Evidence that heparin affects protein levels of three extracellular matrix glycoproteins

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Evidence that Heparin Affects Protein Levels of Three Extracellular...
EVIDENCE THAT HEPARIN AFFECTS PROTEIN LEVELS OF THREE EXTRACELLULAR MATRIX GLYCOPROTEINS

by

Kimberly J. Sebo

A Thesis Presented to the Graduate and Research Committee of Lehigh University in Candidacy for the Degree of Master of Science in Molecular Biology

Lehigh University December 1996
This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science.

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ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my advisor Dr. Linda J. Lowe-Krentz for her mentorship and guidance; Dr. Agnes Ayme-Southgate, and Dr. Lynne Cassimeris for serving on my thesis committee; Catherine A. Granzow for providing assistance in acrylamide gel preparation and x-ray film development; Felix Molina for providing cell lines and assistance in the completion of this study; Dr. Christy Dougherty, and Joyce Savage for helpful suggestions concerning data collection and analysis.

I would like to extend special thanks to my fiancé, John McMenamy; and my parents, Elizabeth and George Wean.
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Patience and tenacity of purpose are worth more than twice their weight of cleverness.

Thomas Henry Huxley, English Biologist
ABSTRACT

In this study, the effect of heparin on three extracellular matrix glycoproteins in porcine aortic endothelial cells was examined. For the extracellular matrix glycoproteins, fibronectin, thrombospondin, and laminin, a concentration range of heparin was applied to endothelial cells and then a variety of biochemical techniques were run to determine that a change in both newly synthesized and expressed protein levels was occurring. In addition, the effect of heparin on cell adhesion molecules was also studied. The results obtained for fibronectin and thrombospondin were compared to results published in the literature on human umbilical vein endothelial cells grown in the presence of growth factors and appeared to be similar. Since there is no published information on heparin’s affect of laminin, cycloheximide was also be applied to show that blocking protein synthesis did have an effect on laminin protein levels. Finally, the data that was obtained here may eventually provide important clues to improving the treatment of vessel wall injury in diseased states.
INTRODUCTION

Cardiovascular disease still remains one of the leading causes of mortality in the United States and Western Europe. Atherosclerosis, the principal cause of myocardial and cerebral infarction, accounts for the majority of the deaths (Ross, 1993b).

Atherosclerosis is characterized by large intracellular lipid accumulations, endothelial cell damage, extensive vascular smooth muscle cell proliferation and changes in the glycosaminoglycan composition of the affected vessel walls (Hyslop et. al., 1993).

In the 1970’s, Ross and colleagues proposed the ‘response-to-injury’ hypothesis of atherogenesis. Initially it was believed that any form of injury, whether it be physical, viral, or chemical injury to the endothelial cell lead to platelet adherence to the arterial wall and resulted in the atherosclerotic plaque. Today, more recent versions have stressed the role of local cell-derived factors that are provoked by vascular injury (Dicorleto, 1993).

Since the endothelial cell lies positioned between the circulating blood and vascular smooth muscle cells, endothelial cell injury or activation may 1) alter permeability properties 2) express binding sites for monocyte activators 3) secrete oxygen free radicals that can modify nearby low density lipoprotein (LDL)
or directly damage neighboring cells and finally 4) synthesize and secrete PDGF mitogens and/or chemoattractants for medial smooth muscle cells (Dicorleto, 1993).

However, in healthy endothelium, endothelial cells produce heparan sulfate proteoglycans which are heparin like molecules as well as other molecules with antiproliferative properties and may explain why platelets and monocytes usually do not adhere at the blood vessel wall and why vascular smooth muscle cells neither migrate nor proliferate (Shulman, 1990).

Therefore, exogenous heparin may aid in this function. Heparin, a known anticoagulant and highly anionic molecule has been discussed as a treatment for atherosclerosis. When healthy endothelium is injured, it loses its normal negative electrical charge. Heparin provided by injection restores this protective negativity. Heparin also inhibits the platelet derived growth factor from promoting the over-growth of medial smooth muscle cells which contribute to plaque formation. Therefore, treatment with low doses of heparin administered intravenously, subcutaneously, orally, or by inhalation at regular intervals is able to reduce or reverse atherosclerotic effects (Hyslop, et. al., 1993).

On the other hand, because of other serious complications of heparin treatment such as osteoporosis and internal bleeding, heparin’s effect on the endothelial cell and surrounding environments warrants further study.
The originating event in the progression of atherosclerosis is the injury to the endothelium (Harrison, 1992, and DiCorleto, 1993). For this to occur, monocytes must attach to the endothelium (Davies, et. al., 1993). Cell-adhesion molecules are involved in this interaction. In addition, an important part of the regulation of the endothelial cell behavior is played by adhesive glycoproteins found in the extracellular matrix. These proteins serve specific functions such as controlling the attachment, migration, and synthetic activity of the cells (Uitto, et. al., 1991). During atherosclerosis, several aspects of the cell-matrix interactions as well as cell-cell interactions may be disturbed. However, since heparin has been shown to inhibit several aspects of atherosclerosis, it is important to study the interaction of heparin, endothelial cells, the extracellular matrix, and cell-adhesive molecules in the prevention and treatment of vascular disease.
The objectives of the project were:

1. To study the effect of heparin on cell-adhesion protein levels in porcine aortic endothelial cells.

2. Determine if there were any changes in extracellular matrix protein expression levels of laminin, fibronectin, and thrombospondin as a result of the addition of heparin.

3. Compare my results on porcine aortic endothelial cells grown without the addition of growth factors to results published in the literature on human umbilical vein endothelial cells grown in the presence of endothelial cell growth factor for Fibronectin and Thrombospondin.
Chapter 2

Literature Review

Extracellular Matrix

The major components of the extracellular matrix are collagens, proteoglycans, and adhesive glycoproteins. Collagens are usually the main organic constituents of the connective tissue matrix and are responsible for the strength and form of tissues. Proteoglycans play an important role in tissue resilience and filtering. Some proteoglycans have a capacity to specifically bind other matrix molecules and growth factors while others act as matrix receptors on the surface. An important part of regulation of the cell behavior is played by adhesive glycoproteins belonging to the fibronectin and laminin families (Uitto, et. al., 1991).

Basement membranes are specialized types of extracellular matrices laid down by epithelial cells and endothelial cells. They are composed of a complex and tissue specific mixture of glycoproteins and proteoglycans usually organized into the two layers of basement membrane, lamina lucida and lamina densa. Basement membranes are always found between endothelial cells and connective tissue where they act as a selective barrier, restricting the passage of cells and
molecules between the tissue compartments. Other functions of basement membranes include anchorage of the basal endothelial cells, morphogenesis, tissue differentiation and regeneration (Uitto, et. al., 1991).

Proteoglycans are a large group of extracellular and cell-surface macromolecules that function in regulating cell adhesion, growth, and extracellular matrix formation (Jackson, et. al., 1991). They are composed of a protein core in which serine-glycine sequences serve as attachment sites for one or more glycosaminoglycan chains. Heparan sulfate proteoglycans contain heparan sulfate. In addition to the function in cell adhesion and matrix binding, heparan sulfate proteoglycans regulate cellular growth, lipid metabolism, and basement membrane function (Jackson, et. al., 1991).

Basement membranes contain large heparan sulfate proteoglycans that consist of a core protein of 400 kDa and three to four heparan sulfate chains that are clustered at one end of the molecule. These proteoglycans occur in close association with and are probably bound to other matrix macromolecules such as laminin and fibronectin (Heremans, et. al., 1990).

All tissues have glycoproteins, the major function of which is to bind cells to the extracellular elements. The major adhesive glycoproteins of the endothelial extracellular matrix are laminin, fibronectin, and thrombospondin. In the basement membrane, laminin serves as the major adhesive glycoprotein.
Laminins are glycoproteins of about 850,000 molecular weight composed of three polypeptides chains called A, B1, and B2, that are organized in a crosslike manner. This structural arrangement has three short arms, each with two globular domains and a long arm that ends with a large globular domain. The three chains intersect at the center of the cross, are linked by disulfide bonds and then run together, in parallel to form the rodlike segment of the long arm. The B1 and B2 chains terminate at the end of the rod and are linked together in this region by a single disulfide bond. The carboxyl terminus of the A chain forms the large globule at the end of the long arm. Strongest heparin binding has been observed in the distal globular end of the long arm. Laminin forms a dense network with proteoglycans and other molecules and its function is in anchoring the cells in place. Laminin also has diverse effects on cultured cells because it can alter their growth, survival, morphology, differentiation, and motility (Martin, et. al., 1987).

In connective tissues, fibronectin is the primary adhesive molecule. Fibronectin is composed of two similar but not identical polypeptide chains attached at their carboxy termini by two disulfide bonds to form a dimer of 550 kDa (Hermans, et. al., 1990). The interaction of glycosaminoglycans with fibronectin occurs on at least two different sites along the polypeptide chain. Skorstengaard et. al. (1986) and Garcia-Pardo et. al. (1987) sequenced a heparin binding proteolytic fragment from the carboxy terminus suggesting that these
residues are important for heparin binding. Each of the fibronectin polypeptides is composed of 5 or 6 domains that have specific affinity to cells, collagen, fibrin, and heparin. It is suggested that, in tissues, fibronectin forms tracks along which cells migrate. Fibronectin also binds to some complement components and participates in the clearance of tissue debris.

Thrombospondin is synthesized and secreted by cells in vitro, where it is then able to bind to various cell receptors and become incorporated into the extracellular matrix. It is a disulfide-linked trimer (450,000 daltons) present in connective tissues and platelet α granules. This trimeric form of the protein accounts for its ability to promote cell-substratum interactions. These interactions occur by binding to cell surface heparan sulfate proteoglycans and are mediated through a heparin-binding domain at the amino terminus of thrombospondin (Hantai, et. al., 1991). Thrombospondin stimulates the proliferation of fibroblasts and smooth muscle cells and in contrast, it inhibits the proliferation of endothelial cells. Thrombospondin may also serve as both an attachment protein and an anti-adhesive molecule as shown by its ability to cause disassembly of focal adhesions in endothelial cells. In addition, thrombospondin interacts with fibronectin and plasminogen during clot formation.
THE EXTRACELLULAR MATRIX'S ROLE IN VASCULAR DISEASE

At initial stages of the disease process, local cell-derived factors such as low density lipoproteins (LDL) may act on the endothelial cells, resulting in degradation of the cell surface and adhesion molecules. As a result, the endothelial cells may react with increased proliferation and production of extracellular hydrolytic enzymes leading to local degradation of the subendothelial basement membrane. Therefore, massive infiltration of leukocytes and other cells takes place. Proteases and other mediators released from the macrophages results in the destruction of matrix molecules of the endothelium. Thus begins the cascade of events that lead to vascular disease such as atherosclerosis. In addition, a number of investigators have isolated proteoglycans from the arterial wall and have shown that they precipitate LDL in vitro. Along with trapping LDL in the extracellular space of the arterial wall, extracellular matrix components contribute to the development of the atherosclerotic foam cell lesion by enhancing LDL uptake by macrophages. The interaction of fibronectin with heparin also enhances the uptake of LDL by macrophages. Therefore, the ability of cells to attach to an underlying matrix is
crucial to growth and repair of injury. Finally, studying the interactions of endothelial cells, matrix components, and heparin is essential to understanding endothelial repair processes.

HEPARIN'S THERAPEUTIC PROPERTIES

Heparin, a highly negatively charged glycosaminoglycan, is a major component of mast cell granules. In human umbilical vein endothelial cells, heparin is able to stimulate the biosynthesis of heparan sulfate in a time and dose dependent manner (Nader, et al., 1989). This stimulation of heparan sulfate production could provide more substrate for the action of platelet-derived heparitinase, and therefore, a higher concentration of antiproliferative molecules since the release of heparan sulfate is primarily to the interstitial space where it may modulate smooth muscle cell proliferation (Hyslop, et al., 1993). It is also possible that the platelet-derived heparitinase may act here to release more heparan sulfate with antiproliferative activity. In addition to stimulating heparan sulfate formation, heparin alters the sulfation pattern of this molecule (Hyslop, et al., 1993). This proteoglycan specific response to heparin may explain some of
its beneficial effects in atherosclerosis. Therefore, there is clinical and experimental evidence that treatment with low doses of heparin at regular intervals is able to reduce atherosclerotic effects (Andriouli, et al., 1990). Also, part of the therapeutic effect of exogenous heparin is its ability to stimulate endothelial cell growth and migration (Terranova, et al., 1985).

It has been suggested that one of the possible causes of atherosclerosis is a loss of the vessel content of heparan sulfate because it has been observed that in atherosclerotic portions of the human aorta, the content of heparan sulfate decreases while that of chondroitin sulfate/dermatan sulfate increases (Hollman, et al., 1989). This is also correlated with age and cholesterol content.

Finally, heparin may also have a protective effect on vascular cells by scavenging oxygen-derived free radicals which originated from cellular metabolism. The presence of these reactive molecules can stimulate the oxidation of low-density lipoproteins by macrophages which are found at the site of injured endothelium (Hiebert, et al., 1991).
CELL ADHESION AND VASCULAR DISEASE

The migration of monocytes and leukocytes into tissues is the central event in the inflammatory response leading to vessel injury. In vascular diseases such as atherosclerosis, it is one of the initiating events.

For endothelial cell injury to occur, monocytes must attach to the endothelium. Little is known of the actual sequence of events leading to monocyte adhesion in vivo, however, attachment between these cells is mediated by cell-adhesion molecules (CAM's) present on the surface of both cells. Intercellular adhesion molecule-1 (ICAM-1), a major cell-adhesion molecule found on the surface of endothelial cells binds to the $\beta 2$ integrin LFA-1 which is present on both mononuclear and polynuclear leukocytes (Poston, et. al., 1992).

The expression of ICAM-1 is upregulated by cytokines released from monocytes and T-lymphocytes such as interleukins, (IL) 1 and 4, and tumor necrosis factor alpha, TNF- $\alpha$ (Poston, et. al., 1992).

Human atherosclerotic lesions of all subtypes except fibrous plaques have recently been demonstrated to show an enhanced expression of ICAM-1 (Davies, et. al., 1993). The high frequency of ICAM-1 expression both on the endothelium
and on macrophages is unclear, but it may facilitate both antigen recognition by T-cells and antigen presentation by macrophages (Davies, et. al, 1993).

P-selectin, another adhesion molecule belonging to the selectin family, binds carbohydrate ligands on neutrophils, monocytes, and memory T-cells (Subramaniam, et. al., 1993). P-selectin is involved in the initial rolling of neutrophils along endothelial cell monolayers which will eventually result in their attachment and allow emigration (Smith, 1993). Upregulation of P-selectin occurs with histamine and is seen within 5 minutes of stimulation, but transient, where it localizes back to the Weibel-Palade bodies of endothelial cells (Smith, 1993). Jones, et. al., (1992) recently demonstrated that histamine stimulation of endothelial cell monolayers promoted neutrophil rolling that was entirely blocked by anti-P-selectin monoclonal antibodies. P-selectin’s role is in the earliest stages of neutrophil localization while ICAM-1 is thought to be involved in the attachment process. Therefore, one clue to treatment possibilities in vascular disease could be in blocking either ICAM-1 or P-selectin to reduce the number of neutrophils that localize at sites of inflammation.
Chapter 3

CULTURE AND IDENTIFICATION OF PORCINE AORTIC ENDOTHELIAL CELLS

INTRODUCTION

This section reviews the isolation, culture, and identification of porcine aortic endothelial cells.

Culture Media

Dulbecco’s Modified Eagle’s Medium (DMEM) with 4500mg/L Glucose and L-Glutamine without Sodium Bicarbonate (Sigma, St. Louis, MO) was supplemented with sodium bicarbonate. The media was completed with 10% fetal bovine serum (FBS) from Atlanta Biological (Norcross, GA) or Gibco (Gaithersburg, MD) which was heat inactivated at 65° C for 1 hour. In addition,
2% glutamine and 1% penicillin/streptomycin solution (Sigma, St. Louis, MO) were added to the culture medium. When the endothelial cells needed to be passaged, a 10x stock of trypsin/EDTA (Sigma, St. Louis, MO) was diluted in 1X phosphate-buffered saline (PBS).

**Endothelial Cell Isolation and Identification**

Porcine aortas are sectioned under sterile conditions. Since endothelial cells are the intimal layer and would slough off first, a brief collagenase digestion was then performed at 37°C. To optimize endothelial cell recovery, cells were plated onto 24 well gelatinized plates. Gelatin is required for endothelial cell growth, but smooth muscle cells may also grow on these plates. Cells were allowed to grow to confluency in DMEM media supplemented with 10% heat inactivated FBS, glutamine and penicillin/streptomycin. Only wells that contain cells with a cobblestone appearance and a confluent monolayer which is characteristic of endothelial cells were continued. The cells from each of these wells were maintained as a separate cell line. These wells were eventually passaged to 150mm plates by trypsin/EDTA treatment and were routinely
subcultured. If after a period of time, the cells piled up on each other, these wells were discarded since there was evidence of smooth muscle cell contamination.

The endothelial cells may be stained with antifactor VIII to also determine their characteristics such as a cobblestone appearance and a confluent monolayer. Endothelial cells may be passaged approximately 20 times before they are discarded.
CHAPTER 4

EXPERIMENTATION TECHNIQUES APPLIED TO DETERMINE THE EXTRACELLULAR MATRIX PROTEIN LEVELS

INTRODUCTION

This section reviews the experimental techniques and assays utilized to determine heparin's effect on the extracellular matrix glycoproteins laminin, fibronectin and thrombospondin. Two additional techniques will be discussed which were applied in an attempt to study the cell-adhesion molecules: P-selectin and Intercellular Adhesion Molecule (ICAM-1). The following techniques were used for the study of the extracellular matrix glycoproteins: Immunoblots, immunoprecipitations, dot blots and radioimmunoprecipitations. For the study of the cell-adhesion molecules, immunofluorescence and the ELISA assay were also done. These experimental techniques were applied to determine changes in
newly synthesized and expressed protein levels at the cell surface and protein released into the media.

**Immunoblots**

Endothelial cells were grown to confluency in 150 mm tissue culture plates at 37°C. Next, a concentration range of heparin was added for a 24 hour period at the same temperature. The concentration range of heparin applied was 50 μg/ml, 100 μg/ml and 200 μg/ml, however, one plate did not contain heparin and served as a control. When fibronectin was going to be examined, the media was replaced with heat inactivated serum supplement (HISS, JRH Biosciences, Lenexa, KS) at the time the heparin was added to prevent the primary antibody from reacting with the FBS in the media. When cycloheximide was applied, heparin was applied for as long as the cycloheximide was added i.e. 4 hours. Cycloheximide (Sigma, St. Louis, MO) was added at a 1:1000 dilution from a concentration of 1 mg/ml in selected experiments.

After the incubation time, the media was removed and set aside for further analysis. The cells were harvested by adding 1X PBS and scraping the surface of the plates with a rubber policeman. The cellular material was then collected by
centrifugation and resuspended in 2X sample buffer. In addition, 0.1% bromophenol blue was added as a tracking dye. The samples were vortexed, boiled for 5 minutes, pulsed in a microfuge and loaded onto the gel. The cell-surface proteins were analyzed and identified by SDS-PAGE using the Sturdier apparatus (Hoefer Scientific Instruments, San Francisco, CA). The proteins were transferred electrophoretically onto nitrocellulose for 4 hours at 60 volts using the Transphor device (Bio-Rad Laboratories, Richmond, CA). For larger molecular weight proteins, a longer transfer was required. (See Figure 4.1 page 32).

After transfer, the nitrocellulose was blocked overnight in blotto containing 5% non-fat milk, 3% bovine serum albumin, sodium azide, and 10% 10X Tris-Buffered Saline, (TBS). After blocking, one lane was cut from the nitrocellulose and kept in block to be used as a secondary control. After blocking, the other piece of nitrocellulose was rinsed three times for 10 minutes with 1X Tris-buffered saline and 0.1% tween-20 (TBST) at room temperature. Next, to the blotto was added the commercially available polyclonal primary IgG antibody developed in rabbit to either laminin or fibronectin (Sigma, St.Louis, MO) at a 1:1000 dilution for 2 hours at 37°C. The primary Ab was then rinsed off five times for five minutes with TBST at room temperature. At this time the secondary control lane was also rinsed with TBST. Both pieces were then placed
in blotto containing donkey anti-rabbit biotinylated secondary antibody (Sigma, St. Louis, MO) at a dilution of 1:10,000 overnight on a rocker at 4°C.

The following day, the nitrocellulose pieces were again washed three times for 10 minutes with TBST at room temperature and incubated for a minimum of two hours with Extra-Avidin Phosphatase (Sigma, St. Louis, MO) at a dilution of 1:10,000 in TBST also at room temperature. Again, blots were washed in TBST, three times for 10 minutes at room temperature. Blots were then placed in alkaline phosphatase buffer for 5 minutes at room temperature and then this buffer was discarded. For the development, 50 µl of BCIP (Sigma, St. Louis, MO) and 100 µl of NBT (Sigma, St. Louis, MO) was added to 50 mls of alkaline phosphatase buffer and allowed to develop in the dark for a 1 to 4 hour time frame. (See Figure 4.2 page 33).

Blots were then scanned on an LKB Bromma Ultrosan XL Enhanced Laser Densitometer (Bromma, Sweden) to quantitate the amount of protein present. Each lane was scanned and the baseline absorbance unit value (AU) for a set of lanes was averaged together. This value was either added or subtracted from each AU result for the individual lanes depending on the intensity of the lane. In addition, the secondary control AU value was subtracted from each result.
Immunoprecipitations and Dot Blots

The media from the 150 mm plates was dialyzed for a minimum of 48 hours at 4°C with at least four milli-Q water changes to dialyze out the salts in the media. One milliliter of media from each sample was used to immunoprecipitate the proteins of interest while the rest was evenly run through a dot blot apparatus (Bio-Rad Laboratories, Richmond, CA). The dots were then developed as described above for immunoblots and scanned on the densitometer.

For the immunoprecipitation, 15% protein A Sepharose beads (Sigma, St. Louis, MO) and a nonimmune IgG rabbit antibody (Sigma, St. Louis, MO) was added at a dilution of 1:1000 to 1ml of media. This was placed on a rocker at 4°C overnight. The following day, the samples were centrifuged and the beads were discarded. To the supernatant, 15% protein A Sepharose beads were added along with the commercially available polyclonal IgG antibody developed in rabbit to either laminin or fibronectin at a 1:1000 dilution. These samples were again incubated overnight at 4°C on a rocker. However, one sample remained in the nonimmune IgG Ab for the rest of the protocol and served as a control.
The next day, the samples were centrifuged again and the supernatant was discarded. The beads were then washed with 30 times their volume with 1X TBS. After each washing, the samples were centrifuged and the supernatant was discarded. After the final rinse, 2X sample buffer was added to each sample plus 0.1% Bromophenol blue was also added as a tracking dye. These samples were then analyzed by SDS-PAGE, transferred to nitrocellulose and then developed as described previously for immunoblots. In addition, these blots were also scanned on the densitometer to quantitate the amount of protein present.

**Radioimmunoprecipitation**

To perform the radioimmunoprecipitation, endothelial cells were grown to confluency in 6 well tissue culture plates. To these cells, 50 μCi/well of $^{35}$S-met, (Dupont, Boston, MA) low-methionine media, (Dulbecco's Modified Eagle's Medium DMEM), and a concentration range of heparin as previously described in the immunoblot section was added for a 24 hour period. If fibronectin was the protein of interest,
HISS was again added to the medium instead of fetal bovine serum since the antibody to fibronectin would also recognize and bind to the fibronectin found in the media. The next day, the media was separated from the cells and the cells were harvested with 1X PBS containing 1% CHAPS. Then the protocol was followed according to the immunoprecipitation procedure for both the media, and cellular material. For the thrombospondin protein, a nonimmune mouse IgG (Sigma, St. Louis, MO) was added at a 1:1000 dilution, followed by a 1:1000 dilution of monoclonal IgG antibody to thrombospondin from mouse ascites fluid (Sigma, St. Louis, MO). In addition, for this assay one sample remained in the nonimmune Ab and served as a control for the rest of the procedure. Also, 0.5 M PMSF, 0.5 M benzamide, and 5 mM caproic acid (all Sigma, St. Louis, MO) protease inhibitors were added to prevent protein degradation. After the proteins were separated by SDS-PAGE, the gel was soaked in Destain I which contains 50% methanol, 10% acetic acid and 2% glycerol for 30 minutes, dried with the gel slab dryer (Biorad, Richmond, CA), and then exposed to X-ray film (Kodak, Rochester, NY) at -70°C for one month. The film was developed in a dark room using commercially available developer (Kodak, Rochester, NY) for 3 minutes, followed by 2 minutes in a stop bath consisting of 3.5% acetic acid and
then finished with fixer (Kodak, Rochester, NY) for 3 minutes. The protein bands that developed were also scanned on a densitometer to quantitate the amount of protein present. (See Figure 4.3 page 34).

Immunofluorescence

In addition, to the protocols previously described, the immunofluorescence and the ELISA assay were also employed to attempt to study P-selectin and ICAM-1.

Endothelial cells were grown on 0.5% gelatinized coverslips. Since P-selectin is a protein which is only expressed during endothelial cell damage or injury, activation is required. Once the cells were confluent, activation was performed with 5 units of IL-1β, initial concentration of $5 \times 10^6$ for a 5 hour time frame. Some coverslips were activated and also exposed to 200 μg/ml of heparin. When activation was unsuccessful, histamine was used as an activator at a 0.0001M solution, according to Subramaniam, et. al., (1993) for 5 minutes.

Following activation, P-selectin is only expressed on the cell-surface for a short time before its transferred to the Weibel Palade bodies of endothelial cells. Cells were washed 3 times for 5 minutes with cold
1X PBS. Next, the cells were solubilized with methanol for a maximum of 5 minutes and fixed with commercially available fixative (Amresco, OH). This was followed by 3 washes for 5 minutes with cold 1X PBS. The cells were then incubated for 1 hour at 37°C with primary monoclonal P-selectin IgG antibody (Becton Dickinson, San Jose, CA). Again the cells were washed 3 times for 5 minutes with 1X PBS. After the washes, the coverslips were incubated in the dark at room temperature for 1 to 2 hours with a 1:160 dilution with Dichlorotriazinyl Amino Fluorescein (DTAF) conjugated affinity purified goat anti-mouse IgG, H+L secondary antibody (Jackson Biological Laboratories, West Grove, PA). Next, 3 washes for 5 minutes with 1X PBS were performed in the dark at room temperature. Finally, coverslips were mounted on slides with 30 μl of tris buffered Mowiol 4-88 (Calbiochem-Novabiochem Corporation, La Jolla, CA), ph 8.5/slide. Slides were stored in the dark and were viewed within 24 hours with a Nikon microscope equipped with epifluorescence. For controls, some coverslips were exposed to 2° Ab only, while some were exposed to no antibodies at all.
**ELISA**

Endothelial cells were grown in 96 well tissue culture plates. Again, cells were activated with either 5 units of IL-1 \( \beta \) or 5 minutes with histamine at a concentration of \( 1 \times 10^{-4} \) (Sigma, St. Louis, MO). Media was removed, dialyzed, and run through a dot blot (Biorad, Richmond, CA) and developed as previously described in the immunoblot section. In addition, in some experiments media was also analyzed by the ELISA assay. The media was allowed to sit in a microtiter plate for several days, removed, and commercially available fixative (Amresco, Solon OH) was applied to retain the proteins that were released from the media. The protocol was then followed as described here. Following activation, cells were fixed with commercially available fixative. Cells were then exposed to primary monoclonal IgG P-selectin antibody developed in mouse (Becton Dickinson, San Jose, CA) at a 1:1000 dilution in blocking buffer containing 5% non-fat dry milk, 6 mM sodium azide, 3% bovine serum albumin and 10X tris buffered saline for 1 hour at 37°C. This was followed by 5 washes for 5 minutes with 1X TBS at room temperature. Next, goat anti-mouse biotin conjugated secondary
antibody (Sigma, St. Louis, MO) was then added to the cells at a 1:10,000 dilution in blocking buffer overnight at 4°C. The following day, the secondary antibody was removed and the cells were washed with 1X TBS, 3 times for 10 minutes at room temperature. Cells were then placed in Extra-Avidin Phosphatase (Sigma, St. Louis, MO) at a 1:10,000 dilution for 1 hour in 1X TBS at 37°C. The Extra-Avidin Phosphatase was removed and the cells were again rinsed with 1X TBS, 3 times for 10 minutes at room temperature. For the development, 50 µl of para-nitrophenyl phosphate (Zymed, San Francisco, CA) was added to 250 µl of 10X 1 M 2-amino-2methyl-1,3-propanediol pH 10.0 (Zymed, San Francisco, CA) per 2.5 ml of milli-Q water. Controls consisted of blank wells which contained 1X TBS only, positive controls which consisted of the enzyme and substrate and finally wells with secondary antibody only. Plates were read on the spectrophotometer (MR700 Dynatech Laboratories, Chantilly, VA) set at 410 nm. When this development was unsuccessful, development was tried with using the enzyme Extra-Avidin Peroxidase (Sigma, St. Louis, MO) and then adding the substrate which was 1 tablet of 2,2 Azino-Bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis, MO) in 100 ml of 0.05 M phosphate-citrate buffer pH5.0 and then 25 µl of 50% H2O2 was added.
Intercellular Adhesion Molecule (ICAM-1) is also a protein which is only expressed during endothelial cell damage or injury. However, following activation, the protein should be located on the cell-surface from 6 hours to 24 hours according to Hahne et. al., (1993). Activation was attempted with Tumor Necrosis Factor α (Sigma, ST. Louis. MO) at a 10 ng/ml concentration (Ritchie, et. al., 1991), NiCl₂ 2mM solution according to Goebeler, et. al., (1993) and PMA 100 ng/ml (Ritchie, et.al.,1991). Following activation, the protocols previously described were followed in an attempt to study ICAM-1 protein levels.

**Northern Analysis**

Total cellular RNA was isolated according to the Gentra Systems., Inc. (Minneapolis, MN) protocol with reagents provided. The amount of RNA isolated was determined with Absorbance readings at 260 nm and purity of the RNA was determined from the ratio of $A_{260}/A_{280}$. 9 μg of total cellular RNA was loaded onto a 1% Agarose gel with 15% formaldehyde added and 10% 10X MOPS buffer which contains MOPS, 3 M sodium acetate, and 0.5 M EDTA. Gel was electrophoresed overnight at 25 volts. Next day, the gel was soaked in 0.1 M
Tris-Cl, for 30 minutes and then placed in a solution of 50 mM NaOH plus 10 mM NaCl for also 30 minutes. Finally, before transfer the gel was placed in 20X SSC which contains 3 M NaCl plus 0.3 M citric acid for 30 minutes. Transfer to a nylon membrane was carried out by electroblotting (Bio Rad, Richmond, CA) at 1mA overnight in 0.5X TBE which contains 44.5 mM Tris-base, 44.5 mM Boric acid, and 1.25 mM EDTA at 4°C. The nylon membrane was crosslinked under a UV light and then placed in 30ml of pre-hybridization solution (KH₂PO₄, 2M, 5X SSC, 5X Denhardt’s solution, salmon sperm DNA, and 50% formamide) at 37°C. The following day, the biotinylated cDNA (Oligos etc., Wilsonville, OR) was restored with 100 μl of DEPC H₂O at a concentration of 1 mg/ml, boiled for 5 minutes and then added to the pre-hybridization solution along with t-RNA baker’s yeast (Sigma, St. Louis, MO) at a 1:200 dilution. Since the Tₘ of the probe was 50°C and the hybridization occurs at 5-15°C below the Tₘ according to Farrell, R.E., (1993), all steps from the pre-hybridization to the development occurred at 37°C. Following hybridization for 96 hours at 37°C, the nylon membrane was washed 2 times for 15 minutes with 1XSSC plus 0.1 %SDS followed by 2 rinses with 0.25XSSC plus 0.1% SDS. The nylon membrane was then placed in lectin block which is made up of 1% fish gelatin, 1% PVP, 0.1% sodium azide, 0.1% Tween-20, and 10X TBS for 24 hours. The next day, the lectin block was removed and the nylon membrane was washed three times for 10
minutes with TBST. This was followed by Extra-Avidin Phosphatase (Sigma, ST. Louis, MO) added at a 1:10,000 dilution in TBST at 37°C overnight. Extra-Avidin Phosphatase was removed and the nylon membrane was again washed three times for 10 minutes with TBST. The nylon membrane was then placed in alkaline phosphatase buffer for 5 minutes and then the buffer was discarded. For the development, 50 µl of BCIP and 100 µl of NBT was added to 50 mls of alkaline phosphatase buffer and allowed to develop in the dark for a 1 to 4 hour time frame.
Figure 4.1: Isolation of Cell-Surface Proteins and Proteins Released into the Media for Immunoblots, Dot Blots, and Immunoprecipitations
Figure 4.2: Development for Immunoblots, Dot Blots, and Immunoprecipitations
Figure 4.3: Protocol for Radioimmunoprecipitations
CHAPTER 5: RESULTS AND DISCUSSION

To study the effect of heparin on the cell-adhesion molecule P-selectin, porcine aortic endothelial cells were stimulated with IL-1β for a 5 hour stimulation time. The cells were harvested, and the results of the immunoblot are provided in Figure 5.1. P-selectin is a membrane protein, Mr 140 kDa, according to Kunzendorf et al. (1993). Since there was not any significant change from the control lanes, to the stimulated lanes of IL-1β, and IL-1β plus 200 μg/ml of heparin treatment applied, it was determined from literature reviews that P-selectin is being expressed on the cell-surface for a brief time, approximately 5 minutes and then transferred back into the Weibel-Palade bodies of endothelial cells. In addition, according to Subramaniam, et al., (1993) histamine appeared to be a better stimulator of endothelial cells to get the P-selectin protein released from the Weibel-Palade bodies and expressed on the cell surface. Histamine was also applied since IL-1β is developed in humans and activation may not have occurred since the assay was run on porcine aortic endothelial cells. Therefore, using a chemical such as histamine, activation should occur since there is no
species interaction occurring. Another immunoblot was performed (Figure 5.2). Again, there was not a change between the unstimulated cell lanes, to the stimulated cell lanes, and to lanes stimulated with histamine and 200 $\mu g/ml$ of heparin treatment. Activation was also attempted with TNF-\(\alpha\) for P-selectin according to Hahne et. al (1993). Maximum surface expression for P-selectin should be between 4 and 8 hours after stimulation. Therefore, endothelial cells were stimulated with TNF-\(\alpha\) for 4 hours, harvested and the results of the immunoblot are provided (Figure 5.3). Again, there appeared to be no change in protein levels from the control lane, to the activated lane, and to the activated lane with the addition of 200 $\mu g/ml$ of heparin treatment. No further immunoblots were performed.

An immunofluorescence protocol was then performed using histamine as an activator to see if the location of the molecule could be followed. Unfortunately, no fluorescence was observed only autofluorescence of the endothelial cells themselves in the control slides was observed. No data is provided for this protocol.

Next, an ELISA assay was run since activation of endothelial cells would result in the expression of this protein on the cell surface. This assay detects expressed protein only as opposed to the immunoblot and immunofluorescence
which detect both newly synthesized and expressed protein. In addition, a time course from 2 minutes to 1 hour with histamine was done to possibly determine an optimum time for stimulation. From Experiment 1, 7 minutes appeared to be the optimum time and was the stimulation time applied for Experiments 2 through 4. Data is provided in TABLE 5.1.

Table 5.1. ELISA results for P-selectin Adhesion Molecule

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
<th>Exp. 4 read 24hrs later</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells without stimulation</td>
<td>0.3± 0.1</td>
<td>0.031± 0.003</td>
<td>0.020± 0.005</td>
<td>0.025± 0.003</td>
<td>0.15± 0.08</td>
</tr>
<tr>
<td>cells with 7 minute histamine stimulation</td>
<td>0.9± 0.2</td>
<td>0.033± 0.005</td>
<td>0.026± 0.003</td>
<td>0.025± 0.002</td>
<td>0.09± 0.04</td>
</tr>
<tr>
<td>cells with 7 minute histamine stimulation + 200 μg/ml heparin</td>
<td>N/A</td>
<td>0.029± 0.001</td>
<td>0.300± 0.005</td>
<td>0.042± 0.005</td>
<td>0.23± 0.07</td>
</tr>
<tr>
<td>2° Ab control</td>
<td>0.7± 0.2</td>
<td>0.031± 0.003</td>
<td>0.017± 0.001</td>
<td>0.036± 0.002</td>
<td>0.19± 0.08</td>
</tr>
<tr>
<td>positive control</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>0.023</td>
<td>0.052</td>
</tr>
<tr>
<td>2 minute histamine stimulation</td>
<td>0.7± 0.1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>5 minute histamine stimulation</td>
<td>0.9± 0.1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>10 minute histamine stimulation</td>
<td>0.8± 0.1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>20 minute histamine stimulation</td>
<td>0.8± 0.2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>30 minute histamine stimulation</td>
<td>0.7± 0.2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1 hour histamine</td>
<td>0.7±</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
As one can see there was not enough difference between the values to notice any significant changes. Therefore, no further experimentation was pursued for this molecule.

Activation is also required for the cell-adhesion molecule, ICAM-1. According to Hahne, et al., (1993), cell surface expression of ICAM-1 is still elevated 24 hours after activation with TNF-α in human umbilical vein endothelial cells. Porcine aortic endothelial cells were activated with TNF-α for 4 hours, one plate was exposed to TNF-α and 200 μg/ml of heparin treatment, and one plate served as a control (Figure 5.3). Unfortunately, there did not appear to be any change in protein levels from the control lane, to the activated lane with TNF-α, and the activated lane with TNF-α and 200 μg/ml of heparin treatment. ICAM-1 is a protein with M_r from 76-114 kDa according to Becton Dickinson Advanced Cellular Biology, (1993). There did not appear to be any protein band in this range of molecular weight. For the media from these cells, an ELISA was performed. The results will follow the immunoblots in TABLE 5.2.
Another immunoblot was run using TNF-α as the activator for 6 hours and 24 hours to see if there is an optimum time for activation of the ICAM-1 protein on the cell surface. The endothelial cells were harvested and the immunoblot is provided (Figure 5.4). Only one molecular weight protein band developed in the 6 hour lanes, that did not appear on the secondary antibody positive control lane, or the 0 hour lanes, however, the molecular weight is approximately 120 kDa and is out of the range for the molecular weight of the ICAM-1 protein superfamily. The media from these cells was also analyzed by the ELISA method and will follow in TABLE 5.2.

Since activation was minimal, activation was attempted with a 2mM NiCl₂ solution according to Goebeler, et. al., 1993. Unlike TNF-α which is a human recombinant, chemical activation with NiCl₂ would eliminate the species difference that is occurring. Stimulation was done for 6 hours with NiCl₂ and TNF-α. Endothelial cells were harvested and the immunoblot is provided (Figure 5.5). Again, there did not appear to be any difference between the 0 hour lane, the NiCl₂ treated lane, the TNF-α treated lane, the NiCl₂ treated lane plus 200 μg/ml of heparin treatment lane, and the TNF-α treated lane plus also 200 μg/ml of heparin treatment applied. The media from these cells was also run on an ELISA and the results are also provided in TABLE 5.2.
Finally, a phorbol ester called PMA, phorbol-12-myristate 13-acetate, was applied according to Ritchie et al., (1991). In addition, some of the lanes were run nonreduced. According to the Hedman et al., (1992) article, ICAM-1 Mₐ is approximately 200 kDa under nonreduced conditions. The results of the immunoblot are provided (Figure 5.6). Again, no difference was observed between the 0 hour lane, the stimulated lanes, and the secondary antibody control lanes. All activators, NiCl₂, PMA, and heparin were applied for 24 hours. The results of the ELISA are provided below in TABLE 5.2

**TABLE 5.2: ELISA Results of ICAM-1 released into the media**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
<th>Exp. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells without stimulation</td>
<td>.06±</td>
<td>.074±</td>
<td>.073±</td>
<td>.058±</td>
<td>.04±</td>
</tr>
<tr>
<td>.01</td>
<td>.008</td>
<td>.004</td>
<td>.002</td>
<td>.02</td>
<td></td>
</tr>
<tr>
<td>4 hour stimulation with TNF-alpha</td>
<td>N/A</td>
<td>N/A</td>
<td>.076±</td>
<td>.060±</td>
<td>N/A</td>
</tr>
<tr>
<td>with TNF-alpha plus 200 µg/ml heparin</td>
<td>N/A</td>
<td>N/A</td>
<td>.081±</td>
<td>.068±</td>
<td>N/A</td>
</tr>
<tr>
<td>6 hour stimulation with TNF-alpha</td>
<td>.08±</td>
<td>.076±</td>
<td>N/A</td>
<td>N/A</td>
<td>.022±</td>
</tr>
<tr>
<td>with TNF-alpha plus 200 µg/ml heparin</td>
<td>.01</td>
<td>.004</td>
<td>.005</td>
<td>.002</td>
<td></td>
</tr>
<tr>
<td>6 hour stimulation with NiCl₂</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>.023±</td>
</tr>
<tr>
<td>with NiCl₂ plus 200 µg/ml heparin</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>6 hour stimulation with NiCl₂</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>.026±</td>
</tr>
<tr>
<td>24 hour stimulation</td>
<td>.061±</td>
<td>.076±</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

40
with TNF-alpha | .009 | .004
---|---|---
positive control | .9± | .5± | .23± | .13± | 1.3±
control | .1 | .2 | .02 | .03 | .2
secondary antibody | .069± | .04± | .06± | .035± | .015±

Values are Means ± SED for quadruplicate wells except for the positive control. Results from 5 different experiments are provided. Absorbance readings were read at 410nm.

No further work was performed on this protein. Next, the extracellular matrix proteins fibronectin, thrombospondin, and laminin were studied.

Fibronectin is an extracellular matrix glycoprotein with M$_r$ of 220 kDa under reduced conditions. A radioimmunoprecipitation was performed for fibronectin with endothelial cells exposed to 100 $\mu$g/ml of heparin treatment for a 24 hour incubation time. The results are provided (Figure 5.7). Fibronectin protein levels are elevated with heparin treatment as compared to the control lane.

The media was also analyzed by radioimmunoprecipitation (Figure 5.8). With 100 $\mu$g/ml of heparin treatment, released newly synthesized protein levels are also elevated. by comparing the control lanes to the heparin treated lanes.

Next, to observe if there was a concentration dependent effect when heparin treatment was applied, a concentration range of heparin treatment was applied to endothelial cells for a 24 hour period. A radioimmunoprecipitation assay was used to determine if heparin treatment was affecting the newly synthesized protein levels at the cell-surface (Figure 5.9). When 50, 100, and 200
μg/ml of heparin were applied, an increase in protein levels at the cell-surface is observed by comparing the heparin treated lanes to the control lane. In addition, the media was also analyzed by radioimmunoprecipitation (Figure 5.10). The newly synthesized released protein levels are also elevated by comparing the treated lanes, to the control lane. Next, fibronectin was analyzed by an immunoblot to determine if both the newly synthesized and expressed protein levels are increasing (Figure 5.11). Again by comparing the control lane, to the heparin treated lanes, one can see that protein levels are increasing. The media from these cells was analyzed by immunoprecipitation (Figure 5.12). Protein levels are again increased by comparing the heparin treated lanes, to the control lane. Two bar graphs are provided from the densitometric AU values obtained from these experiments (Figures 5.13 and 5.14). Error bars are the standard error of deviation. One bar graph is for the protein on the cell-surface and the other is for the protein released into the media.

Next, the extracellular matrix glycoprotein thrombospondin was analyzed. Thrombospondin is a protein that migrates as a single band of M, 450 kDa during SDS-PAGE under nonreduced conditions. A radioimmunoprecipitation was performed with endothelial cells exposed to 100 μg/ml of heparin treatment for a 24 hour incubation time. Refer back to Figure 5.7. When heparin treatment was
applied, newly synthesized cell-surface protein levels are reduced as compared to the control lanes. In addition, when the media was analyzed from this experiment, it was observed that heparin treatment reduced released protein levels as compared to the control lanes by referring back to Figure 5.8 and comparing treated lanes, to the untreated lanes. To determine if there was a concentration dependent effect of heparin treatment on thrombospondin protein levels, a concentration range of heparin was applied.

Therefore, endothelial cells were exposed to a heparin concentration range from 50 µg/ml to 200 µg/ml for a 24 hour period. Two radioimmuno-precipitations were performed for the cell-surface proteins and proteins released into the media (Figures 5.15 and 5.16). For both experiments, newly synthesized protein levels are decreasing at the cell-surface and also the protein released into the media when heparin treatment is applied. Two bar graphs are provided with the standard error of deviation as the error bars (See Figures 5.17 and 5.18).

Third, the extracellular matrix glycoprotein laminin was studied. The laminin molecule is composed of three polypeptide chains, A, B1, and B2 with a Mr of 350-400 kDa, 215 kDa, and 205 kDa, respectively. Depending on the degree of glycosylation, the A chain has variability in its molecular weight and
also the B1 chain and B2 chain may migrate as a single band also depending on glycosylation and the assay applied.

When heparin treatment is applied, laminin protein levels are affected. A concentration range of heparin treatment from 50 μg/ml to 200 μg/ml of heparin treatment was applied to the endothelial cells for a 24 hour period. Laminin was analyzed by immunoblots for the cell-surface proteins and immunoprecipitations for the released protein in the media. When heparin treatment was applied, newly synthesized protein and expressed protein levels are increased at the cellular level for both the A and B chains (Figure 5.19). For the protein released into the media, levels remained about the same regardless if heparin was applied (Figure 5.20). The 400 kDa A chain subunit did not appear in the media. Another immunoblot was run for the cell-surface proteins and another immunoprecipitation was run for the protein in the media (Figure 5.21 for the cell-associated protein). Again the treated lanes have an increase in protein levels for both the A and B chains. The 400 kDa A chain did not transfer as well though. The media was again analyzed by immunoprecipitation and for the protein released into the media, levels remained about the same. Refer back to Figure 5.12 and compare treated lanes, to the untreated lane. Next, a radioimmunoprecipitation was performed for both the cell-associated protein and
protein released into the media (Figure 5.22 for the laminin cell-associated protein). Again newly synthesized laminin protein levels are increasing when heparin treatment was applied. When the media was analyzed, the newly synthesized 400 kDa protein subunit is increasing while the 200 kDa protein subunit is decreasing when heparin treatment is applied (Figure 5.23). Two bar graphs are provided for the laminin cell associated protein and protein released into the media. Error bars are the standard error of deviation (Figures 5.24 and 5.25). However, sometimes when heparin treatment is applied, laminin protein levels at the cell surface also decrease.

For example, when two radioimmunoprecipitations were performed on cell-associated proteins on the same cell line, heparin treatment decreased newly synthesized laminin protein levels (Figure 5.26). Two radioimmunoprecipitations were also performed for the proteins released into the media, and results were consistent with previous experiments i.e. heparin treatment decreased newly synthesized 200 kDa subunit protein levels, but increased the 400 kDa subunit protein (Figure 5.26).

When experiments were run on the same endothelial cell line that was nonconfluent as opposed to a confluent cell line, similar results were obtained. Two immunoblots were performed on the same endothelial cell line with heparin
treatment applied, however, in one experiment the cells were subconfluent (Figure 5.27) and in the other experiment, the cells were confluent (Figure 5.28). In both cases, newly synthesized and expressed protein levels decreased at the cell surface. The 400 kDa protein did not appear in Figure 5.27 though. The media from these two experiments was analyzed by immunoprecipitation, laminin expressed protein levels and newly synthesized protein levels remained about the same for the proteins released into the media which was consistent with previous experiments. No Figure is provided for the media results since the data is consistent with previous experiments.

Since there is no published information in the literature on laminin protein level changes as a result of heparin treatment and because the results were so varied not within the same cell line but between cell clones and were not affected by changes in serum or heparin lots, more experimentation was performed.

Endothelial cells were exposed to heparin treatment and cycloheximide to determine if blocking new protein synthesis would affect laminin protein levels (Figure 5.28). When heparin and cycloheximide treatments were applied, laminin protein levels are reduced as compared to the control lanes, change was observed at the 400 and 200 kDa protein band. A bar graph is provided for the
cycloheximide data (Figure 5.29). The released laminin protein in the media was not affected by cycloheximide and therefore no figure is provided.

Next, RNA was isolated to determine if heparin was affecting RNA levels since heparin affected laminin protein levels. Total cellular RNA was isolated and run on a 1% agarose gel (Figure 5.30). The RNA was stained with ethidium bromide however, when the gel was transferred to a nylon membrane and probed with a cDNA complementary to the B1 region of the laminin molecule, no banding pattern was observed for the mRNA.
Immunoblot of P-selectin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and probed for P-selectin with commercially available antibody. The samples applied were: Lane 1, biotinylated molecular weight marker, Lanes 2 and 5, 0 hour controls, no stimulation of endothelial cells, Lanes 3 and 6, 5 hour stimulation of endothelial cells with II-1β, and Lanes 4 and 7, endothelial cells were stimulated with heparin and II-1β for 5 hours.

Figure 5.1: An Attempt to Probe for P-selection
Immunoblot of P-selectin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4. the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and probed for P-selectin with commercially available antibody. The samples applied were: Lane 1, biotinylated molecular weight marker, Lanes 2 and 5, 0 hour controls, no stimulation of endothelial cells, Lanes 3 and 6, 5 hour stimulation of endothelial cells with IL-1β, and Lanes 4 and 7, endothelial cells were stimulated with heparin and IL-1β for 5 hours.

Figure 5.1: An Attempt to Probe for P-selection
Immunoblot of P-selectin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for P-selectin with commercially available antibody. The samples applied were: Lane 1, biotinylated molecular weight marker, Lanes 2 and 5, 0 hour controls, no stimulation of endothelial cells, Lanes 3 and 6, stimulation of endothelial cells with histamine, and Lanes 4 and 7, endothelial cells were stimulated with heparin and histamine.

Figure 5.2: An Attempt to Probe for P-selection
Immunoblot of P-selectin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for P-selectin with commercially available antibody. The samples applied were: Lane 1, biotinylated molecular weight marker, Lanes 2 and 5, 0 hour controls, no stimulation of endothelial cells, Lanes 3 and 6, stimulation of endothelial cells with histamine, and Lanes 4 and 7, endothelial cells were stimulated with heparin and histamine.

Figure 5.2: An Attempt to Probe for P-selection
Immuno blot of P-selectin and ICAM-1. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for P-selectin (Lanes 3-5) and ICAM-1 (Lanes 6-8) with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3 and 6, 0 hour controls, no stimulation of endothelial cells, Lanes 4 and 7, endothelial cells were stimulated with TNF-α for 4 hours and Lanes 5 and 8, endothelial cells were stimulated with TNF-α and 200 μg/ml of heparin for 4 hours.

**Figure 5.3: An Attempt to Probe for P-selection and ICAM-1 Cell-Associated Protein**
Immunoblot of P-selectin and ICAM-1. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for P-selectin (Lanes 3-5) and ICAM-1 (Lanes 6-8) with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3 and 6, 0 hour controls, no stimulation of endothelial cells, Lanes 4 and 7, endothelial cells were stimulated with TNF-α for 4 hours and Lanes 5 and 8, endothelial cells were stimulated with TNF-α and 200 μg/ml of heparin for 4 hours.

Figure 5.3: An Attempt to Probe for P-selection and ICAM-1 Cell-Associated Protein
Immunoblot of ICAM-1. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for ICAM-1 with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3 and 6, 0hour controls, no stimulation of endothelial cells with TNF-α. Lanes 4 and 8, endothelial cells were stimulated with TNF-α for 6 hours, Lanes 5 and 8, endothelial cells were stimulated with TNF-α for 24 hours, and Lane 9, secondary antibody control.

Figure 5.4: An Attempt to Probe for ICAM 1
Immunoblot of ICAM-1 SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for ICAM-1 with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3 and 6, 0 hour controls, no stimulation of endothelial cells with TNF-α. Lanes 4 and 5, endothelial cells were stimulated with TNF-α for 6 hours. Lanes 5 and 8, endothelial cells were stimulated with TNF-α for 24 hours, and Lane 9, secondary antibody control.

Figure 5.4: An Attempt to Probe for ICAM 1
Immunoblot of ICAM-1 SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for ICAM-1 with commercially available antibody. The samples applied were: Lane 1, biotinylated molecular weight marker. Lane 2, 6 hour control, no stimulation of endothelial cells. Lane 3, endothelial cells were stimulated with TNF-α for 6 hours. Lane 4, endothelial cells were stimulated with TNF-α and 200 μg/ml of heparin for 6 hours. Lane 5, endothelial cells were stimulated with NiCl₂ for 6 hours. Lane 6, endothelial cells were stimulated with NiCl₂ and 200 μg/ml of heparin for 6 hours, and Lane 7, secondary antibody control.

Figure 5.5: Cell-Surface Stimulated with Tumor Necrosis Factor Alpha and Nickel Chloride.
Luminoslot of ICAM-1 SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoreses were carried out in 8% reducing polyacrylamide gel and probed for ICAM-1 with commercially available antibody. The samples applied were: Lane 1, ionized molecular weight marker. Lane 2, no control, no stimulation of endothelial cells. Lane 3, endothelial cells were stimulated with TNF-α for 6 hours. Lane 4, endothelial cells were stimulated with TNF-α and 200 μg ml of heparin for 6 hours. Lane 5, endothelial cells were stimulated with NGF for 6 hours. Lane 6, endothelial cells were stimulated with NGF and 200 μg ml of heparin for 6 hours, and Lane 7, secondary antibody control.

Figure 5.5: Cell-Surface Stimulated with Tumor Necrosis Factor Alpha and Nickel Chloride.
Immunoblot of ICAM-1. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% polyacrylamide gel and probed for ICAM-1 with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3, 0 hour controls, no stimulation of endothelial cells. Lane 4, endothelial cells were stimulated with NiCl₂ for 24 hours and were run nonreduced, Lanes 5, endothelial cells were stimulated with PMA for 24 hours and were run nonreduced, Lane 6, endothelial cells were stimulated with NiCl₂, 1μg/ml of heparin for 24 hours and were run nonreduced, Lane 7, endothelial cells stimulated with NiCl₂ for 24 hours and were run under reducing conditions, Lane 8, endothelial cells stimulated with PMA for 24 hours and were run under reducing conditions, Lane 9, endothelial cells stimulated with NiCl₂ and 200 μg/ml of heparin for 24 hours and run under reducing conditions, and Lanes 10-14, secondary antibody controls.

**Figure 5.6: An Attempt to Probe for ICAM-1**
Immunoblot of ICAM-1: SDS-PAGE was run on cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 8% polyacrylamide gel and probed for ICAM-1 with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3, 0 hour controls, no stimulation of endothelial cells. Lane 4, endothelial cells were stimulated with NiCl₂ for 24 hours and were run nonreduced. Lanes 5, endothelial cells were stimulated with PMA for 24 hours and were run nonreduced. Lane 6, endothelial cells were stimulated with NiCl₂, 100 µg/ml of heparin for 24 hours and were run nonreduced. Lane 7, endothelial cells stimulated with NiCl₂ for 24 hours and were run under reducing conditions. Lane 8, endothelial cells stimulated with PMA for 24 hours and were run under reducing conditions. Lane 9, endothelial cells stimulated with NiCl₂ and 200 µg/ml of heparin for 24 hours and run under reducing conditions, and Lanes 10-14, secondary antibody controls.

Figure 5.6: An Attempt to Probe for ICAM-1
X-ray film of Thrombospondin and Fibronectin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and the radioimmunoprecipitation of TSP with commercially available antibody was Lanes (1, 2, 5, and 6), and radioimmunoprecipitation of Fn with commercially available antibody was Lanes (3 and 4). The samples applied were: Lane 1, untreated endothelial cells, Lane 2, endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lane 3, untreated endothelial cells, Lane 4, endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lane 5, untreated endothelial cells, and Lane 6, endothelial cells treated with 100 μg/ml of heparin for 24 hours.

Figure 5.7: Fibronectin and Thrombospondin Cell Associated Proteins
As Cat. of Thrombospondin and Fibronectin, SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel. The radioimmunoprecipitation of FSP with commercially available antibody was lanes 1, 2, 5, and 6; and radioimmunoprecipitation of Fb with commercially available antibody was lanes 3 and 4. The samples applied were Lane 1, untreated endothelial cells; Lane 2, endothelial cells treated with 100 μg/ml of heparin for 24 hours; Lane 3, untreated endothelial cells; Lane 4, endothelial cells treated with 100 μg/ml of heparin for 24 hours; Lane 5, untreated endothelial cells; and Lane 6, endothelial cells treated with 100 μg/ml of heparin for 24 hours.

Figure 5.7: Fibronectin and Thrombospondin Cell-Associated Proteins
Fibronectin and Thrombospondin proteins released into the media. SDS-PAGE was run of proteins released into the media as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and the radioimmunoprecipitation of FN with commercially available antibody was Lanes (1, 2, 5, and 6), and radioimmunoprecipitation of TSP with commercially available antibody was Lanes (3, 4, 7 and 8). The samples applied were: Lanes 1, 3, 5 and 7 untreated media of endothelial cells, and Lanes 2, 4, 6, and 8, media of endothelial cells treated with 100 µg/ml of heparin for 24 hours.

Figure 5.8: Fibronectin and Thrombospondin Proteins Released into the Media
No. as seen, fibronectin and thrombospondin proteins released into the media. SDS-PAGE was run of proteins released into the media as described in Chapter 4. the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and the radiommunoprecipitation of FN with commercially available antibodies was lanes (1,2, 5 and 6), and radiimmunoprecipitation of TSP with commercially available antibody was lanes (3, 4, 7 and 8). The samples applied were lanes (3, 5 and 7) untreated media of endothelial cells, and lanes (2, 4, 6, and 8) media of endothelial cells treated with 100 ng/ml of heparin for 24 hours.

Figure 5.8: Fibronectin and Thrombospondin Proteins Released into the Media
Figure 5.9: Fibronectin Cell Associated Proteins
Figure 5.3: Fibronectin Cell Associated Proteins
X-ray film of Fibronectin protein released into the media. SDS-PAGE was run of proteins released into the media as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and the radio-immunoprecipitation of FN was performed with commercially available antibody. The samples applied were: Lane 1, untreated media of endothelial cells, Lane 2, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 3, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours, and Lane 4, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours.

Figure 5.10: Fibronectin Proteins Released into the Media
X-ray film of Fibronectin protein released into the media. SDS-PAGE was run of proteins released into the media as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and the radio-immunoprecipitation of FN was performed with commercially available antibody. The samples applied were: Lane 1, untreated media of endothelial cells. Lane 2, media of endothelial cells treated with 50 µg/ml of heparin for 24 hours. Lane 3, media of endothelial cells treated with 100 µg/ml of heparin for 24 hours, and Lane 4, media of endothelial cells treated with 200 µg/ml of heparin for 24 hours.

Figure 5.10: Fibronectin Proteins Released into the Media
Immunoblot of Fibronectin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and probed for Fibronectin with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, untreated endothelial cells, Lane 3 endothelial cells treated with 50 µg/ml of heparin for 24 hours, Lane 4 endothelial cells treated with 100 µg/ml of heparin for 24 hours, and Lane 5, endothelial cells treated with 200 µg/ml of heparin for 24 hours.

Figure 5.11: Fibronectin Cell Associated Proteins
Immunoblot of Fibronectin - SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 10% reducing polyacrylamide gel and probed for Fibronectin with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, untreated endothelial cells, Lane 3, endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 4, endothelial cells treated with 100 μg/ml of heparin for 24 hours, and Lane 5, endothelial cells treated with 200 μg/ml of heparin for 24 hours.

Figure 5.11: Fibronectin Cell Associated Proteins
Immunoblot of Fibronectin and Laminin proteins released into the media.

Immunoprecipitation of proteins released into the media was performed as described in Chapter 4, the experimentation section. SDS-PAGE was run of proteins released into the media. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for Fibronectin, Lanes (3-6), and Laminin, Lanes (7-10) with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lanes 3, and 7, untreated media of endothelial cells, Lanes 4, and 8, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lanes 5 and 9, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lanes 6 and 10, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours, and Lane 11, secondary antibody control.

Figure 5.12: Fibronectin and Laminin Proteins Released into the Media
Immunoblot of Fibronectin and Laminin proteins released into the media

Immunoprecipitation of proteins released into the media was performed as described in Chapter 4, the experimentation section. SDS-PAGE was run of proteins released into the media. Electrophoresis was carried out in 8% reducing polyacrylamide gel and probed for Fibronectin, Lanes (3-6), and Laminin, Lanes (7-10) with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker. Lane 2, biotinylated molecular weight marker. Lanes 3, and 7, untreated media of endothelial cells. Lanes 4 and 8, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours. Lanes 5 and 9, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours. Lanes 6 and 10, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours. Lane 11, secondary antibody control.

Figure 5.12: Fibronectin and Laminin Proteins Released into the Media
FIGURE 5.13: FIBRONECTIN CELL ASSOCIATED PROTEIN LEVELS AS A RESULT OF HEPARIN TREATMENT

AU Value vs. Micrograms of Heparin
$y = 0.1165x + 0.035$

$R^2 = 0.5854$

FIGURE 5.14: FIBRONECTIN PROTEIN RELEASED INTO THE MEDIA AS A RESULT OF HEPARIN TREATMENT

AU Value vs. Micrograms of Heparin Added
Figure 5.15: Thrombospondin Cell Associated Protein and Protein Released into the Media
Figure 5.15: Thrombospondin Cell-Associated Protein and Protein Release into the Media
Radioimmunoprecipitation of Thrombospondin cell-surface protein and protein released into the media, and SDS-PAGE was carried out as described in Chapter 4, the experimentation section. Electrophoresis was run in 5% reducing polyacrylamide gel, and the radioimmunoprecipitation of TSP was performed with commercially available antibody. The samples applied were: Lane 1, untreated media of endothelial cells; Lane 2, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours; Lane 3, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours; Lane 4, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours; Lane 5, untreated endothelial cells; Lane 6, endothelial cells treated with 50 μg/ml of heparin for 24 hours; Lane 7, endothelial cells treated with 100 μg/ml of heparin for 24 hours; and Lane 8, endothelial cells treated with 200 μg/ml of heparin for 24 hours.

Figure 5.16: Thrombospondin Cell Associated Protein and Protein Released into the Media
Specifically, the radiomunoprecipitation of Thrombospondin cell-surface protein and protein released into the media, and SDS-PAGE was carried out as described in Chapter 4, the experimentation section. Electrophoresis was run in 5% reducing polyacrylamide gel, and the radiomunoprecipitation of TSFs was performed with commercially available antibody. The samples applied were: Lane 1, untreated media of endothelial cells, Lane 2, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 3, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lane 4, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours, Lane 5, untreated endothelial cells, Lane 6, endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 7, endothelial cells treated with 100 μg/ml of heparin for 24 hours, and Lane 8, endothelial cells treated with 200 μg/ml of heparin for 24 hours.

Figure 5.16: Thrombospondin Cell Associated Protein and Protein Released into the Media
\[ y = -0.2400x + 1.2775 \]
\[ R^2 = 0.7254 \]

**FIGURE 5.17:** THROMBOSPONDIN CELL ASSOCIATED PROTEIN LEVELS AS A RESULT OF HEPARIN TREATMENT

AU Value vs. Micrograms of Heparin Added
FIGURE 5.18: THROMBOSPONDIN PROTEIN RELEASED INTO THE MEDIA AS A RESULT OF HEPARIN TREATMENT
AU Value vs. Micrograms of Heparin Added

y = -0.2855x + 2.9275
$R^2 = 0.819$
Immunoblot of Laminin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 5% reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, secondary antibody control, Lane 2, endothelial cells treated with 200 μg/ml of heparin for 24 hours, Lane 3, endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lane 4, endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 5, untreated endothelial cells, Lane 6, biotinylated molecular weight marker, and Lane 7 rainbow molecular weight marker.

Figure 5.19: Laminin Cell Associated Protein
Immunoblot of Laminin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 5% reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, secondary antibody control. Lane 2, endothelial cells treated with 200 μg/ml of heparin for 24 hours. Lane 3, endothelial cells treated with 100 μg/ml of heparin for 24 hours. Lane 4, endothelial cells treated with 50 μg/ml of heparin for 24 hours. Lane 5, untreated endothelial cells. Lane 6, biotinylated molecular weight marker, and Lane 7 rainbow molecular weight marker.

**Figure 5.19: Laminin Cell Associated Protein**
Immunoblot of Laminin protein released into the media. Immunoprecipitation of Laminin protein released into the media was performed according to Chapter 4, the experimentation section with commercially available antibody. SDS-PAGE was run of proteins released into the media. Electrophoresis was carried out in 5% reducing polyacrylamide gel and probed for Laminin. The samples applied were: Lane 1, secondary antibody control, Lane 2, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours, Lane 3, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lane 4, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours, and Lane 5, media of untreated endothelial cells.

**Figure 5.20:** Laminin Protein Released into the Media
Immunoblot of Laminin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 6% reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker, Lane 2, biotinylated molecular weight marker, Lane 3, untreated endothelial cells, Lane 4, endothelial cells treated with 50 µg/ml of heparin for 24 hours, Lane 5, endothelial cells treated with 100 µg/ml of heparin for 24 hours, Lane 6, endothelial cells treated with 200 µg/ml of heparin for 24 hours, and Lane 7, secondary antibody control.

**Figure 5.21: Laminin Cell-Associated Protein**
Radioimmunoprecipitation of Laminin cell-associated protein was performed according to Chapter 4, the experimentation section with commercially available antibody. SDS-PAGE was run of cell-associated protein. Electrophoresis was carried out in 5% reducing polyacrylamide gel. The samples applied were: Lane 1, untreated endothelial cells, Lane 2, endothelial cells treated with 50 μg/ml of heparin for 24 hours, and Lane 3, endothelial cells treated with 200 μg/ml of heparin for 24 hours.

Figure 5.22: Laminin Cell Associated Protein
A Western blot of Laminin. Radioimmunoprecipitation of Laminin protein released into the media was performed according to Chapter 4, the experimentation section. SDS-PAGE was run of proteins released into the media. Electrophoresis was carried out in 10% reducing polyacrylamide gel. The samples applied were: Lane 1, untreated media of endothelial cells; Lane 2, media of endothelial cells treated with 50 µg/ml of heparin for 24 hours; Lane 3, media of endothelial cells treated with 100 µg/ml of heparin for 24 hours; and Lane 4, media of endothelial cells treated with 200 µg/ml of heparin for 24 hours.

**Figure 5.23**: Laminin Proteins Released into the Media
FIGURE 5.24: LAMININ CELL ASSOCIATED PROTEIN LEVELS AS RESULT OF HEPARIN TREATMENT
AU Value vs Micrograms of Heparin
FIGURE 5.25: LAMININ PROTEIN RELEASED INTO THE MEDIA AS A RESULT OF HEPARIN TREATMENT
AU Value vs. Micrograms of Heparin
X-ray film of Laminin. Radioimmunoprecipitation of Laminin cell-associated protein and protein released into the media was performed according to Chapter 4, the experimentation section with commercially available antibody. SDS-PAGE was run of cell-associated protein and protein released into the media. Electrophoresis was carried out in 5% reducing polyacrylamide gel. The samples applied were: Lane 1, untreated endothelial cells, Lane 2, endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 3, endothelial cells treated with 100 μg/ml of heparin for 24 hours, Lane 4, endothelial cells treated with 200 μg/ml of heparin for 24 hours, Lane 5, untreated media of endothelial cells, Lane 6, media of endothelial cells treated with 50 μg/ml of heparin for 24 hours, Lane 7, media of endothelial cells treated with 100 μg/ml of heparin for 24 hours, and Lane 8, media of endothelial cells treated with 200 μg/ml of heparin for 24 hours.

**Figure 5.26: Laminin Cell Associated Protein and Protein Released into the Media**
X-ray film of Laminin. Radioimmunoprecipitation of Laminin cell-associated protein and protein released into the media was performed according to Chapter 4, the experimentation section with commercially available antibody. SDS-PAGE was run of cell-associated protein and protein released into the media. Electrophoresis was carried out in 5% reducing polyacrylamide gel. The samples applied were Lane 1, untreated endothelial cells. Lane 2, endothelial cells treated with 50 µg/ml of heparin for 24 hours. Lane 3, endothelial cells treated with 100 µg/ml of heparin for 24 hours. Lane 4, endothelial cells treated with 200 µg/ml of heparin for 24 hours. Lane 5, untreated media of endothelial cells. Lane 6, media of endothelial cells treated with 50 µg/ml of heparin for 24 hours. Lane 7, media of endothelial cells treated with 100 µg/ml of heparin for 24 hours, and Lane 8 media of endothelial cells treated with 200 µg/ml of heparin for 24 hours.

Figure 5.26: Laminin Cell Associated Protein and Protein Released into the Media
Immunoblot of Laminin. SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in 5% reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker; Lane 2, biotinylated molecular weight marker; Lane 3, untreated endothelial cells; Lane 4, endothelial cells treated with 200 μg/ml of heparin for 24 hours, and Lane 5, secondary antibody control.

Figure 5.27: Laminin Cell-Associated Protein
Laminar blot of Laminin SDS-PAGE was run of cell-surface proteins as described in Chapter 4, the experimentation section. Electrophoresis was carried out in a reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, rainbow molecular weight marker Lane 2, non-reduced molecular weight marker Lane 3, untreated endothelial cells, Lane 4, endothelial cells treated with 20 ng/mL of laminin for 24 hours, and Lane 5, secondary antibody control.

Figure 5.27: Laminin Cell Associated Protein
Immunoblot of Laminin. SDS-PAGE was run of cell-surface proteins as describe in Chapter 4, the experimentation section. Electrophoresis was carried out in 5% reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, untreated endothelial cells, Lane 2, endothelial cells treated with 200 μg/ml of heparin, Lane 3, endothelial cells treated with cycloheximide, Lane 4, endothelial cells treated with cycloheximide and 200 μg/ml heparin, and Lane 5, secondary antibody control.

Figure 5.28: Laminin Cell Associated Protein
Immunoblot of Laminin. SDS-PAGE was run of cell-surface proteins as describe in Chapter 4, the experimentation section. Electrophoresis was carried out in 5% reducing polyacrylamide gel and probed for Laminin with commercially available antibody. The samples applied were: Lane 1, untreated endothelial cells, Lane 2, endothelial cells treated with 200 μg/ml of heparin, Lane 3, endothelial cells treated with cycloheximide, Lane 4, endothelial cells treated with cycloheximide and 200 μg/ml heparin, and Lane 5, secondary antibody control.

**Figure 5.28: Laminin Cell Associated Protein**
FIGURE 5.29: THE EFFECT OF CYCLOHEXIMIDE ON NEWLY SYNTHESIZED LAMININ PROTEIN LEVELS AT THE CELL SURFACE
AU Value vs. Treatment of Endothelial Cells

$y = -0.689x + 4.655$
$R^2 = 0.9712$
Ethidium bromide stain of rRNA. Total cellular RNA was isolated according to Chapter 4, the experimentation section. 9 μg of RNA was loaded per well and run on a 1% agarose gel. Gel was stained the following day for ribosomal subunits with ethidium bromide.

Figure 5.30: Ethidium Bromide Stain of rRNA
During the initial stages of atherosclerosis, the integrity of the vessel wall is compromised. The endothelium is damaged resulting in the attachment of monocytes and the destruction of the extracellular matrix. Since heparin has been observed to reverse atherosclerotic effects, it is important to study the effect of heparin on extracellular matrix glycoproteins and cell adhesion protein levels.

To study the cell-adhesion molecules P-selectin and ICAM-1, activation was required. Unfortunately, it was difficult to obtain data. It is hard to believe that activation of the endothelial cells did not take place since three different activators were applied to study both proteins according to information published in the literature, however, activation may not have occurred. The problem may lie in the antibody recognition since the antibody was developed in human cells and work was performed on porcine endothelial cells. In addition, according to Bonfanti, et. al., (1989) P-selectin can be localized to the Weibel Palade bodies of human umbilical vein endothelial cells by immunofluorescence regardless of whether or not activation has occurred. However, when immunofluorescence was performed on porcine cells, no fluorescence was observed on either activated or unactivated cells.

Speculation as to what should have been observed by the addition of heparin, one should see a decrease in both P-selectin and ICAM-1 protein levels possibly by slowing or reducing the synthesis of these two molecules with the addition of heparin working through its receptor. In theory, if heparin is discussed as a possible treatment for
atherosclerosis and P-selectin and ICAM-1 are involved in the initial stages of vessel injury, therefore, through its receptor, heparin will increase the electronegativity of the endothelium which should result in decreased protein levels of these two molecules.

The extracellular matrix glycoproteins, fibronectin, thrombospondin, and laminin were then studied. These glycoproteins are involved in adhesion, also. With the addition of heparin, fibronectin protein levels increased at both the cell-surface and the proteins released into the media. To study the protein levels at the cell surface, immunoblots were performed to determine the change in newly synthesized protein and expressed protein levels while radioimmunoprecipitations were also performed which analyze only newly synthesized protein. For both experiments, heparin treatment resulted in an increase in fibronectin protein levels. When the media from these cells was analyzed by immunoprecipitation and radioimmunoprecipitations, the fibronectin protein released into the media also showed increased protein levels. The difference between the heparin treated lanes and untreated lanes was statistically significant as determined by regression analysis ($\alpha = 0.05$).

In addition, when these result were compared to Lyons-Giordano et. al. (1990), the results appeared similar to the published information when endothelial cell growth factor was not added. However, according to Lyons-Giordano et. al. (1990), when growth factor was added to the culture medium, fibronectin protein levels decreased with heparin treatment. If growth factors were added to our culture medium, it would be difficult to ascertain whether the change in protein levels was occurring from the growth
factors or heparin alone since growth factors are known to work through receptors and cause changes in the proliferation of endothelial cells.

Fibronectin protein levels may be increasing in the presence of heparin treatment in part because of its functional role in spreading and migration.

Thrombospondin was the next extracellular matrix glycoprotein studied. Because of the high molecular weight of the thrombospondin protein, 450 kDa, transfer to nitrocellulose would be difficult using the immunoblot technique, therefore, only radioimmunoprecipitations were performed to study heparin’s effect on newly synthesized protein levels. Thrombospondin protein levels decreased at both the cell-surface and in the media with the addition of heparin treatment, which also compares similarly to the Lyons-Giordano article. The difference observed between the heparin treated lanes and the untreated lanes was also statistically significant using regression analysis ($\alpha = 0.05$). Thrombospondin’s main function is in angiogenesis, however, it can also serve as an adhesive and anti-adhesive molecule. Therefore, because of its functional role as an anti-adhesive molecule, it may in part explain why protein levels are decreasing.

The last extracellular matrix glycoprotein studied was laminin. When heparin treatment was applied to endothelial cells, laminin protein levels were affected at the cell-surface. Protein levels sometimes increased using one endothelial cell line and sometimes decreased using another cell line at the cell surface for the 400 kDa A chain and also for the B1, B2 chains which usually migrate as a single protein band. Variability occurred between endothelial cell lines but did not occur within a particular cell line.
Laminin protein levels were not affected by confluency of cell cultures or passage numbers. In addition, changes in lots of serum or heparin did not affect laminin protein levels since heparin induced increases in protein levels occurred one week while decreases in laminin protein levels using a different cell line occurred the following week.

When cycloheximide and heparin were applied, both induced changes of newly synthesized laminin protein levels at the cell surface by the observed reduction in the 400 and 200 kDa subunits. An attempt to study the laminin mRNA expression pattern when heparin treatment was applied was unsuccessful and therefore no information could be obtained. The cDNA that was obtained may not have been exactly complementary to the B1 region of porcine laminin since the probe was developed complementary to the B1 region of human laminin. In addition, according to Webersinke, et. al. (1992), the laminin B1 may not have been detected because of an extremely rapid turnover of laminin B in the cell due to the presence of a destabilizing 3' untranslated region in the laminin B message.

When immunoblots were performed on the laminin protein released into the media, newly synthesized and expressed laminin protein levels of the 200 kDa subunit remained the same regardless of whether or not heparin treatment was applied. The 400 kDa subunit did not appear on the immunoblots and results cannot be compared to the radioimmunoprecipitation data for this same subunit. Consistent with our results, it has been observed in astrocytes, Schwann cells, and schwannomas that these cells produce and release the B chains, but not the 400 kDa, A isoform of laminin (Chiu, et. al., 1991).
Also, in skeletal muscle it has been observed that the B chains are always expressed but the levels of the A chains are low and often undetectable. It is becoming increasingly clear that the chain composition of laminin varies from tissue to tissue and, even within a particular tissue, as a function of development (Boot-Handford et. al., 1987).

When radioimmunoprecipitations were performed on the laminin protein released into the media after the addition of heparin treatment, newly synthesized 400 kDa A chain increased while the newly synthesized 200 kDa, B1,B2 chain decreased. The 400 kDa subunit may be controlling the availability of the amount of the 200 kDa subunit that is synthesized in this case. When cycloheximide was applied, laminin protein released into the media was unaffected.

Changes in laminin protein levels, after heparin treatment was applied, could be due to variability between endothelial cell lines, but not within a particular cell line since similar results were obtained from the same cell line, regardless of passage number or confluency of the endothelial cell line. Variability in the results at the cell surface may be due to low levels of smooth muscle cell contamination of the cell line which was undetectable at the time the experiments were carried out. While low levels of contamination may not have an effect on fibronectin or thrombospondin, laminin might be affected by it.

In addition, as stated above, the 400 kDa subunit may not have appeared due to the possibility that during development and differentiation of the matrix of the cell line, expression of this isoform was not detected by the polyclonal Ab commercially purchased for these experiments. Unless particular isoforms for laminin are obtained, it
is difficult to determine what results would have been obtained. When regression analysis was run for laminin, heparin induced changes were found to be statistically significant for the changes observed for both subunits ($\alpha = 0.050$).

Finally, the information presented here demonstrates that heparin's effect on endothelial cell growth and protein biosynthesis may be particularly important in the repair of the vessel wall following injury, an early step in the development of vascular disease particularly atherosclerosis. Therefore, an understanding of heparin's action on the endothelial cells and these molecules may eventually provide clues to restoring vessel wall integrity in diseased states.
BIBLIOGRAPHY


Becton Dickinson Advanced Cellular Biology, 1992. CAMfolio is a trademark of Becton Dickinson and Company.


Appendix A

Buffers and Solutions for Protein and Antibody Work.

**Unless otherwise noted all solutions in this appendix are made with 18 M ohm/cm water, are 0.2 \( \mu m \) filtered and can be stored at room temperature.

Alkaline Phosphate Buffer, pH 9.5

- This buffer can be made as a 10X stock solution.

Components

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<td>Tris-Base, 100 mM</td>
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<tr>
<td>NaCl, 100 mM</td>
<td>5.84 g</td>
</tr>
<tr>
<td>MgCl(_2) ( 6H_2O ), 5 mM</td>
<td>1.01 g</td>
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To make working solution add 50 \( \mu l \) of NBT and 25 \( \mu l \) of BCID to 25 ml of 1X buffer.

BCIP Stock

- not 0.2 \( \mu m \) filtered
- stored at - 20° C

Components

- 50 mg BCIP/ 1.0 ml of water
**NBT Stock**

-stored at \(-20^\circ\text{C}\)

Components

50 mg NBT/1.0 ml of 70% DMF

**DESTAIN I**

Components

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<td>Glycerol 2%</td>
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**Phosphate Buffered Saline (PBS), pH 7.4**

-This buffer can be made 10x as a stock solution.

Components

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<th>Component</th>
<th>Amount/Liter</th>
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<tr>
<td>Na(_2)HPO(_4), 8.2 mM</td>
<td>1.15 g</td>
</tr>
</tbody>
</table>

**Phosphate Buffered Saline and 1% CHAPS**

Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/Liter</th>
</tr>
</thead>
</table>
PBS 1x
CHAPS, 1.0%

Blotto

-This solution is heated to 20° C for one hour when first made to fully dissolve all the milk protein.
-Stored at 4° C and is not filtered

Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fat Dry Milk, 5%</td>
<td>50.0 g</td>
</tr>
<tr>
<td>NaN₃ 6 mM</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Bovine-Serum Albrimin, 3%</td>
<td>30.0 g</td>
</tr>
<tr>
<td>10x Tris Buffered Saline-TBS, 10% (See Below)</td>
<td>100.0 mL</td>
</tr>
</tbody>
</table>

Tris Buffered Saline (TBS), pH 7.5

-This buffer can be made 10x as a stock solution.

Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Base, 50 mM</td>
<td>6.06 g</td>
</tr>
<tr>
<td>NaCl, 150 mM</td>
<td>8.77 g</td>
</tr>
</tbody>
</table>

Tris Buffered Saline and 0.1% Tween-20 (TBST)

-This buffer can be made 10x as a stock solution.

Components
TBS, 1x

Tween-20, 0.1%  

Transfer Buffer

Components

Tris-Base, 12.5% mM  
Glycine, 96 mM  

Bromophenol Blue

Components

Bromophenol Blue, 0.1%  

Phosphate-Citrate Buffer, pH 5.0

Components

0.2 M Dibasic Sodium Phosphate  
0.1 M Citric Acid  

To make working buffer, add 10 tablets of ABTS in 1L of this buffer, then add 250 mL of 50% H₂O₂ (make working buffer fresh)

Dibasic Sodium Phosphate

Components

Na₂HPO₄, 0.2 M  

amount/liter

1000 mL

1.0 g

amount/liter

1.5 g

7.2 g

amount/liter

1.0 g

amount/liter

257 mL

243 mL

28.4 g
### Citric Acid

**Components**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric Acid, 0.1 M</td>
<td>19.2 g</td>
</tr>
</tbody>
</table>
Appendix B

Buffers and Solutions for Laemmli Style SDS-GEL Electrophoresis

**All solutions were prepared with 18 M ohm cm water

*Commercially available Acrylamide-Bis-Acrylamide

*Acrylamide Stock, 37.5:1 - Acryl:BIS (Amresco, Solon, OH)

Acrylamide, 38.96%
BIS - Acrylamide, 1.04%

*Resolving Gel Buffer, pH 8.8

Components amount/liter
Tris-Base, 1.5 M 181.5 g

*Stacking Gel Buffer, pH 6.8

Components amount/liter
Tris-Base, 0.5 M 60.0 g

*SDS Stock, 10%

Components amount/liter
SDS, 10% 100.0 g
Sample Buffer (2X), pH 6.8

Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/10mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Base, 12.5 mM</td>
<td>1.513 g</td>
</tr>
<tr>
<td>SDS Stock, 4%</td>
<td>4.00 mL</td>
</tr>
<tr>
<td>2-Mercaptoethanol, 15 mM</td>
<td>1.00 mL</td>
</tr>
</tbody>
</table>

(2-Mercaptoethanol may be excluded to make a non-reduced sample buffer)

Tank (Running) Buffer, pH 8.3

Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Base, 25 mM</td>
<td>3.00 g</td>
</tr>
<tr>
<td>Glycine, 192 mM</td>
<td>14.40 g</td>
</tr>
<tr>
<td>SDS-Stock, 0.4%</td>
<td>4.00 mL</td>
</tr>
</tbody>
</table>

Recipe for Standard Gel (15 cm x 15 cm x 1.5 mm - 10% Separating; 4% Stacking)

Separating Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acryamide Stock</td>
<td>7.5 mls</td>
</tr>
<tr>
<td>Separating Gel</td>
<td>7.5 mls</td>
</tr>
<tr>
<td>Stock SDS</td>
<td>0.3 mls</td>
</tr>
<tr>
<td>Water</td>
<td>14.5 mls</td>
</tr>
<tr>
<td>Ammonium Persulfate*</td>
<td>150 μl</td>
</tr>
<tr>
<td>TEMED*</td>
<td>10 μl</td>
</tr>
<tr>
<td>Component</td>
<td>Volume</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Acrylamide Stock</td>
<td>1.3 mls</td>
</tr>
<tr>
<td>Stacking Gel Buffer</td>
<td>2.5 mls</td>
</tr>
<tr>
<td>Stock SDS</td>
<td>0.1 mls</td>
</tr>
<tr>
<td>Water</td>
<td>6.1 mls</td>
</tr>
<tr>
<td>Ammonium Persulfate*</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED*</td>
<td>5 μl</td>
</tr>
</tbody>
</table>

For polymerization a solution of 0.1 g Ammonium Persulfate per 1 ml of water was made.

*Added to the degassed gel mix immediately before casting.

For a recipe of 8% separating gel substitute 6.0 ml of acrylamide stock and 16.0 mls of water.

For a recipe of 6% separating gel substitute 4.5 mls of acrylamide stock and 17.5 mls of water.

For a recipe of a 5% separating gel substitute 3.75 mls of acrylamide stock and 18.25 mls of water.
**Appendix C**

**Buffers and Solutions for RNA Work**

**All solutions in this appendix are made with DEPC-treated water.**

10X MOPS Buffer [3-(N-morpholino)-propanesulfonic acid] pH 7.0

-Filtered using Whatman #1 filter paper.

Components | amount/liter
--- | ---
MOPS | 41.8 g
3M DEPC-treated Sodium acetate | 16.6 ml
0.5M DEPC-treated EDTA, pH 8 | 20.0 ml

Sodium acetate, pH 5.2

Components | amount/liter
--- | ---
CH₃COONa3H₂O, 3M | 408.0 g

EDTA, 0.5M pH 8.0 (ethylenediamine tetraacetic acid)

Components | amount/liter
--- | ---
Na₂EDTA2H₂O | 186.1 g
Agarose gel, 1%

Components

agarose 
10.0 g

formaldehyde, 37% 
150.0 ml

10X MOPS, 10% 
100.0 ml

Formaldehyde loading buffer

Components

Bromophenol blue, 0.25% 
0.375 g

Xylene Cyanol, 0.25% 
2.5 g

Glycerol, 50% 
500.0 ml

SSC, pH7.0

-This buffer can be made 5X, 10X, and 20X as a stock solution.

Components

NaCl, 150mM 
8.75 g

C₅H₅Na₃O₇·2H₂O, 15mM 
4.4 g
Pre-hybridization solution

Components……………………………………..amount/liter

KH₂PO₄, 2M…………………………………….25.0 ml

5X SSC (see above)…………………………….250.0 ml

5X Denhardt's solution (see below)…………50.0 ml

Salmon sperm DNA, 5mg/ml………………10.0 ml

Formamide, 50%………………………………500.0 ml

To make 30.0 ml of Northern hybridization solution, add 150 μl t-RNA baker's yeast
and cDNA probe (1mg/ml)

Lectin Block

-Solution is heated to 65 °C for one hour when first made to fully dissolve the
components.

-Store at 4 °C.

-Not filtered.

Components……………………………………..amount/liter

Cold Water Fish Gelatin, 1%………………10.0 g

PVP, 1%………………………………………..10.0 g

NaN₃, 0.1%…………………………………….1.0 g

Tween-20, 0.1%………………………………1.0 g

10X Tris Buffered Saline-TBS (see above), 10%…………100.0 ml
*Denhardt solution*

This solution can be made 100X as a stock solution.

Filtered using a Whatman #1 filter paper.

Store at -20°C.

**Components**

<table>
<thead>
<tr>
<th>Component</th>
<th>amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll 400</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

**0.1M Tris Cl, pH 7.5**

**Components**

<table>
<thead>
<tr>
<th>Component</th>
<th>amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Base</td>
<td>12.1 g</td>
</tr>
</tbody>
</table>

**NaCl-NaOH Buffer**

**Components**

<table>
<thead>
<tr>
<th>Component</th>
<th>amount/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH, 50mM</td>
<td>2.0 g</td>
</tr>
<tr>
<td>NaCl, 10mM</td>
<td>0.584 g</td>
</tr>
</tbody>
</table>
**TBE Buffer, Tris-Borate-EDTA, pH 8.3**

This buffer can be made 10X as a stock solution.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base, 89mM</td>
<td>10.8 g</td>
</tr>
<tr>
<td>Boric acid, 89mM</td>
<td>5.5 g</td>
</tr>
<tr>
<td>EDTA-Free Acid, 2.5mM</td>
<td>0.74 g</td>
</tr>
</tbody>
</table>
APPENDIX D
REGRESSION ANALYSIS

Figure 5.24  200 subunit

SUMMARY OUTPUT

<table>
<thead>
<tr>
<th>Regression Statistics</th>
<th>Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple R</td>
<td>0.852659492</td>
</tr>
<tr>
<td>R Square</td>
<td>0.72702821</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.129264402</td>
</tr>
<tr>
<td>Observations</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
</tr>
<tr>
<td></td>
<td>X Variable</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Significance F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.089006429</td>
<td>0.089006429</td>
<td>5.326764417</td>
<td>0.147340508</td>
</tr>
<tr>
<td>2</td>
<td>0.033418571</td>
<td>0.016709286</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.122425</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>micrograms of heparin</th>
<th>AU value</th>
<th>AU value</th>
<th>Mean</th>
<th>Deviation about the Mean ( (x_i - \bar{x}) )</th>
<th>Squared Deviation about the Mean ( (x_i - \bar{x})^2 )</th>
<th>standard error of deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.26</td>
<td>0.06</td>
<td>0.16</td>
<td>0.1</td>
<td>0.01</td>
<td>0.14</td>
</tr>
<tr>
<td>50</td>
<td>0.3</td>
<td>0.18</td>
<td>0.24</td>
<td>0.06</td>
<td>0.0036</td>
<td>0.08</td>
</tr>
<tr>
<td>100</td>
<td>0.54</td>
<td>0.57</td>
<td>0.555</td>
<td>0.015</td>
<td>0.000225</td>
<td>0.02</td>
</tr>
<tr>
<td>200</td>
<td>0.54</td>
<td>0.53</td>
<td>0.535</td>
<td>0.005</td>
<td>0.000025</td>
<td>0.007</td>
</tr>
</tbody>
</table>

\[
S^2 = \frac{\sum (x_i - \bar{x})^2}{n-1} = \frac{.01+.01}{4} = .02
\]

\[
\bar{x} = \sqrt{s^2} = 0.14
\]
\[
\begin{array}{|c|c|c|c|c|c|c|}
\hline
x_i & y_i & x^2 & xy & \hat{y}_i = \frac{0.196 + 0.002017x}{2} & y_i - \hat{y}_i & (y_i - \hat{y}_i)^2 \\
\hline
0 & 0.16 & 0 & 0 & 0.196 & -0.036 & 0.001296 \\
50 & 0.24 & 2500 & 12 & 0.29685 & -0.05685 & 0.003231923 \\
100 & 0.555 & 10000 & 55.5 & 0.397 & 0.1573 & 0.02474329 \\
200 & 0.535 & 40000 & 107 & 0.5994 & -0.0644 & 0.004147 \\
\hline
\Sigma x_i = 350 & \Sigma y_i = 1.49 & \Sigma x^2 = 52500 & \Sigma x y_i = 174.5 & SSE = 0.033419 \\
\bar{x} = \frac{\Sigma x_i}{n} = 87.5 & \bar{y} = \frac{\Sigma y_i}{n} = 0.3725 & \end{array}
\]

\[
b_1 = \frac{\Sigma x_i y_i - (\Sigma x_i \Sigma y_i) / n}{\Sigma x^2 - (\Sigma x_i)^2 / n} = \frac{174.5 - (350)(1.49)/4}{52500 - 350^2/4} = 0.002017
\]

\[
b_0 = \hat{y} - b_1 \bar{x} = 0.372 - (0.002)(87.5) = 0.196
\]

\[
x = \frac{\Sigma x_i}{n} = 350 = 87.5 & \bar{y} = \frac{\Sigma y_i}{n} = 1.49 / 4 = 0.3725
\]

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
x_i & y_i & (y_i - \bar{y}) & (y_i - \bar{y})^2 & (\hat{y}_i - \bar{y}) & (\hat{y}_i - \bar{y})^2 \\
\hline
0 & 0.16 & -0.2125 & 0.04515625 & -0.1765 & 0.03115225 \\
50 & 0.24 & -0.1325 & 0.01775555 & -0.07565 & 0.005722923 \\
100 & 0.555 & 0.1825 & 0.03330625 & 0.0252 & 0.00063504 \\
200 & 0.535 & 0.1625 & 0.02640625 & 0.2269 & 0.05148361 \\
\hline
\end{array}
\]

\[
R^2 = \frac{SSR}{SST} = \frac{0.122425}{0.089006} = 0.727028
\]

72.7% of the observed increase in AU values can be explained by the linear relationship between the micrograms of heparin added and the AU values.

\[
\pm \sqrt{R^2} = 0.852659
\]

The sample correlation coefficient is positive 0.852659 since the slope is positive and conclude there is a strong positive linear association between micrograms of heparin added and the AU value.

\[
MSE = \frac{SSE}{n - 2} = \frac{0.033419}{2} = 0.0167095 & MSR = \frac{SSR}{1} = \frac{0.089006}{1} = 0.089006
\]
Standard Error of Estimate = \sqrt{\frac{MSR}{MSR}} = \sqrt{0.0167095} = 0.129264

F = \frac{MSR}{MSR} = \frac{0.089006}{0.16709} = 5.326764

Reject H_0 if F > F_{\alpha}

F_{\alpha} = 0.147341

\therefore reject null hypothesis

We conclude that we have a significant relationship between micrograms of heparin added and the observed increase in AU values.
Vita

Kimberly Jean Sebo was born to Elizabeth Olexa and Stephen Sebo on January 6, 1967 in Lansdale, Pennsylvania. She graduated with honors from Whitehall High School in 1984. In June of 1984, she began undergraduate study at Pennsylvania State University. As an undergraduate she conducted an independent study project under the guidance of Dr. Stephens. While at Penn State she was employed by the university as a tutor, and made the dean’s list for four semesters. She was a member of the Microbiology Club and became a lifetime member of the American Society of Microbiologists. In 1988, she graduated with a Bachelor of Science in Microbiology and Biology from the Pennsylvania State University. Following graduation, she was employed at Roche Diagnostic Labs as a technologist for a year. During this time, she performed microbial identifications of specimens that were sent to the lab for urinalysis testing and blood culture screening. She then became employed at Merck and Company as a laboratory technician in the quality control microbiology department. While at Merck, she was involved in testing the sterility of injectable vaccines and the intravenous pharmaceutical products and also the identification of environmental microorganisms by gas chromatography. Kim joined the Department of Molecular Biology at Lehigh University as a part time graduate student with associate status in 1991. She petitioned for graduate status and was accepted as a part time graduate student. On December 25 1993 she became engaged to John M. McMenamy whom she met in her sophomore year at Penn State. In the Spring of 1994 she began working with Dr. Linda J. Lowe-Krentz.