of 50x.

Figure 1, concluded
the cells were checked for Factor VIII related antigen by immunoperoxidase
73 [Figure 2] (see Section 2.1.4, Factor VIII Identification).

2.1.1.2 Umbilical Vein [HUVE]

Human umbilical cords were collected following normal vaginal delivery
or Caesarean Section (Department of Ob/Gyn, Labor and Delivery). The
cords were cut from the placenta, drained of blood, and put into a sterile
container with MEM (see Appendix B: Formularies) plus antibiotics and
antimycotics. Cords can not be squeezed or stretched because this loosens the
endothelium from the intima. Cords were picked up every 12 hours and
processed immediately. The cords were checked for clamp marks and any
tissue with clamp marks were discarded. The following is a modification of
the original techniques of Gimbrone6 and Jaffe74. The umbilical vein was
canulated and rinsed with cold PBS to remove the blood clots. The outside
of the cord was also rinsed to removed as much blood as possible. The vein
was distended with 0.1% collagenase (Type II, Cooper Biomedical) in cold
PBS for 20 minutes, then massaged to loosen the endothelial cells from the
intima. The cells plus collagenase were collected in a 50 ml sterile
polypropylene centrifuge tube (Corning), and the vein was flushed with
M-199 plus 10% FBS (1 ml per inch). Each cord was treated as a separate
culture. The cells plus medium were spun at 900 x g in a Beckman model
GLC table top centrifuge (4ºC) for 10 minutes. Each pellet was plated on
either a 25 sq. cm tissue culture flask (Corning) or a 30 mm tissue culture dish (Corning) which had been coated with human fibronectin 5 ug/cm², in growth medium and incubated. The endothelial cells were washed 6 hours after plating and refed. After approximately 10 days the cells were ready to be passaged, Figure 1b. Following the second passage, the cells were checked for Factor VIII related antigen73 [Figure 2].

2.1.1.3 Aorta [HAE]

HAE were derived from either cadaver organ donors or aortic punches at the time of bypass surgery. The whole arteries were handled in the same manner as the saphenous veins14. The aortic punches were placed endothelial cell side down on 10 ug/cm² human fibronectin coated 16mm Corning 24 well pack in growth medium. The cultures were incubated as above. After 3 days the punches were removed and the adherent cells were rinsed in incomplete M-199 and refed with growth medium. HAE were also isolated by scraping the aorta13. A number 11 scalpel blade was gently drawn across the intima. The cells plus residual connective tissue were placed into a sterile centrifuge tube containing M-199 plus 10% FBS and spun. The pellet was then handled in the same manner as the SVE. The yield from arteries was consistently lower than the saphenous vein isolates. It took approximately 3 weeks before the cells were passaged [Figure 1c]. After the third passage the cells were checked for Factor VIII related antigen73 [Figure 2].
2.1.2 Routine Cell Maintenance

2.1.2.1 Passaging of Cells

All cells were fed with endothelial growth food every other day (48 hours). The cultures were routinely passaged at 90 - 95% confluence (approximately 1-3 mitotic figures per 10x field, Nikon Diaphot with a UFII camera attachment). At the time of passaging, the medium was saved for RIA of 6-keto-PGF$_{1\alpha}$, a relatively stable metabolite of PGI$_2$. The cells were rinsed twice with cold PBS; cold 0.01% trypsin (Cooper Biomedical) and 0.02% EDTA (Sigma) were added. The cells were released from the substrate within 30 seconds. The cell/trypsin/EDTA mixture was trituated and inactivated with 10 times the volume of M-199 plus 10% FBS. This mixture was spun at 100 x g (4°C) for 10 minutes. The supernatant was aspirated, and the cells were plated in the appropriate flasks.

2.1.2.2 Cryopreservation

Endothelial cells were stored for future use in liquid nitrogen. The cells were frozen at a 1:1 split (equivalent to a 25 sq. cm. flask). They were frozen in growth medium containing 10 % dimethyl sulfoxide (Fisher Scientific). Cells were first placed in liquid nitrogen vapors before storage in the cryotanks. The endothelial cells had a 70% viability (determined by tyrpan blue exclusion) upon thawing and it took approximately 1 week to achieve confluence.
2.1.3 Cell Enumeration

2.1.3.1 Coulter Counter

Usage of the Coulter Counter only allowed counting the total number of cells. It does not afford the opportunity for differentiation between viable and non-viable cells. One half (0.5) ml of the inactivated enzyme/cell suspension was added to 9.5 ml of Isoton (Coulter) and counted on a Coulter model ZBI counter with an aperture current setting of 2, amplication of 1, lower and upper threshold limits of 10 and 20 %, respectively. The average of 3 counts was used to calculate cell density (see Appendix C: Calculations).

2.1.3.2 Trypan Blue Exclusion

Trypan blue exclusion allows one to differentiate between viable and non-viable cells. A 0.5 ml aliquot of the cell suspension was added to an equal volume of 0.4% trypan blue in buffered normal saline (The Ohio State University, Reagent stores). The viable (non-stained) versus non-viable (stained) cells were counted on a hemocytometer. A total of 10 squares were counted on both sides of the hemocytometer. The total cell number was calculated as follows: \# cells (n) x (dilution factor) x 10^3 x total ml in which the cells were suspended. To calculate cells per cm^2: the total number of cells was divided by the area of their growing surface (sq. cm.).
2.1.4 Factor VIII Identification

Endothelial cell cultures were checked for purity using a modification of the Hsu-ABC Method for Factor VIII related antigen by immunoperoxidase. This was done by the Immunopathology Laboratory under the supervision of Dr. Hari Sharma, Director of Anatomical Pathology, The Ohio State University. Cells were grown on precleaned standard glass slides (Thomas), which had been coated with 10 ug/cm² of humanfibronectin for 1 hour at room temperature. The cells were applied to the slide in 0.1 ml growth medium and were allowed to sit, undisturbed, for 5 minutes before placing into the incubator. For processing, the cells were rinsed 3 times in PBS at room temperature and then fixed in reagent grade acetone (Fisher) at room temperature for 10 minutes. The slides were then taken to the Immunopathology Laboratory for Factor VIIIr Ag processing. Cells were counter stained with hematoxylin and eosin. All cells used in these experiments were shown to be greater than 90% pure endothelial cells (passage 4) by this technique. Endothelial cells in culture displayed numerous morphologies [Figure 2]. As can be seen in the micrographs, F-VIIIr Ag is perinuclear and has been associated with Weibel-Palade bodies [Figure 3] which are diagnostic of endothelial cells⁷³.
Cells were plated on fibronectin coated glass microscope slides and grown in standard growth medium until confluent. Cells were rinsed in PBS and then fixed in acetone. Determination of FVIII was done using the HSU-ABC Method by the Immunopathology laboratory (Department of Pathology, OSU). Cells are counterstained with H and E. Factor VIII is perinuclear. Endothelial cells have multiple morphologies in culture. Magnification 315x.

Figure 2: Factor VIIIr Antigen Positive Human Derived Endothelial Cells
Endothelial cells were pelleted, and sectioned. Weibel-Palade Body has tight striations. Next to the WP body is a mitochondria. The WP is representative of what was seen in all the endothelial cell types. This W/P was from a HAE. M = Mitochondria, WP = Weibel-Palade Body.

**Figure 3:** Transmission Electron Micrograph of Weibel-Palade Body
2.1.5 Preparation Endothelial Cell Growth Factor

Endothelial cell growth factor (ECGF) was prepared according to Maciag\textsuperscript{18}. Whole cow brain was purchased from Karn's Meat Packers (Columbus, Ohio) and used within hours of slaughter. The brain was kept iced until processing. The large blood clots and any bone fragments were removed and the brain was rinsed numerous times in ice cold PBS, then drained and weighed. The brain was homogenized in Waring blender with cold 0.15 M NaCl (1250 ml per kilogram of tissue). The homogenate was stirred for 2 hours at 4°C. The extract was centrifuged at 13,000 x g (Sorval, model RC2-B with a SS-34 fixed angle rotor) for 40 minutes at 4°C. The supernatant was carefully decanted, the amount recorded, and the pellet discarded. Three (3) grams of streptomycin sulfate (Grand Island Biological) per 0.41 kilograms of brain were dissolved in 40 ml of water (pH 8.0) and precooled in an ice bath. The streptomycin was added dropwise to the stirring extract over a period of 5 minutes, pH was adjusted to 7.0. The mixture was stirred for an additional 20 minutes in an ice bath, and then placed at 4°C overnight. The mixture was centrifuged as above and the supernatant was a translucent pink (approximately 1/3 the volume of the original homogenate). An aliquot was used to determine the total protein using Bio-Rad protein assay. Total protein was read at an absorbance of 280 and a volume equivalent to an A 280 of 15 was recorded. That amount was pipetted into pre-cleaned serum vials, and lyophilized. Endothelial cell
growth factor has a shelf life of about 6 months when kept either at 4°C or -80°C.

2.1.6 Preparation of Fibronectin

Human fibronectin was a gift of Dr. Mitchell from Central Michigan Red Cross, Blood Products Division. Fibronectin was prepared from Cohn fraction IV.

The fibronectin was weighed and dissolved in PBS (1 mg/ml) at room temperature. The fibronectin/PBS solution was filter sterilized through a 0.2 um Millipore Sterivex filter, aliquoted into 1 ml samples, and frozen at -20°C until needed. When used, the fibronectin was diluted to 100 ug/ml PBS and stored at 4°C. Glass and plastic growth surfaces were coated with fibronectin at concentrations ranging from 5 - 20 ug/cm² (depending on the surface and cell type).

2.1.7 Labeling Index

HAE, HUVE and SVE at passages 6, 10; 2, 9, 11 and 6, 9, 13, respectively were used for the labeling index, a method developed to determine the in vitro age of cells. On sterile human fibronectin coated (10 ug/sq. cm.) glass coverslips (11 x 22 mm, Thomas) endothelial cells were plated in growth medium at a density of 1 x 10⁴ cells/sq. cm.
Twenty-four (24) hours later, 0.1 uCi of $^3$H Thymidine ($^3$H TdR, sp. act. 6.7 Ci/mmol, New England Nuclear) was added per ml of growth medium. At specific time intervals, coverslips were removed from the labeled medium and washed 3 times with PBS to remove residual medium. Then the cells were fixed 3 times in Modified Carnoy's Fixative (3 MeOH : 1 glacial acetic acid, v/v) for 5 minutes. Coverslips were rinsed in 5% TCA (Fisher Scientific) for 5 minutes. Then they were dipped several times in 70% EtOH and then in 95% EtOH. The coverslips were allowed to air dry overnight, and the following day were mounted onto standard precleaned glass slides (Thomas) with xylene diluted picolyte (The Ohio State University Laboratory Stores). Slides were dried overnight and were then ready for autoradiography.

2.1.7.1 Autoradiography

In a photographic dark room a graduated cylinder containing distilled water, a beaker for dipping slides and another beaker for melting the gelled emulsion were placed into a 41°C water bath for approximately 30 minutes. When glassware was warmed, approximately 10 ml of emulsion (Kodak NTB-2) was placed in one of the beakers and allowed to melt (20 minutes). Removal of the emulsion and all the following procedures were done in total darkness. Equal amounts of the melted emulsion and the distilled water were gently mixed to remove any lumps. After the emulsion was put into the dipping beaker, the slides were dipped 3 times and placed on brown
paper to drain off excess emulsion and dry. Three (3) hours later the slides
were placed in light-tight boxes with Drierite (Fisher Scientific), sealed, and
placed in a -20°C freezer. The slides were checked 7 days later. Slides were
developed using Kodak D-19 for 6 minutes, rinsed in tap water, then fixed
in Kodak rapid fixer for 12 minutes and rinsed again. Slides were stained
for 10 minutes in freshly filtered Toluidine Blue (The Ohio State University
Laboratory Stores) and air dried. One hundred (100) cells per time point
were counted under a flat-field, 40x objective using a Nikon Optiphot
microscope. The percent of labeled cells was used to determine the labeling
index for each cell type at specific passages, (see Appendix C: Calculations).

2.2 Phospholipid Composition

Human endothelial cells derived from varying vascular beds responded
to wounding of the monolayer differently (Section 3.3 Wound Healing
Results). Also, the baseline production of 6-keto-PGF$_{2\alpha}$ was different. Since
PG's are metabolites of arachidonic acid, which is esterified to PI, PC, PS
and PE, the total phospholipid composition of the varying endothelial cells
was analyzed to see if the varying cellular response was due to phospholipid
content.
2.2.1 Cell Culture

Aortic-, saphenous vein- and umbilical vein-derived endothelial cells were grown on either 100 or 60 mm Kimex or Pyrex dishes. The dishes were hand washed, air dried and autoclaved before coating with human fibronectin 20 ug/cm² for at least 1 hour. The cells were plated at a split ratio of 1:3.5 and fed with standard growth medium. It took approximately 7 days for the cells to reach 95%+ confluence (less than 1 mitotic figure per 10x field). Each cell type was grown in this manner for analysis of phospholipids and repeated at least 9 times.

2.2.2 Extraction

The medium was saved for analysis of arachidonic acid metabolites (see Section 2.3.2.1). The cells were rinsed twice with PBS. The petri dish was placed on a block of dry ice for approximately 2 minutes until the cells were frozen. Two (2-3) ml hexane:isopropanol (HIP, 3:2, v/v)²⁶,²⁷ was added and the cells were removed from the dry ice. Then the cells plus HIP were allowed to defrost to slush. The cells were scraped with a Teflon scraper (Nalgene) and the HIP-cell extract was put into a glass 18 x 150 mm test tube. Another 2-3 ml HIP was added, then vigorously tritutated, the fluids were pooled with the cell extract. The extract was filtered through glass wool in a glass 9.5 inch Pasteur pipet to remove the large debris. To remove the smaller debris, the volume of HIP was reduced under a stream of nitrogen, then filtered through a 0.2 um optimal pore size syringe filter.
2.2.3 HPLC

The solvent was removed from the sample under nitrogen. The extracted material was then brought back up in 100 ul of HIP (3:2, v/v) plus 5.5% water. Twenty-one (21) ul was injected into the HPLC. The samples were run on Dupont Zorbax-sil column (5-6 microns) using a H$_2$O gradient from initial 45% n-hexane : 2-propanol, 3:2 (v/v) with 5.5% H$_2$O by volume to 100% over 105 minutes; fractions were collected at preset times where specific phospholipid standards did elute$^{78}$. The HPLC is composed of an Altex pump model 100A, an ISCO v4 absorbance detector, a Jones chromatography heating block set at 34°C, a Beckman 421 controller, an Altex microprocessor 420, a Kipp and Zonen BD41 chart recorder, and a Nelson analytical 760 series integrator interfaced with a Hewlett Packard HP 85.

2.2.4 Phosphorus Determination

Phosphorus was determined according to Rouser et al.$^{79}$. Briefly, samples were evaporated to dryness and 0.5 ml of H$_2$O plus 0.5 ml of HClO$_4$ was added to each sample. The samples were then incubated for 1 hour at 100°C and cooled to room temperature. Then 3.3 ml of H$_2$O plus 0.5 ml of ammonium molybdate were added, the tubes were vortexed, and the 0.5 ml of L-ascorbic acid was added. The samples were heated in boiling water for 5 minutes, they were cooled, and the absorbance read at 797 nm.
2.2.5 Transmission Electron Microscopy

There was a decreased amount of cardiolipin in HAE versus SVE. Cardiolipin is associated with mitochondria; therefore transmission electron micrographs were examined to determine if there was a qualitative difference between the cell types.

HUVE, HAE and SVE (passage 10) were released from the substrate and pelleted. The cell pellet was resuspended and washed with PBS (3 times). After the third washing the PBS was replaced with gluteraldehyde and kept at 4°C until embedding in epon. The cell pellets were processed, sectioned and photographed by the TEM Laboratory under the supervision of Dr. Hari Sharma, Department of Pathology, The Ohio State University.

2.3 Stress Studies of Endothelial Cells

2.3.1 Physical - Wound Healing
2.3.1.1 Culture

Wound healing in vitro is a demonstration of the regenerative capacity of an intact sheet of cells to physical damage. Endothelial cells derived from different mammals have different reproductive capacity when physically damaged. Human derived endothelial cells were examined to determine their ability to repopulate a "wound". HAE, HUVE and SVE were plated on Corning 16mm 24 well pack tissue culture dishes coated with 5 to 10 ug/ml human fibronectin (See Section 2.1.6). Upon reaching stationary confluence (approximately 1 mitotic figure per 10 x field) the cultures were rinsed with room temperature PBS and photographed using a Nikon camera attached to a Nikon Diaphot Inverted Phase microscope. A 3-4 mm wound was made across a diameter of the well with a plastic micropipet tip. The cells were rinsed with room temperature M-199, refed with growth medium, photographed and then placed back in the incubator. Matched unwounded controls were handled in the same manner.

2.3.1.2 Photography

The endothelial cell monolayers were photographed before the start of the experiment, immediately after wounding, and every 6 hours for the first 48 hours. Then the cells were photographed every 12 hours for the next 24 hours or until the wound was completely repopulated.
2.3.1.3 Serum vs. ECGF and Heparin

Wound healing in HUVE has been shown to be a 2 stage phenomenon\textsuperscript{35,36}: migration and the division. This 2-stage process was observed in HAE, HUVE and SVE cultures. To see if there was an affect of FBS or ECGF/Na-heparin on the infilling of the wound; stationary confluent cells were washed, scored with a micropipet tip, rewashed and the experimental medium was added. Cells were divided into 4 categories: 1) complete growth medium, 2) M-199 + FBS, 3) M-199 + ECGF and heparin, and 4) M-199. These experiments were done on all 3 cell types. Cell activity was recorded as in Section 2.3.1.2 and tritiated thymidine was added as in Section 2.3.1.4.

2.3.1.4 DNA Labeling

In selected cultures at the time of wounding the medium was replaced with growth medium containing 0.1 uCi/ml tritiated thymidine (sp. act. 6.7 mCi/mmol, New England Nuclear)\textsuperscript{38}. Medium of the unwounded matched controls were also replaced with medium containing tritiated thymidine. At appropriate times the cells were harvested and saved for measurement of tritiated thymidine incorporation.
2.3.1.5 Harvesting

If tritiated thymidine had been added to the cells, at the time of photography, 4 wells of cells were harvested (control and experimental). The wells were washed twice with PBS, then 0.1 ml of 0.01% trypsin plus 0.1 ml of 0.02% EDTA were added to the wells. The 24 well pack was placed back into the incubator for 3 minutes in order to release the cells. The cells plus trypsin/EDTA were trituted 4 times with a 1 ml polystyrene serological pipet (Falcon) and put into a 3 ml polystyrene pop-top tube (Kew Scientific). The wells were washed 3 times with 0.2 ml of PBS and the wash was combined with cell suspension. Then the wells were rinsed with ice cold 10% TCA (trichloracetic acid) such that the final concentration of TCA plus the cells was 5% which was stored at 4°C.

2.3.1.6 Liquid Scintillation Counting

Cell suspension was dried onto 2.4 cm glass microfibre Whatman GF/B filters. The cold cell suspension was washed onto the filter and the tube was rinsed 3 times with ice cold 5% trichloroacetic acid (TCA). The tube was poured into the filter holder (Millipore stainless steel 47 mm filter holder) in the same direction each time. The filter holder was rinsed 3 times with ice cold 95% ethanol to rinse down any residual material onto the filter. The dried filter was then put into a 15 ml glass scintillation vial (Kimax), and 10 ml of Aquasol II (New England Nuclear) was added. All
filters were placed into the vials facing the same direction. A blank filter was prepared for background counts. The radioactivity was measured using a Beckman liquid scintillation counter 6800 and quench was monitored by H#.

2.3.2 Chemical - A 23187, a Calcium Ionophore

2.3.2.1 Baseline Production of 6-keto PGF$_1$alpha

Human umbilical vein-derived endothelial cells under non-stimulated conditions do not produce 6-keto-PGF$_{1\alpha}$\textsuperscript{58}. Therefore, to determine if endothelial cells derived from different vascular beds produced the same or varying amounts of PGI$_2$, growth medium was analyzed for PGI$_2$ and measured as 6-keto PGF$_{1\alpha}$ a relatively stable metabolite by RIA. An aliquot of medium was removed from the tissue culture flask (or petri dish) and frozen at -80°C until analyzed. The following parameters were recorded: passage number, total amount of medium, total growing surface in square centimeters, stage of growth, and portion of cell cycle (lag, log or stationary). The number per squared centimeter was calculated when cells were passaged.
2.3.2.2 A 23187 Experimental Protocol

A 23187, a Ca$^{+2}$ specific ionophore, causes an increase in the production of 6-keto PGF$_{1\alpha}$ in endothelial cells$^{43,56}$ and does not allow for the down regulation of cAMP$^{67}$. To determine the effect of A 23187 on the production of 6-k-PGF$_{1\alpha}$, PGF$_{2\alpha}$ and cyclic nucleotides by HAE, HUVE and SVE I used the following protocol. Cells were plated on precoated 60 mm glass pyrex petri dishes (500ug/ P60 human fibronectin) and grown to at stationary confluence. The cells were grown in standard growth medium (see Section 2.1.1.1) which takes approximately 1 week. Cells were washed with room temperature M-199 and refed with growth medium 24 hours before addition of 1 $\mu$M A23187 (Sigma). Twenty-four (24) hours later the medium was removed, saved and frozen at -80°C for RIA determination of PGF$_{2\alpha}$ and 6-keto PGF$_{1\alpha}$. At the beginning of the experiment, the medium from one plate was saved and the cells were extracted for cyclic nucleotides (see Section 2.3.2.3). Cells were rinsed with incomplete M-199 (room temperature) and either the control medium or the experimental medium was added. The medium consisted of M-199 plus 5% FBS or M-199 plus 5% FBS plus 150 ug/ml ECGF and 90 ug/ml Na-heparin with or without 1 $\mu$M A 23187. The A 23187 was initially solubilized in M-199 plus 10% injection grade DMSO. The control and experimental media were added to the cells. At time zero the medium was saved from all the control plates. Two (2) control plates were rinsed with PBS and 1 plate of cells was counted; and
the other plate was extracted for cyclic nucleotides. The other 2 plates were washed with room temperature M-199, fed with standard growth medium, and incubated for 24 hours. At that time, 1 plate was counted and the other was extracted. After 60 minutes, the experimental cells were removed from the incubator and treated in the same manner as the controls. The cells were counted with either the Coulter counter ZBI or by hemocytometer.

2.3.2.3 Cyclic Nucleotide Extraction

The endothelial cells were handled as described in 2.3.2.2. The cells were washed twice with PBS and 1 ml of 5% TCA was applied to the dishes. The cells were scraped with a teflon scraper and triturated. The 5% TCA was saved, and another 1 ml of 5% TCA was added, and the plate rescraped. Cell extract was stored at 4°C until assayed.

2.4 Biochemical Studies

2.4.0.1 Determination of 6-keto PGF₁α and PGF₂α by RIA

Determination of PGF₂α and 6-keto PGF₁α (a relatively stable metabolite of PGI₂) was done according to the method of Fertel, et al. 1981 in his laboratory as described by Albrightson. Briefly, the media from the experimental and baseline cultures were saved, frozen at -80°C, and
thawed immediately before assaying. The prostaglandin buffer (PG buffer) was composed of 50 mM Tris, 0.1% BSA, and 0.05% sodium azide at pH 7.5. The 6-keto PGF$_{1\alpha}$ standard (UpJohn Diagnostics) and tritiated 6-keto PGF$_{1\alpha}$ were stored in the PG buffer; and the PGF$_{2\alpha}$ was stored in 95% ethanol at -20°C. A standard curve was run for both PG’s ranging from 0.1 to 10,000 picograms/100 ul. The tritiated PG’s were purchased from Amersham with specific activities of 150 and 180 Ci/mM for 6-keto PGF$_{1\alpha}$ and PGF$_{2\alpha}$, respectively. The labeled PG’s were diluted to give approximately 10,000 cpm/100 ul. The antibodies for both PG’s were developed in the laboratory of Dr. Fertel$^{81}$ and were added to give approximately 35% binding per 100 ul (diluted in PG buffer).

One hundred (100) ul of the sample medium was assayed in duplicate. The samples were incubated with the antibody and label overnight at 4°C. The bound and free fractions were separated using 500 ul of dextran coated charcoal (2.5 g Norit A decolorizing charcoal, plus 250 mg Dextran T-70 in 1 liter of PG buffer) for 15 minutes at 4°C. The samples were spun at 3800 rpm in a Beckman Model J-6B centrifuge for 20 minutes. The supernatant was then decanted into mini-scintillation vials, 2.5 ml of Scinti-Verse II (Fisher) was added, and the vials were shaken vigorously to produce a gel. The samples were counted in a Beckman LS-6800. The results were expressed as percent bound versus the concentration of the unlabeled PG standards. The sample concentrations of the PG’s were determined from the
standard curve. The concentration of the 6-keto PGF$_{1\alpha}$ and PGF$_{2\alpha}$ were expressed as picograms per 1 million cells.

2.4.0.2 Cyclic Nucleotide Assay by RIA

Endothelial cell extracts were analyzed for cGMP and cAMP using the procedure outlined by Albrightson$^{61}$. Briefly, the TCA cell extract was extracted with water-saturated ether and the pH adjusted with 1 M sodium acetate. One hundred (100) ul was assayed for cAMP and was assayed 300 ul for cGMP. The samples were incubated with acetic anhydride and triethylamine to increase the RIA sensitivity. $^{125}$I- labeled cGMP or cAMP ($^{125}$I was purchased from Amersham and the cyclic nucleotides were iodinated in the laboratory of Dr. Fertel) having a specific activity of 5 vCi/ml was diluted with 0.25% bovine gamma globulin; 50 ul were added to give approximately 15,000 cpm per sample. The antibody was diluted in 50 mm sodium acetate buffer, pH 6.3 plus 0.2% bovine serum albumin (Sigma); this produced an absolute binding of 0.30-0.39. The samples were incubated at 4°C overnight. The bound and unbound fractions were separated by precipitation with 60% saturated ammonium sulfate for 20 minutes at room temperature. Following 20 minute centrifugation at 3800 rpm (4°C), the supernatant was decanted and the precipitate counted in a Beckman model 700 Gamma counter. The concentration of cyclic nucleotides in each sample was determined from a 16 point standard curve. Data were expressed in femtomoles per 10$^6$ cells.
Chapter 3
Results

3.1 Labeling Index

3.1.0.1 HAE: Mid and Late Passages

Human arterial-derived endothelial cells at passage 6 had only 70% of the total cells labeled after 96 hours. By passage 10, there was a decrease in the total number of labeled cells to 48% [Figure 4]. Both passages 6 and 10 had about the same percentage of labeled cells for the first 72 hours (54% and 49%, respectively). But, by 96 hours there was an increase in the number of labeled endothelial cells in the passage 6 cells to 70%. Passage 10's total number of labeled cells did not increase after 48 hours [Figure 4].
$^3$H TdR was added at T=0. Coverslips were removed from the labeled medium at specific time intervals and autoradiographed. One hundred cells were counted at each time point and the percent labeled cells calculated.

Squares = Passage 6 and Circles = Passage 10.

**Figure 4:** Human Arterial Derived Endothelial Cells: Labeling Index
3.1.0.2 HUVE: Early, Mid and Late Passages

Human umbilical vein-derived endothelial cells showed a decrease in the total number of labeled cells at 120 hours versus the total number of passages in culture [Figure 5]. Six (6) hours after adding \(^3\)H TdR, there was a definite difference in the total number of labeled cells versus passage. Passage 3 had 29% of the cells labeled whereas Passages 9 and 11 had only 17% of the total cells labeled. There was an increase in the amount of label incorporated with time. By 48 hours, there was a marked difference in the percentage of labeled cells, Passage 3 = 87%, Passage 9 = 67% and Passage 11 = 42%. By 120 hours the Passage 3 cells had 93% of the population labeled, whereas Passage 9 and Passage 11 had only 77% and 63% of the cell population labeled.

3.1.0.3 SVE: Early, Mid and Late Passages

Saphenous vein-derived endothelial cells showed a decrease in the total number of labeled cells as the age in vitro increased [Figure 6]. SVE showed a decline in the total number of labeled cells with increase in the cells in vitro age. Passage 6 cells had a very steep labeling curve. Older passage cells had a typical log phase portion of the curve with a plateauing of the incorporation of the \(^3\)H thymidine label. The maximum number of labeled cells was 86% for passage 6; whereas there was a decrease in the maximum number of labeled for both passages 9 and 13. Up to 48 hours there was no
$^3$H TdR was added at T=0. Coverslips were removed from the labeled medium at specific time intervals and autoradiographed. One hundred cells were counted at each time point and the percent labeled cells calculated. Squares = Passage 3, Circles = Passage 9, X = Passage 11.

**Figure 5:** Human Umbilical Vein Endothelial Cells: Labeling Index
difference in the number of labeled cells for passages 9 and 13 (59% and 62%, respectively). After 72 hours the younger cells reached 74% labeled cells by 120 hours. Passage 9 cells reached their $^{3}\text{H}$ thymidine incorporation plateau at 72 hours. Passage 13 cells reached the maximum labeling at 72 hours of 69% but, there was a decrease in the number of labeled cells after that time indicating a loss of dividing cells in the culture. At 48 hours there was a leveling off of the $^{3}\text{H}$ thymidine incorporation for the passage 13 cells. The older cells ultimately appear to have less labeled cells than the passage 6 cells.

3.1.0.4 Comparison: HAE vs. SVE vs. HUVE

The labeling index has been compared among HAE, HUVE and SVE at Passages 10, 9 and 9, respectively [Figure 7]. There was no difference in the total number of labeled cells for either the SVE or HUVE. But, HAE had only 48% of the total population of the endothelial cells labeled. HUVE and SVE do not appear to have a lag phase. The kinetics of HAE resulted in a more shallow curve. The total number of labeled cells for HAE did not increase after 48 hours, whereas HUVE and SVE plateaued at 96.
$^3$H TdR was added at T=0. Coverslips were removed from the labeled medium at specific time intervals and autoradiographed. One hundred cells were counted at each time point and the percent labeled cells calculated. Squares = Passage 6 and Circles = Passage 9 and $X$ = Passage 13.

**Figure 6:** Saphenous Vein Endothelial Cells: Labeling Index
$^3$H TdR was added at T=0. Coverslips were removed from the labeled medium at specific time intervals and autoradiographed. One hundred cells were counted at each time point and the percent labeled cells calculated. Squares = HAE P 10, Circles = HUVE P 10 and X = SVE P 9.

Figure 7: Labeling Index: Comparison of Human Derived Endothelial Cells
3.2 Membranes and Morphology

The phospholipid assays were done by Eric J. Murphy in the laboratory of Dr. Lloyd Horrocks, Department of Physiological Chemistry, The Ohio State University.

The phospholipid content of the samples were calculated from a linear regression curve of the phosphorus standards. The standard curve was used to convert the absorbence to moles phosphorus per fraction. The amount of phosphorus in each fraction was divided by the total amount of phosphorus in the sample to achieve a molar percent of the total phospholipid content of the cells.

To test for significance the Mann-Whitney U/ Wilcoxon rank sum W test (non-parametric paired t-test) and a one-way analysis of variance (parametric) were used. The cells were compared by passage and by cell type.

3.2.1 Baseline Comparison of Phospholipids

Results are presented in Appendix A.
3.2.2 Comparison of Morphology by Transmission Electron Micrographs of HAE, HUVE and SVE

To determine if the variance in the cardiolipin content of these endothelial cells could be related to their ultrastructure, thin sections were examined by transmission electron microscopy. HUVE and SVE [Figure 8a and b] were more similar to each other than to HAE [Figure 8c]. The venous derived cells seemed to have areas of higher concentrations of mitochondria than HAE. SVE and HUVE appeared to be more reactive than the HAE. This can be noted in the cell shape. The HUVE [Figure 8a] and SVE [Figure 8b] were rounder and not as spread-out as the HAE [Figure 8c]. All cells had Weibel-Palade (W-P) bodies, which are the hallmark of endothelial cells. The W-P bodies are approximately the same size as mitochondria. W-P bodies have very tight striations and are easily differentiated from mitochondria [Figure 3]. HAE had numerous large lipid laden vacuoles, whereas there were very few or none evident in the HUVE and SVE. The lipid laden vacuoles were reflective of the cells metabolic state. Large pseudopodia were extending from one side of the HAE an indicating migratory abilities and a directionality of movement. In higher magnification TEM's extensive areas of microfilaments were seen within the pseudopods. The HUVE and SVE had much smaller and fewer pseudopodia than the HAE.
Figure 8: Transmission Electron Micrographs: HUVE, HAE and SVE
Figure a: HUVE passage 10. The cell has numerous mitochondria (M). The cell has few pseudopodia (P). The cell has no large lipid inclusions. 
N = nucleus; WP = Weibel-Palade.

Figure b: SVE passage 10. The cell has numerous mitochondria (M). Few pseudopodia (P) and no obvious lipid vacuoles (L). Weibel-Palade (WP) bodies are present. N = nucleus.

Figure c: HAE passage 9. Cell has numerous pseudopodia (P) and large lipid inclusions (L). The cell has less mitochondria than SVE or HUVE. Weibel-Palade bodies are evident near the nucleus (WP). N = nucleus; MF = Myelin.

Figure 8, concluded
3.3 Wound Healing

3.3.1 HAE vs HUVE vs SVE

3.3.1.1 Wound "Repair"

HUVE started to repopulate the wound within 6 hours of denuding the area [Figure 9 panel b]. The cells did not show any directed movement into the wound. Near the wound edge there was an increase in the number of large cells having thin wispy looking cytoplasm and/or multiple nuclei. Passage 10 had more large cells than passage 2 [Figure 9 panel b and e]. As the age in culture of the HUVE increased, there was a loss of compactness of the cell sheet near the wound as compared to the passage 2 cells [Figure 9 panel b and e]. But, by 12 hours post-wounding both the "young" and "old" cells had completely repopulated the wounded area [Figure 9 panel c and f]. HUVE passage 2 were tighter at the wound than at passage 10 [Figure 9 panel c and f].

SVE showed minimal migration into the wound for the first 6 hours, but, by 18 hours the wound was not visible [Figure 10 panel c and f]. SVE moved into the wound perpendicular to the wound [Figure 10 panel b and e]. The cells of both passage 2 and 10 seem to spread as a sheet. There was a loosening of the confluent areas of the dish with the cells closer to the
Figure 9: Wound Healing: HUVE
Passage 2 versus Passage 10
Cells were rinsed with PBS and wounded. Rewashed with PBS and fresh medium added. Cultures were photographed every 6 hours until the wound was repaired. Original magnification of 100 x.

Passage 2:
Panel a: $T = 0$ Hr. Cells freshly wounded.
Panel b: $T = 6$ Hrs. Migration of whole sheet toward the wound. Note a loosening of the cell sheet.
Panel c: $T = 12$ Hrs. The wound was completely healed. The cell sheet had lost its compactness upon healing.

Passage 10:
Panel d: $T = 0$ Hr. Cells freshly wounded.
Panel e: $T = 6$ Hrs. Random cellular migration. Note large bipolar cells in the wound. Also, there are large bare areas between the cells. Some multinucleated cells can be seen.
Panel f: $T = 12$ Hrs. The cell sheet has completely repopulated the wound. There are many large cells with "wispy" cytoplasm. Also, sprout cells can be seen.

Figure 9, concluded
Figure 10: Wound Healing: SVE Passage 2 versus Passage 10
Cells were rinsed with PBS and wounded, rewashed with PBS and fresh medium added. Cultures were photographed every 6 hours until the wound was repaired. Original magnification of 100 x.

Passage 2:
Panel a: T = 0 Hr. Cells freshly wounded.
Panel b: T = 12 Hrs. Cells migration perpendicular to the wound edge. There is a spreading out of the cells.
Panel c: T = 18 Hrs. The wound was completely healed. Near the wound the cells are more compact then toward the edge of the culture.

Passage 10:
Panel d: T = 0 Hr. Cells freshly wounded.
Panel e: T = 12 Hrs. The cell sheet has lost its compactness. The cell movement into the wound was more random than passage 2 at 12 hours.
Panel f: T = 18 Hrs. The wound was completely healed. The cell sheet was less compact. The cells had a bipolar morphology. There were gaps between numerous cells.

Figure 10, concluded
wound being much more spread out. This was more evident in passage 10 than passage 2 [Figure 10 panel c and f].

HAE showed minimal movement into the wounded area for the first 12 hours. At first cellular movement into the wound was irregular [Figure 11 panel b]. Complete infilling of the denuded area took 36 hours [Figure 11 panel c]. The least amount of time to infill the wound was 24 hours. At 24 hours many cells were seen to line up parallel to the wound and this was still visible at 36 hours [Figure 11 panel c]. Near the wound the cells were more compact than cells toward the edge of the tissue culture dish.

3.3.1.2 Morphology

A comparison of SVE and HUVE at passage 2 showed differences in their response to physical stress. The saphenous vein-derived endothelial cells [Figure 10 panel c] were the only cell type to retain their typical endothelial cell pavement morphology. The HUVE [Figure 9 panel c] developed an increased number of "giant" multinucleated cells near the "wound" edge. To infill the "denuded" area the endothelial cells appear to become less adhesive to each other. All the cultures lost the tight compactness of typical stationary confluent endothelial monolayer.

HAE, HUVE and SVE at passage 10 showed differences in their morphologies. HAE had more cells with multiple nuclei [Figure 11 panel c],
Figure 11: Wound Healing: HAE Passage 10
Cells were rinsed with PBS and wounded, rewashed with PBS and fresh medium added. Cultures were photographed every 6 hours until the wound was repaired. Original magnification 200 x.

Passage 10:
Panel a:  $T = 0$ Hr. Cells freshly wounded.
Panel b:  $T = 12$ Hrs. The cells spread out and gaps were seen between the cells. Cell movement was random into the wound but, once in the wound the cells lined up parallel to it.
Panel c:  $T = 36$ Hrs. Cells were lined up parallel within the wound.
There was a definite loosening of the cell sheet.

Figure 11, concluded
but their cytoplasm did not show swirls as seen in both the HUVE [Figure 9 panel f] and SVE [Figure 10 panel f]; they therefore appear less stressed than the venous-derived cells. The HAE at passage 10 showed definite directionality into the wound whereas the HUVE and SVE polarity were more random. All the cultures were less compact than the controls. The loss of compact cell sheets appear to be typical for early as well as later passages.

3.3.1.3 Age of Cells *in vitro* vs Repair

The HUVE and SVE wound healing experiments were repeated a total of 6 times with donors and cultures of varying ages. The HAE wound healing experiments were repeated a total of 3 times. The age of the donor or of the culture did not affect the ability of the endothelial cells to repair the denuded area. The *in vitro* age of the cultures varied from Passage 2 to Passage 10. The age of the donors varied from 11 years old to 38 for the HAE and the SVE ages varied between 50 and 70 years old. The HUVE are considered to be new born an age of 0. The HAE and SVE were isolated from males. The sex can not be undetermined for the HUVE.
3.3.2 Two Stages of Wound Healing

3.3.2.1 Wounded vs Unwounded $^3$H TdR Incorporation

There was a decrease in the total amount of $^3$H TdR incorporated into wounded vs. unwounded HAE and HUVE. The unwounded cells initially incorporated more of the label than the wounded monolayers. The unwounded monolayer had $5.6 \times 10^4$ cells/$cm^2$ and the wounded monolayer had $4.47 \times 10^4$ cells/cm$^2$, a 20% decrease in the total number of cells. A 20% decrease in the total number of cells for the wounded monolayer negates the increase in $^3$H TdR for the unwounded monolayer. The unwounded HAE reached maximum labeling 24 hours after the thymidine addition (44844 cpm) [Figure 12]. The wounded HAE reached maximum incorporation at 36 hours (47311 cpm) [Figure 12]. The HUVE unwounded monolayer had a relatively steep curve [Figure 13]. There were approximately 25% less cells in the wounded versus the unwounded monolayer (approx. $3.75 \times 10^4$ and $5 \times 10^4$, respectively). [Figure 13]. The wound caused a depression in the initial ability of the HUVE and HAE to incorporate $^3$H TdR. There was a reversal of the incorporation pattern of SVE wounded and unwounded monolayers [Figure 14]. There seems to be an increase in the cells ability to incorporate the thymidine after wounding the monolayer. At 72 hours after the addition of the precursor the wounded cells had $25307 \pm 6365$ cpm (mean $\pm$ standard deviation); while the unwounded cells had $13839 \pm 4813$ cpm of label incorporated [Figure 14].
The cells were washed with PBS wounded, rewashed with PBS and 0.1 
\(\mu\)Ci \(^3\)H TdR/ml of growth medium was added. At specific times 
cells were washed, trypsinized and diluted with PBS and an equal 
volume of 10% TCA was added. Incorporated \(^3\)H TdR was measured by 
LSC. Squares = unwounded, N =1; circles = wounded, N = 3. A 20% 
decrease in the total number of cells for the wounded monolayer 
negates the increase in \(^3\)H TdR for the unwounded monolayer.

**Figure 12:** Wounded vs. Unwounded HAE: 
\(^3\)H Thymidine Incorporation
The cells were washed with PBS then wounded, rewashed with PBS and 0.1 μCi $^3$H TdR/ml of growth medium was added. At specific times cells were washed, trypsinized, diluted with PBS and an equal volume of 10% TCA was added. Incorporated $^3$H TdR was measured by LSC. $N = 3$. Squares = unwounded and circles = wounded. Wounding caused a depression in the $^3$H TdR incorporation curve.

**Figure 13:** Wounded vs Unwounded HUVE: $^3$H Thymidine Incorporation
The cells were washed with PBS then wounded. Rewashed with PBS and 0.1 nCi/ml of growth medium was added. At specific times cells were washed, trypsinized and diluted with PBS and an equal volume of 10% TCA was added. Incorporated $^3$H TdR was measured by LSC. N = 4. Squares = unwounded and circles = wounded. The wounded monolayer incorporated more label than the unwounded at 72h (p=0.03).

**Figure 14:** Wounded vs Unwounded SVE: 
$^3$H Thymidine Incorporation
A Student’s t-Test was done to determine if there was a significant difference between the wounded and unwounded monolayers ability to incorporate $^{3}$H TdR. There was no significant difference between the wounded and unwounded SVE at 48 hours ($p<0.1$). Seventy-two (72) hours after the addition of the $^{3}$H TdR the wounded monolayer incorporated significantly more $^{3}$H TdR ($p=0.03$). The HUVE at times 0, 24, and 48 hrs were not significantly different ($p > 0.1$). At 12 and 24 hours post addition of the $^{3}$H TdR there was a significant difference ($p < 0.005$). Due to the small sample population of the unwounded HAE a Student’s t-Test was not done. But, the data suggested that there was no significant difference between the wounded and unwounded HAE monolayers.

3.3.2.2 Effects of Serum vs. ECGF and Heparin on Wound Healing

SVE and HUVE responded to the serum by migrating into the wound by 8 hours. Cells grown in the ECGF/heparin took 12 hours before cells were seen bridging the wound. Medium which contained the ECGF and heparin seemed to facilitate complete repopulation of the wound. Wound repopulation was delayed to 48 hours for both cell types. The medium containing only FBS had extensive cellular debris and the wound was still evident after 48 hours. Under the same conditions the HAE responded by detaching from the surface. There was some evidence of migration but no true bridging. By 48 hours most of the HAE cells were no longer attached to the substrate.
Tritiated thymidine was added to these cultures at time zero [Figure 15]. As seen in Figure 15, the cells grown in M-199 and ECGF/heparin had a higher amount of $^3$H thymidine incorporation over M-199 plus FBS or M-199 alone [Figure 15]. Only the cells grown in the ECGF/Heparin medium healed ($T = 48$ hours). The total amount of label incorporated for the ECGF/heparin treated cells was lower for the HAE in comparison to M-199 plus FBS or M-199 alone. The HAE showed a decrease in total tritiated thymidine incorporated at 72 hours [Figure 16]. This was due to cells detaching from the substrate.

3.4 Production of Prostaglandins

The data was manipulated using parametric and non-parametric analysis. The statistical tests used included: ANOVA with an a posteriori Scheffe procedure, Kruskal-Wallis 1-Way ANOVA (non-parametric) and Mann-Whitney U Test (non-parametric equivalent of the t-Test).
Cells were washed with PBS and then wounded. At T=0 the test medium was added with 0.1 μCi/ml ³H TdR. Triangle = M-199 plus ECGF/Heparin, Circle = M-199 plus 20%,FBS and Square = M-199. Solid symbols = SVE and the open symbols = HUVE, N = 2.

No significant difference between the effects of the test media for SVE (p<0.05). There was a significant difference for FBS vs. M-199 at 4, and 72 h (p<0.01); FBS vs. ECGF at 24 and 72 h (p<0.01) and ECGF vs. M-199 at 72 h (p<0.01) as determined by Student’s t Test.

**Figure 15:** Effects of Medium Additives on Wound Healing of HUVE and SVE
Cells were washed with PBS and then wounded. At $T=0$ the test medium was added with 0.1 $\mu$Ci/ml $^3$H TdR. Triangle = M-199 plus ECGF/Heparin, Circle = M-199 plus 20% FBS and Square = M-199. M-199 plus 20% FBS had the highest labeling.

**Figure 16:** Effects of Medium Additives on Wound Healing of HAE
3.4.1 Baseline Data: Prostaglandin Production

The production of 6-keto PGF$_{1\alpha}$ was significantly higher in arterial derived endothelial cells than the umbilical vein at $p < 0.01$ and SVE were significantly different from HUVE at $p < 0.05$. The average production of 6-keto PGF$_{1\alpha}$ per 1 million cells was $1899 \pm 707.11$, $375.5 \pm 84.46$, and $216.91 \pm 61.69$ picograms for HAE, SVE and HUVE under non-stimulated conditions, respectively [Figure 17a]. There was no significant difference between the concentration of 6-keto PGF$_{1\alpha}$ for HAE and HUVE. Due to the large variance among the samples, a comparison was done among cells by passage and stage of confluence. These 2 parameters did not have any effect on the data.

Baseline PGF$_{2\alpha}$ data were taken from the A 23187 experiments. There was no significant difference between the cells in their ability to produce PGF$_{2\alpha}$ [Figure 17b]. The amount of PGF$_{2\alpha}$ produced ranged from 9871 to 7814 picograms per $10^6$ cells. There was an order of magnitude greater production of PGF$_{2\alpha}$ by the venous derived endothelial cells as compared to their production of 6-k PGF$_{1\alpha}$ [Figure 17a and b]. The HAE had a 3 fold greater production of PGF$_{2\alpha}$ over 6-k PGF$_{1\alpha}$. 
Figure 17: Baseline Production of Prostaglandins
Medium was removed from growing culture and the medium frozen at -80°C. PGs were determined by RIA. For 6-k PGF<sub>1α</sub> sample number = 23 for SVE, 42 for HAE and 34 for HUVE. HAE was significantly different from HUVE p < 0.01 and SVE was significantly different from HUVE p < 0.05. For PGF<sub>2α</sub> sample number = 36 for SVE, 45 for HAE and 36 for HUVE. There was no significant difference at p < 0.05. To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis). Data was expressed as picograms plus or minus the standard error of the mean.

Figure 17, concluded
3.4.2 Prostacyclin Production After Addition of A 23187

3.4.2.1 Saphenous Vein Endothelial Cells

There was no significant difference in the amount of 6-k PGF$_{1\alpha}$ produced by SVE with either time or treatment [Figure 18a]. The amount of 6-k PGF$_{1\alpha}$ produced ranged from 366.28±117.81 to 158.73±16.77 (mean ± standard error of the mean) picograms per 10$^6$ cells.

3.4.2.2 Human Arterial-Derived Endothelial Cells

HAE produced significantly more 6-k PGF$_{1\alpha}$ 24 hours after exposure to 1 μM A 23187, p < 0.01 [Figure 18b]. There was a decreased production of 6-k PGF$_{1\alpha}$ after washing the cells and adding the control medium as compared to the baseline of 6-k PGF$_{1\alpha}$ (28.89±5.53 vs 86.76±4.93, respectively). When comparing the effect of either 1 hour of exposure to 1 μM a 23187 or to the control 24 hours later there was no difference in the amount of 6-k PGF$_{1\alpha}$ (67.05±13.14 and 63.89±5.10, respectively).

Twenty-four hours after exposure to 1 μM A 23187 there was 3 fold increase in the amount of 6-k PGF$_{1\alpha}$ produced over the rest of the data [Figure 18b].
Figure 18: 6-k PGF₁α Production by Endothelial Cells

![Graph showing 6-k PGF₁α production by various endothelial cells over time.](image-url)
At \( T=0 \) medium was saved. Cells were washed with M-199 and appropriate test media added. At the designated times, media were saved, cells rinsed, and one plate counted. Fresh growth medium was added and the cells incubated for 24 hrs. And the procedure repeated. To test for significance an analysis of variance with an apriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis).

SVE: \( N= 36 \) for baseline, control =16, 1 \( \mu M \) A23187 = 12, control 24 hrs post = 8 and 1 \( \mu M \) A23187 post-exposure = 8. There was no significant difference at \( p<0.05 \).

HAE: \( N= 45 \) for baseline, control = 20, 1 \( \mu M \) A23187 = 19, control 24 hrs post = 10 and 1 \( \mu M \) A23187 post-exposure = 10. There was a significant difference between the cells 24 hours post-exposure to 1 \( \mu M \) A23187 and the rest of the cells at \( p<0.01 \).

HUVE: \( N= 36 \) for baseline, control =16, 1 \( \mu M \) A23187 = 15, control 24 hrs post = 8 and 1 \( \mu M \) A23187 post-exposure = 8. Baseline and cells 24h post-exposure with 1 \( \mu M \) A23187 produced significantly more 6-k PGF\(_{1\alpha}\) than the control (\( p<0.05 \)).

Open Bar = baseline, left slant bar = control, right slant bar = 1 \( \mu M \) A 23187. * \( p<0.05 \), ** \( p<0.01 \)

Figure 18, concluded
3.4.2.3 Human Umbilical Vein-Derived Endothelial Cells

There was a significant difference in the amount of $6$-k PGF$_{1\alpha}$ produced by HUVE in response to $1$ $\mu$M A 23187 [Figure 18c]. Cells that were washed and refed with standard growth medium produced significantly more $6$-k PGF$_{1\alpha}$ than the control cells at $p < 0.05$. Also, 24 hours after exposure to the ionophore the amount of $6$-k PGF$_{1\alpha}$ produced was twice the baseline production ($824.57\pm176.18$ and $353.77\pm26.16$ picograms, respectively) [Figure 18c].

3.4.2.4 Comparison: SVE, HAE, and HUVE

The A 23187 experimental data were analyzed by cell type, keeping the treatment constant. The cells were washed; the control medium was added and after 15 minutes the medium was saved. SVE and HUVE, after 15 minutes in control medium, produced significantly more $6$-k PGF$_{1\alpha}$ than HAE, $p < 0.001$ [Figure 19 panel a] HAE concentration of $6$-k PGF$_{1\alpha}$ was $86.76\pm4.93$ picograms per $10^6$ cells versus SVE and HUVE concentration of $294.99\pm27.50$ and $353.77\pm26.16$, respectively. After 1 hour exposure to $1$ $\mu$M A 23187 the HAE produced a significantly lower amount of $6$-k PGF$_{1\alpha}$ than the SVE or the HUVE at $p < 0.01$. All 3 cell types produced more $6$-k PGF$_{1\alpha}$ than their respective controls [Figure 19 panel b]. In Figure 19 panel c, 24 hours after exposure to the control medium there was still a significant difference among the cells. HAE produced far less $6$-k PGF$_{1\alpha}$ than
Figure 19: 6-k PGF$_{1\alpha}$ Production: A Comparison by Cell Type
To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis).

Panel A: After 15 min in the control medium there was a significant difference among the cells. HAE was significantly lower than HUVE and HAE at $p<0.001$.

Panel B: After 1 hr in medium containing 1 μM A 23187 the HAE produced significantly lower amount ($p<0.01$).

Panel C: Control 24 hrs post-exposure SVE and HUVE produced significantly more 6-k PGF$_{1\alpha}$ than HAE ($p<0.05$).

Panel D: There was no difference among the cells 24 hrs after exposure to 1 μM A 23187.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 19, concluded
the veins \((p < 0.05)\). But, 24 hours after the removal of the 1 \(\mu\text{M A 23187}\) there was no significant difference among the cells [Figure 19 panel d].

3.4.3 \(\text{PGF}_2\alpha\) Production After Addition of 1 \(\mu\text{M A 23187}\)

3.4.3.1 Saphenous Vein-Derived Endothelial Cells

The amount of \(\text{PGF}_2\alpha\) produced during the 24 hours after exposure to 1 \(\mu\text{M A 23187}\) was significantly higher than either the baseline production, the control or exposure to the ionophore for 1 hour [Figure 20a]. After washing the cells with room temperature M-199 and application of the control medium there was a decrease in the amount of \(\text{PGF}_2\alpha\) by an order of magnitude below the baseline values. But, 24 hours after the exposure to the control medium the \(\text{PGF}_2\alpha\) concentration was almost equivalent to the baseline value. Twenty-four (24) hours after challenge with the ionophore, twice as much \(\text{PGF}_2\alpha\) was released as compared to the baseline (18350±2109 vs. 9871±1076, respectively) [Figure 20a].

3.4.3.2 Human Arterial-Derived Endothelial Cells

The baseline as well as all the experimental cells produced significantly more \(\text{PGF}_2\alpha\) than the controls [Figure 20b]. The baseline cells and the cells 24 hours after exposure to either the control or experimental media were at least 3 times greater than the control. The cells challenged with 1 \(\mu\text{M A}\)
Figure 20: PGF$_{2\alpha}$ Production by Endothelial Cells
At T=0 medium was saved. Cells were washed with M-199 and appropriate test medium added. At the designated times, media were saved, cells rinsed, and one plate counted. Fresh growth medium was added and the cells incubated for 24 hrs, and the procedure repeated. To test for significance an analysis of variance with an apriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis). N = sample number.

SVE: N = 36 for baseline, control = 16, 1 uM A 23187 = 12, control 24 hrs post = 8 and 1 uM A 23187 post-exposure = 8. The control samples was significantly lower than the other treatments at p<0.05.

HAE: N = 45 for baseline, control = 20, 1 uM A 23187 = 19, control 24 hrs post = 10 and 1 uM A 23187 post-exposure = 10. There was a significant difference between the cells 24 hours post-exposure to 1 uM A 23187 then the rest of the cells at p<0.01. Also the control, was significantly lower than the other samples (p<0.01).

HUVE: N = 36 for baseline, control = 16, 1 uM A 23187 = 15, control 24 hrs post = 8 and 1 uM A 23187 post-exposure = 8. Baseline and cells 24h post-exposure with 1 uM A 23187 produced significantly more 6-k PGF$_{1\alpha}$ than the control (p<0.01).

Open Bar = baseline, Left slant bar = control, Right slant bar = 1 uM A 23187. * p<0.05, ** P<0.01.

Figure 20, concluded
23187 showed a 2-fold increase in PGF$_{2\alpha}$ production over its control. There was a significant difference at $p < 0.01$.

### 3.4.3.3 Human Umbilical Vein-Derived Endothelial Cells

Both, baseline production and the amount of PGF$_{2\alpha}$ produced 24 hour post-exposure to 1 $\mu$M A 23187 was significantly different than the control ($p < 0.01$) [Figure 20c]. There was no significant difference between the other samples.

### 3.4.3.4 Comparison: SVE, HAE and HUVE

SVE, HAE and HUVE were compared by treatment [Figure 21]. There was no significant difference observed among the cells by treatment. The cells had the lowest amount of PGF$_{2\alpha}$ after they were washed and the control medium was added for 15 minutes [Figure 21 panel a]. There was an increased production of PGF$_{2\alpha}$ after exposure to the 1 $\mu$M A 23187, but there was not a significant difference among the different endothelial cells [Figure 21 panel b and panel d].
Figure 21: SVE, HAE and HUVE Production of PGF$_{2\alpha}$: A Comparison
To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis).

Panel A: There was no significant difference among the cells after addition of control medium.

Panel B: After 1 hr in medium containing 1μM A23187 there was no significant difference among the cells.

Panel C: 24 hrs post-exposure to the control medium there was no difference among the cells.

Panel D: There was no difference among the cells 24 hrs after exposure to 1 μM A23187.

Figure 21, concluded
3.5 Cyclic Nucleotides

3.5.1 Baseline Production of Cyclic Nucleotides

Prior to initiation of the experiment, cells were extracted for the baseline content of cyclic nucleotides. The data showed that SVE produced significantly more cAMP than HAE ($p < 0.01$). HUVE production was not significantly different from either HAE or SVE [Figure 22a]. There was no significant difference in the amount of cyclic GMP in the endothelial cells. HUVE average production of cGMP was twice that of the HAE and 3 times the SVE cells, but due to the variance within the population there was no significant difference [Figure 22b].

3.5.2 cAMP Production After Exposure to A 23187

3.5.2.1 Saphenous Vein-Derived Endothelial Cells

Application of A 23187 did not have an effect on increasing the production of cyclic AMP over the baseline or the controls [Figure 23a]. After 1 hour of exposure to A 23187 the amount of cAMP did not change as compared to the control or baseline [Figure 23a]. Twenty four hours after exposure to A 23187 there was a slight increase in the amount of cAMP over the matched control. The amount of cAMP produced varied between 3280 to 4593 femtomoles per $10^6$ cells [Figure 23a].
Figure 22: Baseline Production of Cyclic Nucleotides
Medium was removed from confluent cells which were then washed twice with PBS. Cells were extracted with 5% TCA and the cyclic nucleotides were determined by RIA. To test for significance an analysis of variance with an apriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis). N = 3 for SVE, 4 for HAE and 3 for HUVE. SVE produced significantly more cAMP than HAE p<0.01. There were no significant differences in the amounts of cGMP produced by the cells (p<0.05).

** p<0.01.

Figure 22, concluded
Figure 23: Effect of 1 μM A23187 on cAMP Production

- **SVE**
- **HAE**
- **HUVE**

**cAMP PER 10^8 CELLS (FEMTOMOLES)**

**TIME (HOURS)**

- Baseline
- Control
- 1 μM A23187
At time=0 one plate was extracted. The rest of the cells washed with M-199 and appropriate test medium added. At the designated times, cells were rinsed with PBS and extracted with 5% TCA. Fresh growth medium was added and the cells incubated for 24 hrs. And the procedure repeated. These experiments were done in tandem with the PG experiments. To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis). N = sample number.

SVE: N = 3 for baseline, control = 3, 1 μM A23187 = 3, control 24 hrs post = 3 and 1 μM A 23187 post-exposure = 3. There were no significant differences among the samples at p<0.05.

HAE: N = 4 for baseline, control = 4, 1 μM A 23187 = 4, control 24 hrs post = 4 and 1 μM A 23187 post-exposure = 4. There were no significant differences at p<0.05

HUVE: N = 3 for baseline, control = 3, 1 μM A23187 = 3, control 24 hrs post = 3 and 1 μM A 23187 post-exposure = 3. There were no significant differences at p<0.05.

Open Bar = baseline, left slant bar = control, right slant bar = 1μM A 23187.

Figure 23, concluded
3.5.2.2 Human Arterial-Derived Endothelial Cells

As seen in the SVE data, 1 μM A23187 did not have an effect on increasing the amount of cAMP produced [Figure 23b]. The control concentration of cAMP was equivalent to the amount of cAMP seen after addition of the ionophore (1836=149 and 1795=470, respectively). A similar response was seen when comparing the data for 24 hours after the exposure to 1 μM A23187 versus the matched control (2087=980 and 2253=397, respectively).

3.5.2.3 Human Umbilical Vein-Derived Endothelial Cells

Cyclic AMP production by HUVE did not change upon exposure to 1 μM A23187 [Figure 23c]. Twenty four hours after HUVE exposure to 1 μM A23187 there was a decrease in the amount of cAMP as compared to the baseline or matched controls; but this was not significant at p < 0.05 (1587, 3024, and 2746, respectively). This was due to a large variance among the samples.

3.5.2.4 Comparison: SVE, HAE and HUVE

Endothelial cells were compared with treatment being kept constant. In Figure 24 panel a, there was a significant difference between the HAE cells and those of HUVE and SVE after being exposed to the control medium. SVE had a higher concentration of cAMP, 4593 femtomoles per 10^6 cells.
than either HAE (1837) or HUVE (2684). SVE concentration was significantly different from HUVE and HAE (p < 0.01). The other comparisons did not reveal any differences among the cells [Figure 24 panels b,c,d]. Even though there appear to be differences in the average production of cAMP 24 hours after exposure to the ionophore, there was no statistical difference due to the size of the variance of the sample population.

3.5.3 cGMP Production After Exposure to A 23187

3.5.3.1 Saphenous Vein-Derived Endothelial Cells

SVE showed no significant changes in the amount of cGMP detected after treatment with A 23187 [Figure 25a]. The concentration of cGMP varied from 818 femtomoles per $10^6$ cells for the baseline to 2675 femtomoles per $10^6$ cells, 24 hours after exposure to the ionophore. But, there was no significant difference, p < 0.05, due to the variance within the sample population.

3.5.3.2 Human Arterial-Derived Endothelial Cells

There were no significant differences among the different treatments with respect to the production of cGMP. HAE showed an increase in cGMP after addition of A 23187 [Figure 25b] over the matched control. There was, also, an increase in the amount of cGMP 24 hours after exposure to the
Figure 24: Comparison: SVE, HAE and HUVE
Production of cAMP

![Graph showing production of cAMP per 10^6 cells for SVE, HAE, and HUVE.](image)
To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis).

Panel A: SVE produced significantly more cAMP than HAE or HUVE at p<0.01.

Panel B: After 1 hr in medium containing 1μM A 23187 there was no significant difference among the cells.

Panel C: 24 hrs post-exposure to the control medium there were no differences among the cells.

Panel D: There were no differences among the cells 24 hrs after exposure to 1 μM A 23187.

** p<0.01.

Figure 24, concluded
Figure 25: Production of cGMP by Human Endothelial Cells
At \( T = 0 \) one plate was extracted. The rest of the cells were washed with M-199 and appropriate test medium added. At the designated times, cells were rinsed with PBS and extracted with 5% TCA. Fresh growth medium was added, the cells incubated for 24 hrs, and the procedure repeated. These experiments were done in tandem with the PG experiments. To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis). Sample number = \( N \).

SVE: \( N = 3 \) for baseline, control = 3, 1 \( \mu M \) A23187 = 3, control 24 hrs post = 3 and 1 \( \mu M \) A23187 post-exposure = 3. There were no significant differences among the samples at \( p<0.05 \).

HAE: \( N = 4 \) for baseline, control = 4, 1 \( \mu M \) A23187 = 4, control 24 hrs post = 4 and 1 \( \mu M \) A23187 post-exposure = 4. There were no significant differences.

HUVE: \( N = 3 \) for baseline, control =3, 1 \( \mu M \) A23187 = 3, control 24 hrs post = 3 and 1 \( \mu M \) A23187 post-exposure = 3. There were no significant differences among the samples at \( p<0.05 \).

Open Bar = Baseline, Left Slant Bar = Control, Right Slant Bar = 1 \( \mu M \) A23187.

Figure 25, concluded
ionophore versus the matched control. Again, due to the variance within the sample population there was no significant difference at $p < 0.05$.

3.5.3.3 Human Umbilical-Derived Endothelial Cells

There was a decrease in the production of cGMP 24 hours after the application of $1 \mu M$ A 23187 and a decrease was also seen in the matched control as compared to the baseline concentration of cGMP [Figure 25c]. The concentration of cGMP ranged from 3272 ($1 \mu M$ A 23187) to 1670 (24 hours after exposure to $1 \mu M$ A 23187) femtomoles per $10^6$ cells. As with the HAE and SVE, there was no significant difference within the cell population at $p < 0.05$.

3.5.3.4 Comparison: SVE, HAE and HUVE

There was no difference among the cell types when compared by treatment [Figure 26 panels a,b,c,d]. From Figure 26 a it can be seen that due to the large standard error of the mean there was no difference between the HUVE and the SVE or HAE. This was evident in all comparisons by treatment.
Figure 26: Comparison: SVE, HAE and HUVE
Production of cGMP
To test for significance an analysis of variance with an a posteriori Scheffe procedure was done. Cell differences were also analyzed using a nonparametric 1-way ANOVA (Kruskal-Wallis).

Panel A: There were no significant differences among the cell types.

Panel B: After 1 hr in medium containing 1μM A 23187 there were no significant differences among the cell types.

Panel C: 24 hrs post-exposure to the control medium there were no differences among the cell types.

Panel D: There were no differences among the cell types 24 hrs after exposure to 1 μM A 23187.

Figure 26, concluded
Chapter 4
Discussion

4.1 Labeling Index

Labeling indices are a method for determining the proliferative capacity of cells in culture\textsuperscript{75}. The proliferative capacity of cells \textit{in vitro} is a good measure of cellular aging. As the \textit{in vitro} age of a cell culture increases the total number of cells which are able to divide decreases. The cells will also develop signs of \textit{in vitro} ageing, cytoplasm will become less homogeneous and the nucleus becomes more condensed. HAE have a lower proliferative capacity in culture than the SVE or HUVE [Figure 7]. This could be interpreted as there being less cells able to divide in the HAE population than either the HUVE or SVE. At passage 9, SVE and HUVE retain a higher capacity for division than their arterial counterparts at passage 10 [Figure 7]. To avoid the problem of differences in the proliferative capacities
of HAE, HUVE, and SVE, all experimentation was done on stationary confluent cultures.

Arterial-derived endothelial cells do not have the same proliferative capacity as the venous derived endothelial cells under the same environmental conditions. HAE seem to be adversely affected by growth in vitro. These cells develop lipid laden vacuoles and prominent myelin figures by passage 10. The SVE and the HUVE did not have any obvious lipid vacuoles or prominent myelin figures [Figure 8].

There may be several reasons for the differences in the maximum number of labeled cells including nutrition and population doubling levels. The total number of cells harvested from a given artery was much lower. Most arteries received had been on the kidney pump for more than 5 hours. The pressure at which nutrients were passed through the kidney most likely caused sloughing of the endothelium and added to the low yield of cells. With a lower number of cells in the primary cultures, the population doubling level of these cells would be much higher by passage 1.
4.2 Phospholipids

This is the first reported study of the phospholipid composition of human derived endothelial cells. Phosphatidylcholine concentration in human endothelial cells ranged from 37% to 53% of the molar percent phospholipids [Table 1], this was comparable to the ranges reported by Milo\textsuperscript{82} and Rooney\textsuperscript{83} for human and rabbit Type II cells. The percent of phosphatidylethanolamine was equivalent for the human endothelial cells and the human epithelial cells\textsuperscript{82}. Cardiolipin is found in the mitochondria\textsuperscript{42}. It has been suggested that the number of mitochondria is reflective of cellular activity. For instance, the concentration of cardiolipin was found to be higher in rabbit alveolar macrophages and alveolar tumor cells than in the Type II cells\textsuperscript{83}. SVE had a significantly higher concentration of cardiolipin than HAE [Appendix A: Table 1]. The average cardiolipin content for HUVE and SVE was not significantly different. HUVE concentration did not differ significantly from the HAE due to the large variance in the HUVE population. The SVE and HUVE were morphologically very similar [Figure 8]. Transmission electron micrographs showed that the HUVE and SVE had more mitochondria than the HAE. If cardiolipin is a measure of cellular activity it appears that HUVE and SVE are more reactive than HAE. Neither Rooney\textsuperscript{83} or Milo\textsuperscript{82} separated PI from PS and their phosphatidylserine composition varied among the cells. In the present study
both PI and PS were measured and there was a significant difference in PS between HUVE and SVE [Appendix A: Table 1]. Human blood cells (erythrocytes, platelets, and leukocytes) have a higher total amount of PS (10% to 16%) than viscera (1.8% to 9.7%)\(^1\). PS synthesis is possibly influenced by extracellular stimuli. The addition of mitogens to human lymphocytes caused an increase in the total PS\(^1\). Bruni and Toffano\(^1\) showed that lysophosphatidylserine in mast cells interact with nerve growth factor effectively eliciting histamine. This indicates that the deacylated phosphatidylserine may interact with endogenous compounds capable of specific effects at the plasma membrane. In the present study, the differences in the PS could be due to ECGF or serum interacting with the deacylated PS causing the release of chemical mediators which influence the maintenance of normal blood flow.

### 4.3 Wound Healing

Removal of endothelium *in vivo* exposes a surface which is conducive to platelet to adherence. In these studies it was found that endothelial cells derived from various vascular beds differ in their ability to repair physical damage. Umbilical vein-derived endothelial cells migrate and repopulate the wound rapidly [Figure 9]. Saphenous vein [Figure 10] and arterial [Figure 11] derived endothelial cells wounds heal slower than HUVE. A possible
explanation for these differences is that the isolation and culture conditions may have favored the HUVE's with a longer in vitro life span. The fact that these wound repopulation studies were repeated with cell cultures from entirely different vessels decreases this likelihood. Another attractive hypothesis is that wound healing is more inherent in HUVE. However, since wound healing must be a high priority for all vessels, the SVE and HAE may be getting extracellular assistance. HAE and SVE could be releasing factors into the medium which could signal blood borne cells to help in the wound repair process (macrophages)\textsuperscript{84,85}. Recent work by Albrightson et al.\textsuperscript{85} has shown that endothelial cells produce chemotactic factors for macrophages. These factors could be elicited upon stimulation of the cells by exogenous agonists (physical or chemical stress). Also, HAE produce greater amounts of PGI\textsubscript{2} as compared to either HUVE or SVE under standard conditions in vivo\textsuperscript{49} and in vitro [Figure 17]. PGI\textsubscript{2} is an important vasodilator and inhibits platelet aggregation\textsuperscript{47}. An increased production of PGI\textsubscript{2} has been seen in acute inflammation\textsuperscript{25}, causing a loosening of the cell-cell junctions and allowing for cellular spreading\textsuperscript{2,3}. An increased production of PGI\textsubscript{2}, as demonstrated by HAE, could be one of the factors allowing for the delayed wound healing.

The addition of fresh growth medium to confluent cell cultures can cause cells to enter a new round of DNA synthesis. This was seen in both HAE [Figure 12] and HUVE [Figure 13] wounded and confluent non-wounded
cultures. Due to the decreased total number of cells in the wounded HAE culture, however, there appears to be no difference in the amount of $^3$H TdR incorporated between the wounded and unwounded monolayer [Figure 12]. The addition of fresh serum to the wounded HAE does not seem to cause the cells to enter a new round of DNA synthesis immediately. Sholley\textsuperscript{35} did not observe a significant increase in tritiated thymidine incorporation until 36 hours after denuding. HUVE seem to repopulate the wound completely by migration followed later by a round of replication as witnessed by an increase in the number of labeled cells [Figures 9 and 13]. There was no significant difference in the amount of $^3$H TdR incorporated by the wounded or unwounded SVE monolayer until 72 h after the addition of the $^3$H TdR. This suggests that it takes longer for the cells to respond to the damage caused by the wound. These data suggest but do not prove that endothelial cells derived from different vascular beds have differing responses to wound repopulation.

Wound repopulation is a 2 stage phenomenon, migration and then division\textsuperscript{35,37,36}. It appears that medium additives (ECGF/heparin and FBS) control separate effects on wound repopulation. Serum effectively caused early migration of the venous cells into the wound. But, HUVE and SVE grown in M-199 plus FBS did not completely repopulate the wound they had detached from the substrate. Cells grown in M-199 plus ECGF/heparin had delayed entry into the wound. In this medium, complete repopulation of the wound
was delayed by 36 hours for HUVE and 30 hours for SVE. The HAE were detaching from the substrate within 48 hours. It appears that ECGF/heparin and serum separately affect the 2 stages of wound repair. ECGF/heparin, in the HUVE system, seems to have more of an effect on cell division [Figure 15], whereas FBS has more effect on cellular migration. It appears that ECGF and heparin inhibits \( ^3 \)H TdR incorporation of HAE [Figure 16]. Since HAE, HUVE and SVE respond differently to specific medium additives, these data further support the idea that differences between the endothelial cells could be related to their bed of origin as suggested by the wound healing experiments carried out in complete growth medium.

4.4 Prostaglandin Production

Human derived-endothelial cells under non-stimulated conditions produce PGI\(_2\), as measured as 6-keto PGF\(_{1\alpha}\) [Figure 17]. Other laboratories, to date, have not been able to show production of PGI\(_2\) under standard growth conditions\(^{49,86,21,58}\). This is the first data to show that HAE, SVE and HUVE will produce PGI\(_2\) without the addition of an agonist, buffered saline or fresh complete medium\(^{57}\). Under standard growing conditions HAE produced more PGI\(_2\) than SVE or HUVE. Endothelial cells derived from different vascular beds retain their \textit{in vivo} characteristic production of PGI\(_2\) even after prolonged growth in culture\(^{49}\). The ability to continue to produce
PGI₂ after prolonged growth in culture seems to be related to the addition of ECGF and heparin. Most human endothelial cell research is done on primary or early passage cells which do not require the addition of ECGF. Without the addition of ECGF adult human endothelial cells can not be grown past passage 4. Endothelial cells grown in standard medium for 24 hours had a baseline production of PGF₂α, which was far greater than PGI₂ [Figure 17]. The addition of fresh medium with ECGF and FBS stimulated a new round of cell division which was reflected in an increased PGF₂α production. There was no difference among the cells in their ability to produce PGF₂α [Figure 17]. PGE₂ and PGF₂α have been implicated in cellular division and differentiation.⁸⁷,⁵¹,⁵⁵

This is the first reported experimentation to use endothelial cells exposed to A 23187 in medium containing serum, ECGF and heparin. Previously, only primary or low passage cultures (below passage 4) [for example,⁵⁸,⁴⁹,²¹] have been used. HAE and HUVE respond to A 23187 with the production of PGI₂ [Figure 18 panel b and c]. There was no change in the SVE production of PGI₂ after exposure to A 23187. After the addition of A 23187 there was a consistently lower amount of PGI₂ produced by the HAE than the venous endothelial cells [Figure 19]. Human umbilical vein-derived endothelial cells produce more PGI₂ than arteries under stressed conditions⁴⁹. Interestingly, the amount of PGF₂α produced by the cells varied due to treatment and not due to cell type [Figure 21]. The ability to
produce prostacyclin seems to be related to the bed of origin whereas the production of PGF$_{2\alpha}$ seems to be inherently similar in all cultures. Changes in the prostacyclin production would effect vascular tone and normal blood flow. Increased production of PGF$_2$ could cause an increase proliferation of fibroblasts and smooth muscle cells$^{51}$. Thickening of the media would cause narrowing of the vessel and decreased blood flow$^{31}$.

In normal steady state conditions there is no circulating ECGF in human blood$^{17}$. The removal of ECGF from confluent HUVE cultures induces endothelial cell organization$^{88}$. Disassociation of the 3-dimensional capillary-like structure and the addition of ECGF caused the cells to revert to a proliferative state$^{88}$. A similar phenomenon was observed when TPA was added to bovine microvascular endothelial cells (BMEC)$^{89}$. Locally confluent BMEC responded to TPA by formation of capillary-like structures. Removal of the TPA caused the cells to revert to a proliferative state. These data suggests that induction of differentiation in endothelial cells would be reflected in a decrease in PGF$_{2\alpha}$ production and an increase in the production of PGI$_2$. 
4.5 Cyclic Nucleotides

Confluent human endothelial cells, like most confluent cell cultures produce more cAMP than cGMP\(^{64,62}\) [Figure 22]. After the addition of the ionophore there were no changes in the concentration of either cAMP or cGMP vs the controls [Figure 23 and Figure 25]. Also, there was no change in the amount of cAMP produced with the addition of A 23187. Adams Brotherton and Hoak\(^{27}\) observed that thrombin, A 23187, and arachidonic acid did not cause an increased accumulation of cAMP in HUVE (primary) cell cultures over the controls. The addition of 4mM 1-methyl-3-isobutylxanthine (MIX), a phosphodiesterase inhibitor, increased the average production of cAMP by 4 fold\(^{27}\). Without the addition of methyl xanthine the average production of cAMP ranged from 2 to 6 pg [Figure 24]\(^{27,90,91,71}\). Addition of methyl xanthine would not have changed the conclusion that HAE, SVE and HUVE production of cAMP after exposure to A 23187 was the same. When swine aortic endothelial cells were stimulated with adenosine there was a decrease in the amount of intracellular cAMP, but there was an increased amount of extracellular cAMP\(^{90}\). Goldman suggests that extracellular cAMP could participate in information exchange between cells of the vascular wall and the vascular wall and blood cells\(^{90}\). The release of cAMP could be a mechanism of communication with accessory cells, which would help to maintain the normal vascular equilibrium.
Chapter 5
Conclusions

There are fixed heritable differences in endothelial cells from different vascular origins which act in conjunction with the cells environment to describe the cells phenotype. Endothelial cells derived from different vascular beds retain these differences after numerous passages in vitro indicating that these differences are fixed and heritable. There appears to be 2 types of responses elicited from the endothelial cells: vascular and cellular. The ability to produce PGI$_2$ seems to be related to their in vivo function and the production of PGF$_{2\alpha}$ related to cellular response to stress. cAMP and cGMP, also, appear to be inherent responses. It is therefore plausible to assume that PGI$_2$ is related to the state of differentiation of the cell and its phenotypic expression is related to the cells vessel of origin while PGF$_{2\alpha}$, cAMP, and cGMP are responses to/for proliferation, and cell maintenance and are similar in all endothelial cells. Arterial-derived endothelial cells appear to have major differences from the venous-derived endothelial cells, i.e
PGI$_2$ production, wound healing potential, labeling index and morphology. The development of an *in vitro* model for the study of vascular disease it must not be assumed that all endothelial cells are the same. Extrapolation from *in vitro* to *in vivo* should be approached with caution.
Appendix A.
Baseline Comparison of Phospholipids

A.1 Baseline Comparison of Phospholipids

The phospholipid content of human endothelial cells was not highly variable [Table 1]. Phosphatidylserine and cardiolipin were the only phospholipids which were shown to be statistically different. HUVE had a significantly lower concentration of PS as compared to the SVE (6.54±2.85 and 9.36±2.14, respectively) having a p < 0.02. Although there was no significant difference among HAE, HUVE and SVE (8.8±1.98, 6.54±2.85 and 9.36±2.14, respectively). Cardiolipin, which is found in mitochondria, was significantly lower in HAE than SVE at p < 0.05. Due to the large variance in the HUVE sample there was no statistical difference between the HUVE and the HAE [Table 1]. Phosphatidylcholine was the predominant phospholipid and was equivalent among the cell types, ranging from 42-46%.
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<th>HAE</th>
<th>HUVE</th>
<th>SVE</th>
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<td><strong>MEAN ± S.D.</strong></td>
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<tr>
<td>CL</td>
<td>2.01±1.56</td>
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<td>21.00±1.9</td>
<td>19.74±2.82</td>
<td>18.74±2.49</td>
</tr>
<tr>
<td>PG</td>
<td>1.40±1.8</td>
<td>3.20±3.50</td>
<td>2.06±1.84</td>
</tr>
<tr>
<td>PS</td>
<td>8.80±1.98</td>
<td>6.54±2.85</td>
<td>9.36±2.14</td>
</tr>
<tr>
<td>LPE</td>
<td>3.00±2.2</td>
<td>3.61±3.29</td>
<td>5.88±2.45</td>
</tr>
<tr>
<td>PI</td>
<td>4.69±2.7</td>
<td>2.57±3.42</td>
<td>3.37±2.28</td>
</tr>
<tr>
<td>PA</td>
<td>1.93±0.83</td>
<td>2.45±2.85</td>
<td>1.87±1.72</td>
</tr>
<tr>
<td>SM</td>
<td>12.83±4.17</td>
<td>10.62±3.07</td>
<td>11.54±2.72</td>
</tr>
<tr>
<td>PC</td>
<td>44.48±2.24</td>
<td>46.63±7.72</td>
<td>42.22±5.44</td>
</tr>
</tbody>
</table>

Abbreviations: CL= cardiolipin; PE= phosphatidylethanolamine; PG= phosphatidylglycerol; PS= phosphatidylserine; LPE= lysophosphatidylethanolamine; PI= phosphatidylinositol; PA= phosphatidic acid; SM= sphingomyelin; PC= phosphatidylcholine. Sample number (N) = 9, 15, and 11, for HAE, HUVE and SVE, respectively.

Confluent monolayers of endothelial cells were extracted directly and analyzed for total phospholipid content by HPLC. A Mann-Whitney U/Wilcoxon Rank Sum W Test showed that at p<0.05 there was a significant difference between HAE and SVE for cardiolipin and at p < 0.02 there was a significant difference in phosphatidylserine between SVE and HUVE.

**Table 1:** Molar Percent Distribution of Phospholipids in Human Derived Endothelial Cells

Phosphatidylethanolamine and sphingomyelin were next in percent of total composition with a 18.74-21.00% and 10.62-12.83%.
Appendix B.
Formulary

B.1 Media Formularies

_Eagle's Minimum Essential Media_

<table>
<thead>
<tr>
<th>Salts</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>200.00</td>
</tr>
<tr>
<td>KCl</td>
<td>400.00</td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>97.67</td>
</tr>
<tr>
<td>NaCl</td>
<td>7865.00</td>
</tr>
<tr>
<td>NaH₂PO₄ H₂O</td>
<td>140.00</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1500.00</td>
</tr>
</tbody>
</table>

_Essential and Non-Essential Amino Acids_

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>17.80</td>
</tr>
<tr>
<td>L-Arginine HCl</td>
<td>189.00</td>
</tr>
<tr>
<td>L-Asparagine H₂O</td>
<td>26.40</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>26.60</td>
</tr>
<tr>
<td>L-Cystine 2 HCl</td>
<td>46.94</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>29.40</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>292.00</td>
</tr>
<tr>
<td>Compound</td>
<td>Amount</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Glycine</td>
<td>15.00</td>
</tr>
<tr>
<td>L-Histidine HCl H$_2$O</td>
<td>63.00</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>78.00</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>78.00</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>108.75</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>22.50</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>48.00</td>
</tr>
<tr>
<td>L-Proline</td>
<td>23.00</td>
</tr>
<tr>
<td>L-Serine</td>
<td>21.00</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>72.00</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>15.00</td>
</tr>
<tr>
<td>L-Tyrosine (disodium salt)</td>
<td>78.14</td>
</tr>
<tr>
<td>L-Valine</td>
<td>69.00</td>
</tr>
</tbody>
</table>

**Vitamins**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Ca pantothenate</td>
<td>1.50</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.50</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.50</td>
</tr>
<tr>
<td>i-Insitol</td>
<td>3.00</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>1.50</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>1.50</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.15</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.50</td>
</tr>
</tbody>
</table>

**Other Additives**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1000.00</td>
</tr>
<tr>
<td>Phenol red</td>
<td>100.00</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.11</td>
</tr>
</tbody>
</table>

**M - 199**

<table>
<thead>
<tr>
<th>Salts</th>
<th>mg / l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ (anhydrous)</td>
<td>200.00</td>
</tr>
<tr>
<td>Fe (NO$_3$)$_3$ 9H$_2$O</td>
<td>0.72</td>
</tr>
<tr>
<td>KCl</td>
<td>400.00</td>
</tr>
<tr>
<td>MgSO$_4$ (anhydrous)</td>
<td>97.67</td>
</tr>
<tr>
<td>Compound</td>
<td>Price</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>NaCl</td>
<td>6800.00</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$ $\cdot$ H$_2$O</td>
<td>140.00</td>
</tr>
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</table>

**Amino Acids**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Alanine</td>
<td>50.00</td>
</tr>
<tr>
<td>L-Arginine HCl</td>
<td>70.00</td>
</tr>
<tr>
<td>DL-Aspartic Acid</td>
<td>60.00</td>
</tr>
<tr>
<td>L-Cysteine HCl $\cdot$ H$_2$O</td>
<td>0.11</td>
</tr>
<tr>
<td>L-Cystine 2 HCl</td>
<td>26.00</td>
</tr>
<tr>
<td>DL-Glutamic acid $\cdot$ H$_2$O</td>
<td>150.00</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>100.00</td>
</tr>
<tr>
<td>Glycine</td>
<td>50.00</td>
</tr>
<tr>
<td>L-Histidine HCl $\cdot$ H$_2$O</td>
<td>21.88</td>
</tr>
<tr>
<td>L-Hydroxyproline</td>
<td>10.00</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>40.00</td>
</tr>
<tr>
<td>DL-Leucine</td>
<td>120.00</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>70.00</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>30.00</td>
</tr>
<tr>
<td>DL-Phenylalanine</td>
<td>50.00</td>
</tr>
<tr>
<td>L-Proline</td>
<td>40.00</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>50.00</td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>60.00</td>
</tr>
<tr>
<td>DL-Tryptophan</td>
<td>20.00</td>
</tr>
<tr>
<td>L-Tyrosine (disodium salt)</td>
<td>57.66</td>
</tr>
<tr>
<td>DL-Valine</td>
<td>50.00</td>
</tr>
</tbody>
</table>

**Vitamins**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>0.05</td>
</tr>
<tr>
<td>Alpha tocopherol phosphate</td>
<td>0.01</td>
</tr>
<tr>
<td>(disodium salt)</td>
<td></td>
</tr>
<tr>
<td>d-Biotin</td>
<td>0.01</td>
</tr>
<tr>
<td>Calciferol</td>
<td>0.01</td>
</tr>
<tr>
<td>D-Ca pantothenate</td>
<td>0.10</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.50</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.01</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>0.05</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.01</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.025</td>
</tr>
</tbody>
</table>
Niacinamide 0.025  
Para-aminobenzoic acid 0.05  
Pyridoxal HCl 0.025  
Pyridoxine HCl 0.025  
Riboflavin 0.01  
Thiamine HCl 0.01  
Vitamin A (acetate) 0.14  

Other Additives  
Adenine sulfate 10.00  
Adenosinetriphosphate (disodium salt) 1.00  
Adenylic acid 0.20  
Cholesterol 0.20  
Deoxyribose 0.50  
D-Glucose 1000.00  
Glutathione (reduced) 0.05  
Guanine HCl 0.30  
Hypoxanthine (Na salt) 0.354  
Phenol red 20.00  
Ribose 0.50  
Sodium acetate 20.00  
Thymine 0.30  
Tween 80 20.00  
Uracil 0.30  
Xanthine (Na salt) 0.344  

B.2 Phosphate Buffer Saline Formulary  

<table>
<thead>
<tr>
<th>Grams / Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
</tbody>
</table>
in double distilled deionized water, pH 6.8

B.3 RIA Buffers

Tris Buffer:

50 mM Tris pH 7.5  
0.1 % Bovine Serum Albumin (BSA)  
0.05 % Na Azide

Charcoal Solution

in Tris Buffer:  
0.25% Norit A charcoal  
0.025% Dextran T 70
Appendix C.

Calculations

C.1 Viability

Hemocytometer One half (0.5) ml of trypan blue (0.4% in normal saline) was mixed with 0.5 ml of cell suspension (1:2 dilution) and was loaded onto the hemocytometer. The corners and the center grids of each side of the hemocytometer was counted (a total of 10 squares). The number of cells in 10 squares (c), times the dilution factor 2 (0.5 ml of cell suspension to 0.5 ml trypan blue) times $10^3$ equals the number of cells in a milliliter (Equation A). The cell density was calculated as follows: number of cells/ml (C), times the total number of milliliters in which the cells were suspended (M), divided by the total surface area of the growing surface (cm$^2$), equals the number of cells per centimeter squared (Equation B).

\[ c \times 2 \times 10^3 = \text{number of cells} \ / \ \text{milliliter} = C \ (\text{Equation A}) \]

123
C x M / cm² = number of cells per centimeter squared (Equation B)

C.2 Coulter Counter

One half milliliter of cell suspension is added to 9.5 ml of Isoton (Coulter) and inverted 3 times to mix. A blank of pure Isoton is run and the background read, should less than 100. The sample is counted 3 x and the average number of counts (c) used. This number (c) is multiplied by 40 to give the number of cells in 1 ml of fluid. Multiplied by the total volume in which the cells were suspended (V), equation C. This number is divided by the total growing surface in cm² (equation D).

c x 40 x V = total number of cells (Equation C)

V / cm² = number of cell per centimeter squared (Equation D)
List of References


34. Schwartz, SM, Selden, SC and Bowman, P, *Growth Control in Aortic*
Endothelium at Wound Edges, Cold Spring Harbor, New York, 1979, pp. 593-610.


56. Ody, C, Seillan, C and Russo-Marie, F, "6-ketoProstaglandin F1g(a),"


