In Vitro Binding and Transport Regulation by Endothelial Cells: Preliminary Studies looking at FIX and IGF-I

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(ABSTRACT)

Endothelial cells separate the bloodstream from the underlying tissue and play a crucial role in vascular homeostasis. They also form an important barrier for vascular drug delivery. This thesis contains preliminary studies targeted at understanding the mechanisms of binding and transport across endothelial cells cultured *in vitro*. Specifically, the first study investigates how the recombinant source of Factor IX (FIX), a blood coagulant protein used in the treatment of Hemophilia B, impacts surface ligand binding (FIX to its specific receptors) to bovine aortic endothelial cells (BAECs). Competitive binding experiments between ^{125}I-FIX and FIX were undertaken to quantify the interaction of recombinant and transgenic FIX with BAECs and human collagen IV and determine if there was a measurable difference in binding affinity. Results indicate limited specific binding of ^{125}I-FIX to BAECs and no binding to human collagen IV. Concrete conclusions were not drawn from this data due to technical issues during the experimental process. The second study investigates insulin-like growth factor-I (IGF-I) transport across both BAEC and MAC-T cells, a mammary epithelial cell line, cultured on tissue culture inserts. IGF-I is a circulatory growth factor implicated in the regulation of cell division and tissue proliferation. Competitive binding experiments between ^{125}I-IGF-I and unlabeled protein (IGF-I, Y60L-IGF-I, a mutant of IGF-I, and IGF Binding Protein-3 (IGFBP-3)) were undertaken to quantify the binding and transport of IGF-I

under various experimental conditions. Results confirmed earlier work from the Williams' laboratory indicating that ^{125}I-IGF-I transport was enhanced by incubation with its non-receptor-binding analog, Y60L-IGF-I, but cell surface associated ^{125}I-IGF-I was decreased by its presence. Other studies were undertaken but conclusive results could not be drawn.

Dedication

This thesis is dedicated to my loving fiancé, Matthew. Thank you for allowing me to pursue my dreams and working hard behind the scenes to ensure that I realize them! I could not have made it through these last 3 years without your love and constant support.

I love you.

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I would first like to thank my advisor Dr. Kimberly Forsten Williams for her constant support and mentoring throughout this Masters project. I will always appreciate you for giving me a chance to learn and prove myself in the field of chemical engineering. You have been a great mentor, friend, big sister and mother away from home to me.

I would like to thank Dr. R. Michael Akers for his patience and support as I struggled through some of the rough parts of my experiments. Thanks so much for radiolabeling my proteins, donating tons of Mac-T cells and especially for just listening.

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I would also like to acknowledge all of my friends and colleagues in the Williams, Goldstein and Van Cott labs. Thanks so much for your help with my experiments and tending to my cells and other odd jobs in the lab when I could not. Your advice, support, encouragement and friendship mean a lot to me and I will never forget you guys. You kept me sane.

I would especially like to thank my parents and younger brother and sister for all of your love and kind words. I could not have finished without you. Thanks for always believing in me and standing behind me. Special thanks to my mom and dad for sacrificing so much for us and for shaping me into the person I am today.

To my perfect little nephew Markel, I know you are too young to understand this now, but I want you to know how much I love you. When life became too hard for me, I thought of you and that alone is what kept me going. Thank you for being the little angel that I needed to get me through the rough times.

A special thank you to my fiancé Matt. If it weren't for you I would not have stayed in Virginia too long. You are my world.

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Chapter 1: Overview of Thesis

1.1 Introduction

This masters thesis is a culmination of experiments that investigates two important aspects of cellular events: ligand surface binding and transport processes of the cell. Two cell types, bovine aortic endothelial cells (BAECs) and a bovine mammary epithelial cell line (Mac-T) were used in this work as model systems to investigate these cell regulation. Radiolabeled ligands, ^{125}I-Factor IX (FIX) and ^{125}I-Insulin-like Growth Factor-I (IGF-I), were used in this work to monitor and quantify binding and transport. Unlabeled ligands such as FIX, IGF-I, IGF binding protein (IGFBP-3) and Y60L-IGF-I were used to help elucidate mechanisms of action.

The studies presented in this masters thesis are a continuation of previous research in the Williams laboratory at Virginia Polytechnic Institute and State University (Virginia Tech) and the Velander and Van Cott laboratories formerly at Virginia Tech and now at the University of Nebraska-Lincoln. The first study investigates ^{125}I-FIX surface ligand binding to BAECs. FIX is a blood coagulant protein that is used in the treatment of Hemophilia B. Hemophilia B is a hereditary disorder caused by mutations in the FIX gene that result in longer bleeding episodes. Intravenous replacement therapy of plasma derived (pd-FIX) or recombinant FIX (BeneFIX®) is used in the treatment of this disease (51). Unfortunately, pd-FIX and BeneFIX® are in extremely short supply worldwide. Research in the laboratory of Dr. William Velander has resulted in the development of an abundant and economical source of recombinant FIX produced in the milk of transgenic animal bioreactors (51). The motivation behind the research in this thesis is to determine if the binding characteristics of transgenic FIX as compared to recombinant FIX result in

longer retention in the bloodstream. Longer retention time in the bloodstream might make it possible for lower doses and less frequent IV infusions for patients.

The second study investigates ^{125}I-IGF-I transport across BAEC and Mac-T cell monolayers. Endothelial cells form the primary barrier against transport from the blood stream and regulate the delivery of circulating agents to underlying tissues (33). IGF-I is a circulatory growth factor implicated in the regulation of cell division and tissue proliferation. It is used in intravenous infusions to treat various pathological diseases such as diabetes (36) and Gaucher disease (37). Previous work (33) showed that internalization and transport of IGF-I across BAECs *in vitro* were mediated by IGF-I receptors and that cell surface and extracellular matrix IGF binding proteins (IGFBPs), while abundant, did not facilitate transport across the cell layer. This work suggested that IGFBPs function as a reservoir for IGF-I, sequestering it for later release and receptor-mediated transport. The motivation behind this research was to determine if experiments could be designed to show enhanced overall transport of IGF-I due to the presence of IGFBPs.

This chapter opens with a brief literature review on ligand binding and transport experiments. Next, background sections on competitive binding experiments, pulse chase experiments and modes of transport are presented. The chapter closes with an overview of the chapters of the thesis and outlines the studies presented.

1.2 Ligand Binding and Transport Overview

Lauffenburger and Linderman (1993) review major aspects of cellular events such as ligand surface binding and trafficking processes within the cell, and focus on how these events can be analyzed in a quantitative manner. The authors describe five major

approaches to distinguish cell surface binding events from subsequent receptor trafficking processes via endocytosis. Below is an overview of their analysis.

The first approach is to perform experiments at lowered temperature (4 – 15 ˚C). Trafficking processes appear to be drastically slowed at these temperatures allowing one to separate surface from intracellular events. For example, Tomoda *et. al.* (1989) examined endocytosis of ^{125}I-mannose-bovine serum albumin and exocytosis of ^{125}I-mannose-poly-D-lysine by rabbit alveolar macrophages as a function of temperature. The authors found that ligand degradation does not occur below 20 ˚C and internalization of surface-bound ^{125}I-mannose-BSA is negligible below 10 ˚C. The rate constant for internalization increased dramatically above 20 ˚C. A more recent study from Vinayek *et. al.* (1994) showed that the binding of ^{125}I-IGF-I to human esophageal epithelial cells was temperature dependent (52). This group found that binding at 37 ˚C was twofold lower than at 23 ˚C and fourfold lower than at 4 ˚C. This method of reduced temperature is a convenient and simple approach to use; however, there is still some trafficking of ligand that occurs at lowered temperatures that may have an effect on surface binding events.

The second approach is to use pharmacologic agents that inhibit trafficking events. Phenylarsine oxide (PAO) is one example that is thought to work by inhibiting internalization of cell surface receptors via its specific inhibitory effect on endocytosis (14). Endocytosis is an energy requiring process, and PAO affects this process by exerting reducing effects on cellular energy stores (14). For example, Low *et. al.* (1981) examined the interaction of protease-PN complexes with human fibroblasts using phenylarsine oxide to inhibit endocytosis (30). This group determined that PAO did not

affect cell surface binding and showed that the thrombin-protease-nexin (Th-PN) complexes must be internalized before they are degraded. They showed this by noting that the thrombin portion of cell bound ^{125}I-Th-PN was degraded to free amino acids by the fibroblasts. This was measured by determining the amount of free ^{125}I-Tyr in the medium. After 30 minutes of incubation, ^{125}I-Tyr release from untreated (no phenylarsine oxide treatment) fibroblasts was detectable, and further release occurred by 120 minutes of incubation. PAO-treated cells did not release ^{125}I-Tyr into the medium even after 120 min of incubation. Therefore, ^{125}I-Th-PN complexes must be internalized before they are degraded to amino acids. A drawback of this method arises from the fact that the inhibitory property of PAO is concentration and cell type specific. Gibson *et. al.* (1989) note that all cell types are similarly affected by PAO, however, its use as an inhibitor of endocytosis in other cell types should be preceded by careful evaluation of its effects on that cell's metabolism (14).

The third method is to use isolated membranes that possess receptors. The use of these membranes eliminates molecules that reside in the cytoplasm or on intracellular organelle membranes that may possess specific or nonspecific ligand binding and degradation sites on their membranes (23) that work to internalize the surface bound ligand. The binding obtained from these isolated membranes would not reflect the true dynamic nature (internalizing receptors and recycling them back to the surface) of cell surface binding because they lack the cytoplasmic elements that are needed in the regulation of cell surface binding (25). Furthermore, a given membrane preparation may not be representative of the total plasma membrane as the isolation procedure is a selection process in which it has been estimated that only 10-50% of the total plasma

membrane is obtained (23). However, these membranes do provide a simplified model of surface ligand binding as compared to whole cells because subsequent trafficking of ligand is not an issue.

The fourth method is to develop an experimental protocol that isolates particular steps by time scales or measurement procedure. For example, Wiley and Cunningham (1982) derived an equation that expressed the relationship between the endocytotic rate constant and the relative amount of surface-bound and internalized ligand as a function of time (54). This equation predicts that plotting the ratio of internalized ligand to surface-bound ligand, as a function of time will yield a straight line with a slope that corresponds to the rate constant. In order for this equation to be valid, the amount of surface-bound ligand must approximate a constant, there must be no degradation or loss of internalized ligand during the time course of measurement and the internalized ligand and surface bound ligand can be quantitatively discriminated from each other. The drawback of this approach is that several experiments are needed to validate the equation and its constraints and this method is specific to only one type of experimental protocol.

The fifth approach involves estimating all rate constants together with the use of a complete model and a comprehensive set of kinetic experiments. For example, Waters *et. al.* (1990) measured the kinetic parameters for interaction of epidermal growth factor with fetal rat lung cells under different experimental conditions (53). This group fit all six parameters: the association rate constant for binding of the ligand to the receptor, the dissociation rate constant of the ligand from the receptor, the endocytosis rate constant of ligand-receptor complexes, the hydrolysis rate constant for elimination of degraded ligand from the cells, the turnover rate constant for internalization of free receptors, and

the insertion rate of free receptors onto the cell surface as a set. This group applied sensitivity analysis to see which parameters were well defined. In the first set of experiments (method 1), internalization and dissociation kinetics of ^{125}I-EGF were measured under varied temperature conditions in ligand-free media. The second set of experiments (method 2) was carried out at a constant temperature and the initial free ligand concentration was varied. In both sets of experiments, surface-bound, internalized, and free ^{125}I-EGF was measured as functions of time. According to the authors, the values obtained for the association and endocytotic rate parameters from both methods agreed with other published data. However, their sensitivity analysis indicated that the other parameters (hydrolysis rate, turnover rate, and the insertion rate) couldn't be confidently measured by both experimental methods. A major disadvantage of this approach is that it is very time consuming due to the necessary validation of each rate expression (25).

Each method has merit but temperature is the simplest method for analyzing surface binding events and was the method used in this thesis. In order to use any of these methods, it is necessary to label the ligand itself so that quantification is possible. Lauffenburger and Linderman (1993) suggest four labeling methods: (1) radiolabeled and fluorescently labeled ligands, (2) avidin/biotin coupling, (3) electron dense markers and (4) labeling with small colloidal gold particles. The binding experiments in this thesis made use of radiolabeled ligands to quantify surface binding. Radiolabeling was the preferred labeling technique for this work because of the ease of detecting the label in samples as compared to other types of labeling and because Dr. R. Michael Akers, Dairy

Science, Virginia Tech, routinely uses the technique and generously provided his expertise for this work.

1.3 Competitive Binding Experiments

The goal of competitive binding experiments is to determine the specific amount of radiolabeled ligand bound to the cell surface and was a technique used extensively in this work. In these types of experiments, as shown in figure 1.1, a fixed amount of radiolabeled ligand in the presence of various concentrations of unlabeled material is incubated with the cells for a specified time period. This is followed by the removal of unbound ligand from cells via media aspiration and washes and then measuring the amount of radioactivity remaining in the cells or associated with the cells via counting of the cell lysate. For iodinated proteins such as those used in this thesis, detection using a gamma counter is typically done.

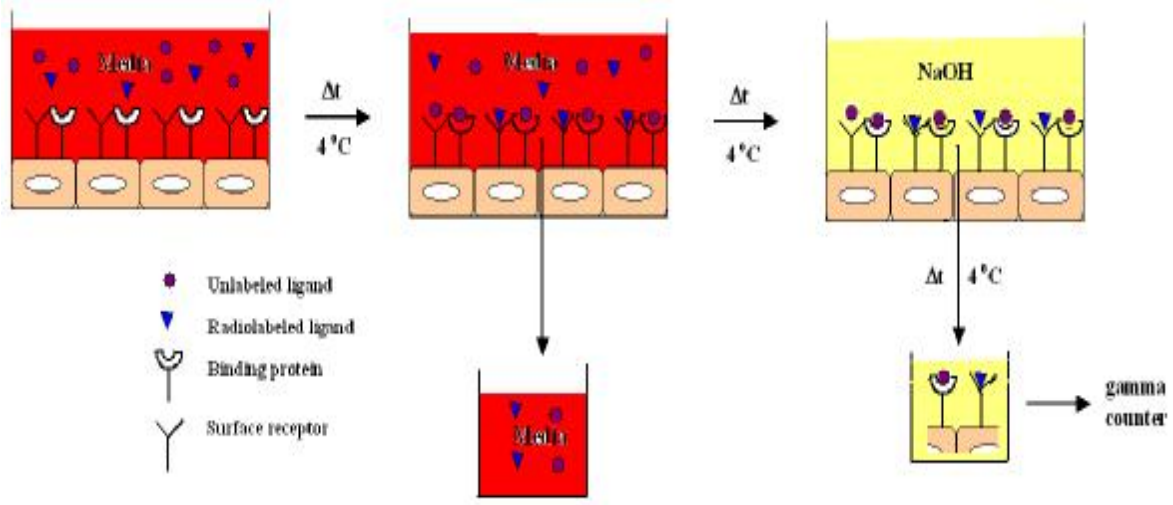

Fig. 1.1 Schematic of a competitive binding experiment. Cells are incubated with labeled ligand and unlabeled material for a specified incubation period. After this incubation period, the media containing unbound material is removed and NaOH is added. After a specified incubation period, the NaOH containing cell lysate is collected and counted using a gamma counter.

The desired result from these experiments is a determination of specific binding radiolabeled ligand to specific cell surface proteins. Specific binding is generally defined as the difference between total binding, the amount of radiolabeled ligand bound in the absence of excess unlabeled ligand, and non-specific binding, the amount of radiolabeled ligand bound in the presence of excess unlabeled ligand. Non-specific binding is caused by association of ligand with membrane constituents or by trapping of ligand in the medium associated with the cell (25, 49). Some of these non-specific sites may be other receptors from the same class or from a different class, surface proteins or the culture dish itself.

Measuring the amount of radiolabeled ligand in the presence of very high concentrations of unlabeled ligand concentrations that saturate receptor sites is the standard way to experimentally determine non-specific binding (25, 49). The idea is that with a fixed amount of radiolabeled ligand, as the concentration of unlabeled ligand increases in the system, the amount of binding of radiolabeled ligand to specific receptor sites decreases due to competition for sites (figure 1.2). A key assumption is that non-specific binding is essentially non-saturable and hence there is only competition for specific binding sites.

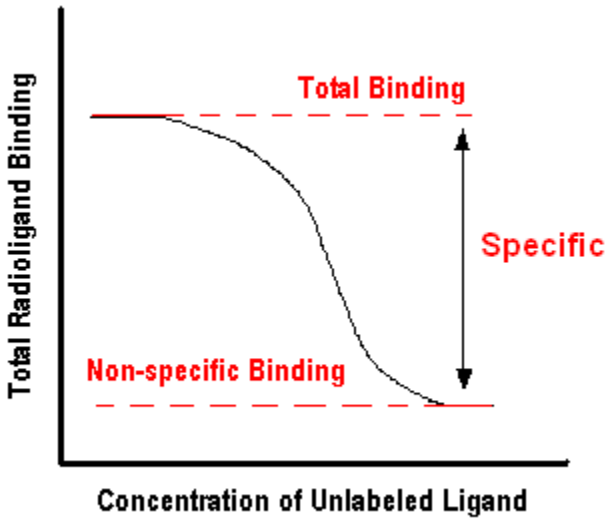

Fig. 1.2 Radiolabeled ligand binding as a function of unlabeled ligand concentration. Hypothetically, as the concentration of unlabeled ligand increases, the amount of binding of radiolabeled ligand to specific receptor sites decreases due to competition for sites.

1.4 Pulse-Chase Experiments

The pulse-chase experiments described in this thesis were designed to investigate radiolabeled ligand binding to cell monolayers and transported across these monolayers within two specific time periods – a period when ligand is added exogenously and a period when ligand in solution has been removed. In these studies, cells are incubated with radiolabeled ligand added to the apical chamber (figure 1.3) for a certain "pulse" time and then the unbound ligand is removed and cells are maintained for a "chase" period. The key feature is that available ligand in the apical chamber for transport during the chase period comes from ligand bound to the cell surface. These types of experiments were used to determine the amount of ^{125}I-IGF-I transported across BAE and Mac-T cell monolayers and is diagramed in figure 1.4.

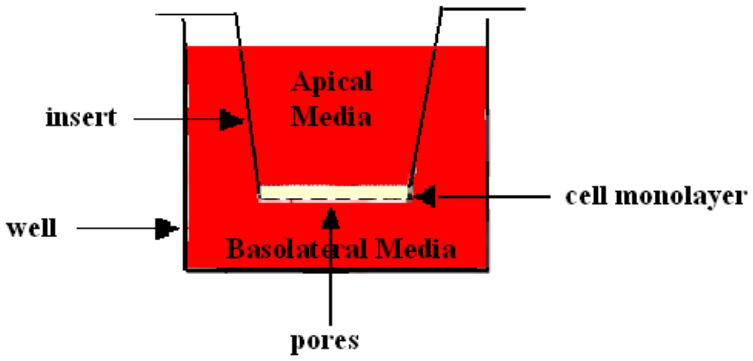

Fig. 1.3 Schematic of a Transwell® plate. Cells are seeded on the porous insert and media is added to the apical and basolateral chambers.

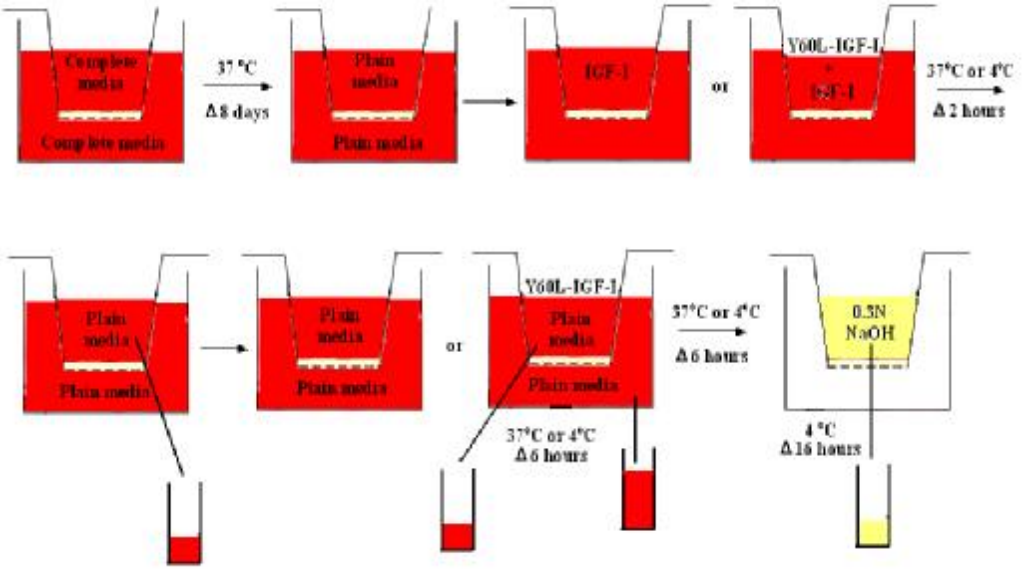

Fig. 1.4 Schematic of a Pulse Experiment. Cells are seeded on the porous insert and maintained in culture for 8 days before initiating the experiment by switching to serum-free media. Radiolabeled ligand is added to the media in the apical chamber and unlabeled material, when appropriate, is added to the apical and basolateral chamber. Cells are incubated with the labeled and unlabeled material for a certain pulse time before collecting the apical and basolateral media. Fresh serum-free media is added to replace the collected material and cells are incubated again for a certain chase period before collecting apical and basolateral media. NaOH is added to the apical chamber to collect cell lysate at the completion of the study. Δ denotes the time period between steps, which were done at 4 ˚C or 37 ˚C.

1.5 Transcellular and Paracellular Transport

Sheets of epithelial cells line all body cavities and the skin (2). Epithelial cells frequently transport ions or small molecules from one side to the other. For example, cells lining the small intestine transport products of digestion into the blood and those that line the stomach secrete hydrochloric acid into the stomach lumen. The plasma membranes are organized into the apical and basolateral regions that allow epithelial cells to carry out their oriented transport functions. Also, specialized regions of the plasma membrane connect adjacent epithelial cells to impart strength and rigidity to the cell-cell junctions. These specialized regions also serve to prevent water and other solutes on one side from freely moving between the cells to the other side.

Epithelial cell transport occurs via the transcellular and paracellular pathways (figure 1.5). Transcellular transport is an active transport pathway that relies on cell-mediated uptake, sorting, and delivery (2). A specific receptor on the cell surface binds to the ligand that it recognizes and the receptor-ligand complex is internalized into the cell where it can be recycled back to the cell surface, in the lysosomes or to the other side of the cell in a process called transcytosis (28). Paracellular transport is a passive transport process in which molecules pass between adjacent cells (2). Cells transiently alter their tight junctions in order to allow solutes and water to pass through the space in between adjacent cells. This transport is important in the absorption of amino acids and monosaccharides from the lumen of the intestine, where their concentration is high enough to drive passive transport.

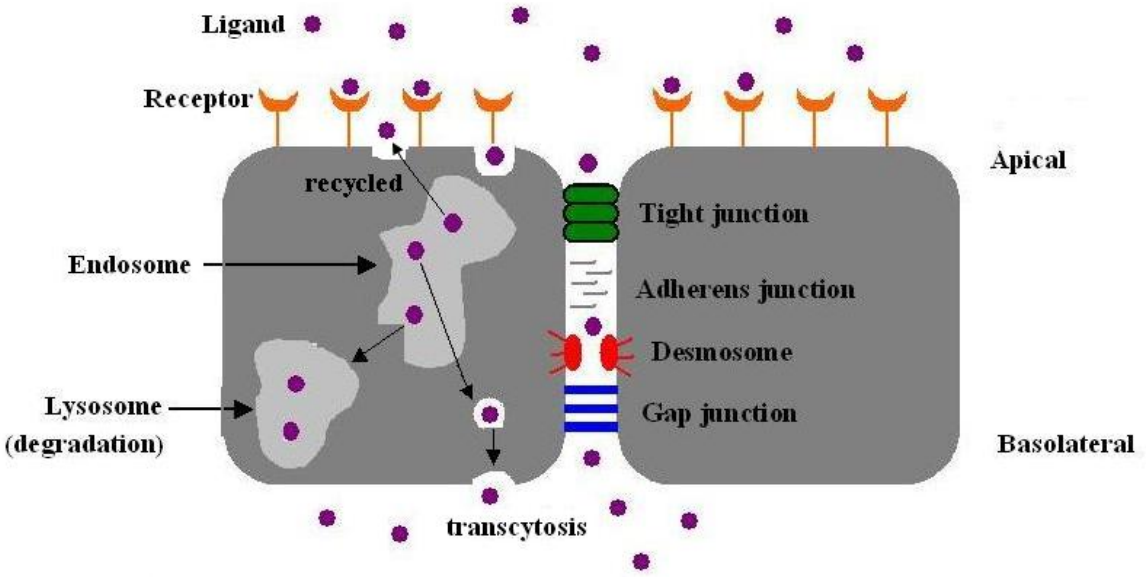

Fig. 1.5 Schematic of Transcellular and Paracellular Transport (Adapted from *Molecular Biology of the Cell, 4th Ed.*) In transcellular transport, an extracellular ligand is imported from one side of the cell (endocytosis) and transported to the other where it is secreted (exocytosis). In paracellular transport, cells alter their tight junctions in order to allow solutes and water through the space between cells.

Degradation analysis by trichloroacetic acid (TCA) and phosphotungstic acid (PTA) precipitation, is one assay that can distinguish between transcellular and paracellular transport. The TCA/PTA soluble material contains protein that was degraded via the transcellular pathway while the TCA/PTA precipitable material contains protein that was not degraded and might be transported via either pathway. The transcellular material would more likely be degraded than the paracellular material because ligand transported via the transcellular pathway is internalized into the cell and might encounter the lysosomes. Ligands that are transported via the paracellular pathway are not internalized into the cell, and therefore would not become degraded by lysosomes. However, some ligand degradation may occur with the paracellular pathway. Cells locally secrete extracellular proteolytic enzymes in order to degrade matrix

components to clear a path through the extracellular matrix and as a requirement for cells to migrate through a basal lamina (2).

1.6 Overview of Thesis

Each chapter is opened with an introduction outlining its contents. Next, a background section reviews relevant literature to the study. The experimental procedures section details the materials and methods used. The results and discussion section presents the results of each experiment and contains an in depth explanation of the results obtained. The chapter is closed with a conclusion and future work section that summarizes the chapter and gives recommendations for future work.

In particular, the second chapter of the thesis focuses on FIX interactions with BAECs and extracellular proteins. Competitive binding experiments between ^{125}I-FIX and FIX were undertaken to quantify the interaction of recombinant and transgenic FIX and determine if there was a measurable difference in binding affinity. The hypothesis was that the transgenic FIX has different binding characteristics from the recombinant FIX due to differences in the glycosylation of the molecules due to their cell source. The transgenic produced material was hypothesized to bind better or have more binding sites and therefore, ultimately exhibit better *in vivo* characteristics. BAECs were used as an economical alternative to human aortic endothelial cells and are a suitable model system for the studies of endothelial function due to these cells retaining non-transformed phenotypes of endothelial cells. Human collagen IV was reported to be a strong candidate for the FIX binding site on BAECs (10). The experiments utilizing collagen were used as an additional assay for quantifying recombinant and transgenic FIX binding to determine measurable differences in binding affinity.

The third chapter focuses on cell surface binding and transport processes of IGF-I. Competitive binding experiments between ^{125}I-IGF-I and unlabeled protein (IGF-I, Y60L-IGF-I and IGFBP-3) were undertaken to quantify the binding and transport of IGF-I to BAECs and Mac-T cells. The experiments presented in this chapter look at how IGF-I binding and transport are affected by certain experimental conditions. Specifically temperature, cell growth period, exogenous binding protein addition, surface binding components and exposure time (pulse-chase) were addressed. The hypothesis is that IGFBPs on the cell surface retain IGF-I on the apical surface during the pulse period, thereby reducing IGF-I transport during that period. However, during the chase period, increased IGF-I in the apical chamber due to dissociation from IGFBPs could lead to increased transport during the chase period. Mac-T cells were also used because they form a better barrier to transport and should have a lower paracellular contribution to the process (33).

The thesis is closed with a conclusions and future work chapter. This chapter summarizes the studies in this thesis and provides recommendations for future work.

Chapter 2: Attempts to Quantify Binding of ^{125}I-Factor IX to Both Cultured Bovine Aortic Endothelial Cells and Human Collagen Type IV

2.1 Introduction

This chapter focuses on studies attempting to quantify factor IX (FIX) binding to bovine aortic endothelial cells (BAECs) and human collagen IV. First, a brief introduction of the properties and characteristics of endothelial cells, FIX and Hemophilia B followed by the purpose of this project is given. Second, the experimental procedures detailing the materials and methods used in this project are given. Next, the results and a discussion of this project are presented. The chapter is closed with conclusions and recommendations for future work.

2.2 Background

2.2.1 *Endothelial Cells*

Endothelial cells form the luminal vascular surface separating the blood flow from the underlying smooth muscle cells (figure 2.1). They line the entire vascular system and control the passage of materials and the transit of white blood cells, into and out of the bloodstream (2). They are also active in maintaining compatibility between the blood and the vessel wall (18). Endothelial cells have the ability to sense shear stress from blood flowing over their surface by mechanoreceptors and signaling this information to surrounding cells (2). By doing this, they enable the blood vessel to adapt its diameter and wall thickness to suit the blood flow and maintain compatibility between the blood and the vessel wall.

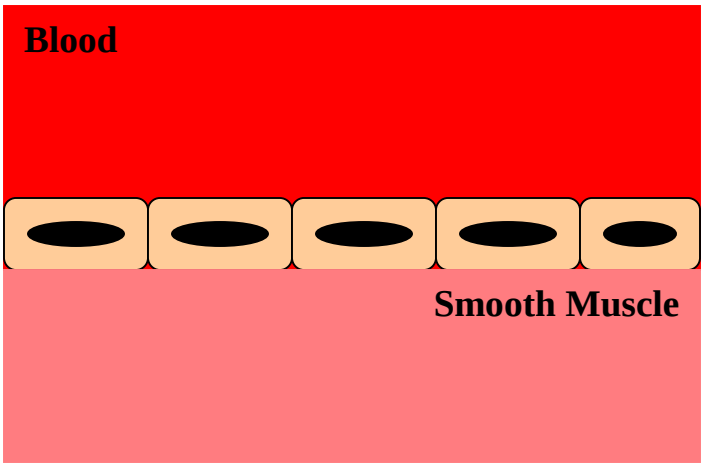

Fig. 2.1. Endothelial cells separate blood flow from the underlying tissue

Localized blood coagulation plays an important role in hemostasis (44, 45). Endothelial cells have the ability to both inhibit and support coagulation. Hemostasis, arrest of bleeding, and thrombosis, blood clot formation, is inhibited by secretion of prostacyclin (an inhibitor of platelet aggregation), plasminogen activator (generates plasmin) and thrombomodulin, which binds to thrombin and alters its substrate specificity. Coagulation is initiated by the generation of two tissue factors: von Willebrand factor, which is involved in platelet adhesion, and thromboxane, an inducer of platelet aggregation.

For the studies in this chapter, BAECs, isolated and cryopreserved at first passage, were purchased from Clonetics (Walkersville, MD) and used between passage 11 and 13. These cells are an economical alternative to human aortic endothelial cell systems and suitable for the studies of endothelial function and endothelial metabolism (7). BAECs are a suitable alternative because they retain non-transformed phenotypes of endothelial cells.

2.2.2 *Factor IX*

Factor IX and its activated form FIXa have been found to bind to confluent cultured bovine aortic endothelial cells (18, 44). Factor IX (Christmas factor) is the zymogen, inactive precursor of an enzyme, of a serine protease active in normal hemostasis (10, 27). The gene is 34 kilobases in size and is comprised of eight exons and seven introns (38). The functional domains (from amino to carboxyl terminus) are: the vitamin K dependent domain containing γ-carboxyglutamic acid (Gla) residues, two epidermal growth factor-like (EGF) domains, an activation peptide region (AP) and the catalytic domain (CD) which confers the protease function (9, 10, 27) (figure 2.2). Gla residues consist of approximately the first 40 amino acids of FIX. There are 12 of these residues that are thought to be responsible for the calcium-mediated binding of this coagulation factor to phospholipids or platelets.

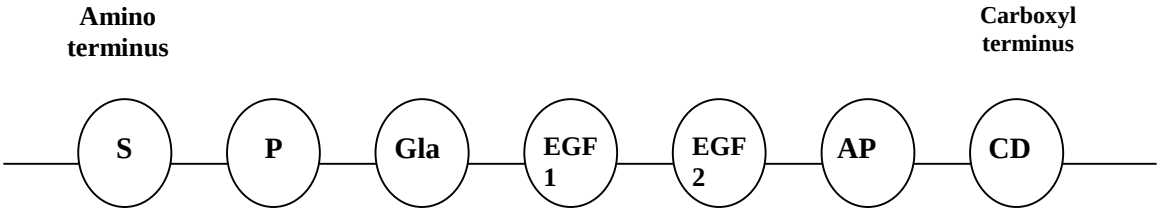

Fig. 2.2. Schematic of FIX functional domains in the protein. 'S' denotes the signal peptide and 'P' denotes the propeptide.

FIX and its activated form FIXa are blood coagulation proteins that bind to a specific receptor shown to be a protein of about 140 kDa on the surface of endothelial cells (9). This binding is calcium dependent, saturable, and reversible. Cheung *et. al.* (1996) reports that collagen IV is a strong candidate for the FIX binding site on endothelial cells (10). Collagen IV is an extracellular matrix protein (ECM) and is one of

the major components of the basement membrane of endothelial cells. Toomey *et. al.* (1992) suggest that the FIX Gla domain mediates the high affinity interaction between FIX and its specific receptor on the surface of endothelial cells. Residues 3-11 of the Gla domain mediate this binding (8). Stern *et. al.* (1983) determined that FIXa retains its procoagulant activity when bound to cultured vascular endothelial cells. This provides a mechanism for the localization of clot promoting activity.

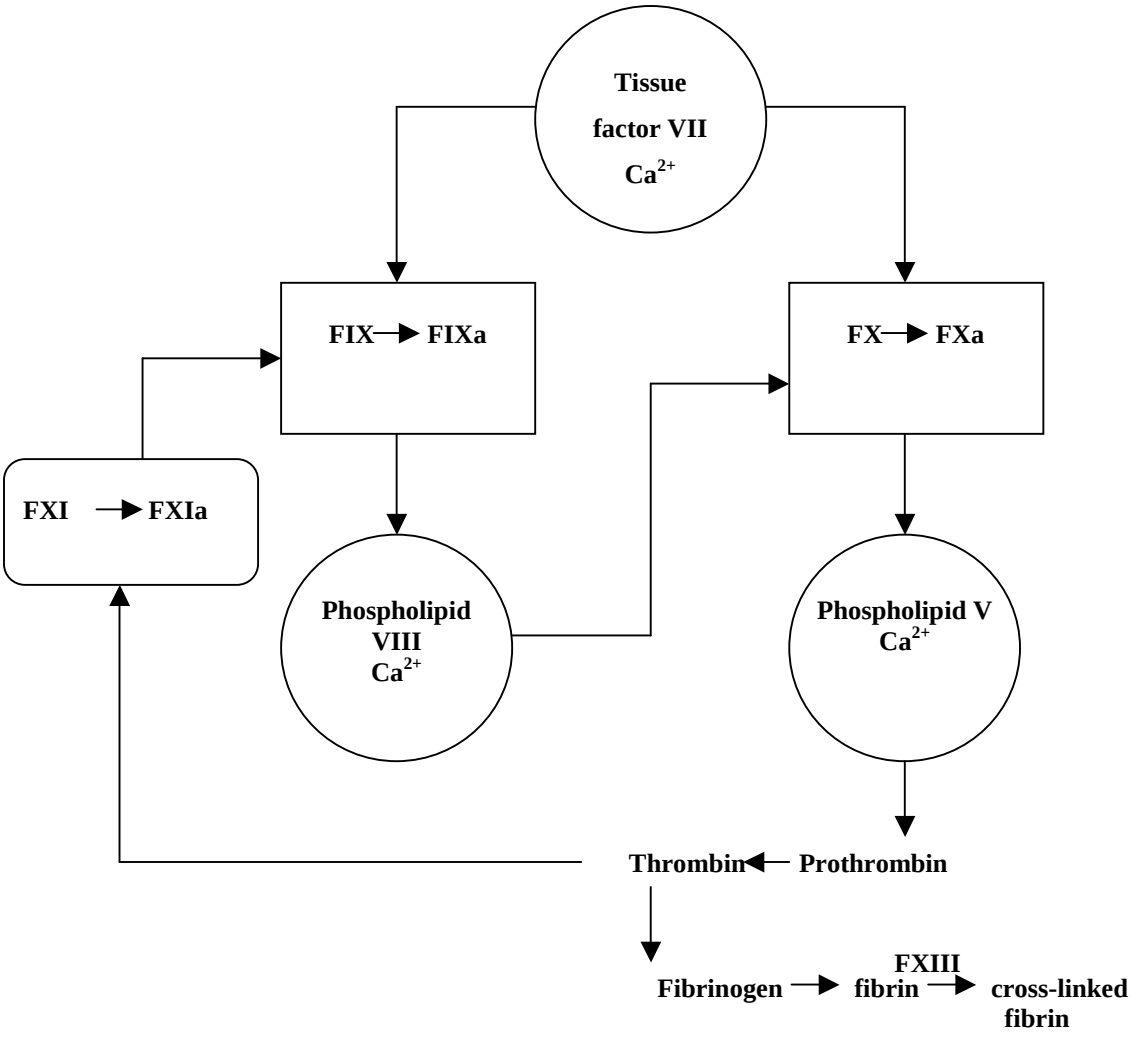

Fig. 2.3. The Coagulation Pathway. Coagulation is initiated when tissue factor is exposed to Factor VII in the presence of calcium. This pathway leads to blood clot formation when monomers of fibrin polymerize to form the fibrin clot that is stabilized by factor XIII.

FIX and FIXa are part of the coagulation pathway (figure 2.3). The end result of this pathway is blood clot formation. In this process, coagulation is initiated when tissue factor, bound to cell membrane of vascular cells, is exposed to Factor VII, localized in blood plasma, in the presence of calcium (41). The activated tissue factor-Factor VII complex activates Factors IX and X to form FIXa and FXa respectively. FIXa production is enhanced by activation of Factor XI. FXI is activated by thrombin to enhance its (thrombin) own generation. FIXa enhances the production of FXa in the presence of Factor VIII and Ca^{2+}. Factor Xa then converts prothrombin to thrombin (Factor IIa). Thrombin cleaves fibrinogen that yields monomers of fibrin, which then polymerizes to form the fibrin clot. Factor XIII, activated by thrombin and Ca^{2+}, stabilizes this clot by forming covalent bonds between the fibrin molecules. Inadequate activity of FIX results in failure of normal coagulation, which leads to the bleeding disease Hemophilia B.

2.2.3 *Hemophilia B*

Hemophilia B is a hereditary hemorrhagic disorder that is inherited as an X-linked recessive characteristic (38). It is the oldest known hereditary disorder that primarily affects males (11). Women rarely have this disease but can be carriers and pass it on to their children. Normally when bleeding occurs, clotting factors in the blood help stop the bleeding. For people with hemophilia B, this mechanism is disrupted and bleeding lasts longer.

Mutations in the FIX gene result in hemophilia B (10). The disease occurs in mild, moderate and severe forms that result from absent or defective FIX molecules (8, 9, 38). The level of clotting factor in the blood determines the severity of the disease (11).

Those with severe hemophilia may bleed internally frequently and without apparent reason. Milder forms of the disease result in rare bleeding episodes. Moderate and severe hemophilia B most likely result from independent mutations of FIX while mild hemophilia B is a result of the presence of an inherited gene variant (38). Roberts (1993) reports that mutations in the signal peptide region can lead to either a mild or a severe form of the disease, mutations in the propeptide region can lead to moderate to severe forms while mutations in the Gla domain lead to severe hemophilia B.

Although there is no known cure for this disease, intravenous replacement therapy of plasma derived (pd-FIX) or recombinant FIX (BeneFIX®) is used to treat bleeding episodes (51). Unfortunately, FIX is in extremely short supply worldwide (32). Its complex structure requires that it be made by recombinant means only in select animal cells (50). Production of recombinant human FIX is confounded by low productivity that leads to high costs, making safe and efficacious treatment nearly impossible (51).

2.2.4 *Purpose of Project*

Research originated at Virginia Tech in the laboratory of Dr. William Velander has resulted in the development of an abundant and economical source of recombinant FIX produced in the milk of transgenic animal bioreactors, hereafter referred to as transgenic FIX (tFIX) (51). The purpose of this project was to quantify the interaction of recombinant (BeneFIX®) and transgenic FIX with endothelial cells and collagen IV to determine if there is a measurable difference in binding affinity. The hypothesis is that the tFIX has different binding characteristics from the BeneFIX® and that the transgenic produced material will exhibit slower dissociation from cells/collagen resulting in longer retention times within the bloodstream. Longer retention time in the bloodstream might

make it possible to use lower doses and less frequent IV infusions of the FIX. From preliminary studies in the laboratory of Dr. Kevin Van Cott, formerly at Virginia Tech and now at the University of Nebraska, it was suggested that the transgenic FIX has a different Gla domain structure than the plasma-derived FIX (Van Cott *et.al. J Chromatogr*, submitted). The Gla domain is the portion of the FIX molecule reported to regulate binding to vascular endothelial cells (48).

2.3 Experimental Procedures

2.3.1 *Materials*

Bovine aortic endothelial cells were purchased from Clonetics (Walkersville, MD) and used between passage 11 and 13. Collagen from human placenta, type IV cell culture tested and BioChemika type IV, Blue dextran, Alexin™, Calcium Chloride and Sepharose CL-6B gel were purchased from Sigma (St Louis, MO). Normal reference plasma and Factor IX deficient plasma were gifts from ProGenetics LLC (Virginia Tech Corporate Research Center Blacksburg, VA). Human collagen IV from placenta was purchased from BD Biosciences Discovery Labware (Franklin Lakes, NJ). Bovine serum albumin (BSA) Fraction V was purchased from Fisher Scientific (Pittsburgh, PA). Dulbecco's modified Eagle's medium (DMEM) and trypsin/EDTA were purchased from Gibco (Grand Island, NY). Dulbecco's phosphate buffered saline (DPBS), penicillin/streptomycin and L-glutamine were purchased from Mediatech (Herndon, VA). Bovine calf serum was purchased from Hyclone (Logan, UT). Iodobeads® were purchased from Pierce (Rockford, IL). Cell culture dishes (100mm x 20mm), 96 removable well and 12 and 24 well plates were purchased from Corning (Cambridge, MA). Heparin Sodium USP (03003) was purchased from Celsus Laboratories Inc.

(Cincinnati, OH). Sephacryl S200 HR gel was purchased from Amersham Biosciences (Piscataway, NJ**)**. BeneFIX® from Chinese Hamster Ovary Cells (CHO), referred to as recombinant factor IX (rFIX) in this thesis, and transgenic factor IX (tFIX) produced in transgenic pig mammary glands were gifts from Professors William Velander and Kevin Van Cott of the University of Nebraska - Lincoln.

2.3.2 *Methods*

Cell Culture – BAECs were seeded at 1 x 10^6 cells/cell culture dish and grown in DMEM containing 10% calf serum, 1% penicillin/streptomycin and 1% L-glutamine. Growth media was changed every 2-3 days after washing with DPBS and subcultured every 4-5 days using 3ml/dish of trypsin/EDTA.

Iodination of Protein – rFIX (5μg) and tFIX (5μg) were first labeled in the laboratory of Dr. R. Michael Akers using the chloramine T method as described previously (1). Subsequent studies used proteins (5μg each) labeled via Iodobeads® according to the manufacturer's instructions (Pierce) also in Dr. Akers' laboratory. For the chloramine T method, the labeling resulted in solutions of rFIX at a concentration of 0.044μCi/μg and 0.022μCi/μg for the tFIX. Using Iodobeads®, the labeling resulted in solutions of the rFIX were 0.014μCi/μg (first iodination) and 0.01μCi/μg (second iodination). The labeling resulted in solutions of the tFIX were 0.022μCi/μg (first iodination) and 0.044μCi/μg (second iodination). The final volumes for each labeling method were 3.5ml.

Biological (Clotting) Assay – The clotting activity of radiolabeled FIX was determined using a MLA™ Electra® 750A Coagulation Timer (Medical Laboratory Automation Inc. Pleasantville, NY) after each iodination. Normal reference plasma

(NRP), lot #113029, was reconstituted in deionized water (1ml) for 15 minutes at room temperature. Factor IX deficient plasma (F9-D), lot #113027, was reconstituted in deionized water (1ml) for 30 minutes at room temperature. Alexin™ (aPTT reagent) and Calcium Chloride solutions (20mM) were warmed for 15 minutes at 37 ˚C in the coagulation timer apparatus. The reconstituted NRP was diluted (1:5, 1:10, 1:40, 1:80) in imidazole buffer (50mM Hepes, 100mM NaCl, 0.1% BSA pH 7.4) and coagulation times for these dilutions were measured and used to construct a standard curve. Dilutions in imidazole buffer of the radiolabeled and unlabeled FIX samples were made to correspond to the clotting times of the 1:10 and 1:40 NRP dilutions from the standard curve. aPTT reagent, F9-D and FIX sample (100µl each) were placed in a coagulation cuvette and incubated at 37 ˚C for 3 minutes in the coagulation apparatus. The cuvette was then placed in the coagulation timer slot to reset the timer and pre-warmed $CaCl_2$ solution (100µl) was added to the cuvette. The time for clot formation was measured by the coagulation timer and recorded. Triplicate readings were taken for each sample.

Binding of Factor IX to Intact BAECs – This procedure was adapted from previous work by Cheung ***et. al***. (1991). BAECs were seeded at 1.5 x 10^5 cells/cm^2 in a 24 well plate or seeded at 2x10^5 cells/cm^2 in a 12 well plate and allowed to grow in DMEM containing 10% calf serum, 1% penicillin/streptomycin and 1% L-glutamine for 48 hours at 37 ˚C and 5% CO_2. The cells were then washed four times with buffer A, 0.5ml, (0.01M HEPES, pH 7.5, 0.137M NaCl, 0.004M KCl, 0.011M glucose) and incubated in incubation buffer, 0.5ml, (buffer A containing 2mg/ml BSA and 0.002M $CaCl_2$) for 15 minutes at 4 ˚C. ^{125}I-FIX (14ng) and various amounts (100, 200, 400, 500 and 800ng) of unlabeled FIX were added to the wells and allowed to incubate for 24

hours at 4 ˚C. The wells were then washed three times with cold wash buffer, 0.5ml, (0.005M Tris-HCl, pH 7.5, 0.14M NaCl, 0.002M $CaCl_2$, 2mg/ml BSA) and cells were collected in dissolving solution, 0.5ml, (0.2M NaOH, 0.01M EDTA, 1% SDS) for at least one hour at room temperature. The contents of each well were counted using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL).

Binding of Factor IX to Collagen Type IV (Cell-free assay) - This procedure was adapted from previous work by Cheung ***et. al***. (1996). Cell culture tested and BioChemika type IV collagens were dissolved in acetic acid according to manufacturer's instructions. The stock collagen IV (cell culture tested was at 2mg/ml and BioChemika was at 5mg/ml) was then dissolved in TBS (10mM Tris-HCl, 150mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, pH 8) for a final concentration of 50µg/ml. Removable wells from a 96 well plate were coated with the collagen solutions, 0.1ml, overnight at 4 ˚C. The wells were washed three times with TBS and blocked with 3% BSA in TBS for three hours at room temperature. The wells were then washed three times with TBS after the three-hour blocking period. Six wells did not receive this additional washing step and blocking solution, 0.1ml, remained in these wells. ^{125}I-FIX and various amounts (3 and 100ng) of unlabeled FIX in the presence (1µg/ml final concentration) or absence of heparin were added to the wells. The final total volumes were 0.1ml in the wells with the additional washing step and 0.2ml for the wells without this step. The wells were allowed to incubate for 2.5 hours at 4 ˚C and then washed six times with TBS (0.1ml). Wells were then separated and bound radioactive material in the entire well was measured using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL). Blank wells were counted as a control.

Statistical Analysis – Statistical comparisons were done by two-tailed t-tests in Microsoft Excel® with significance defined as $p < 0.05$.

2.4 Results and Discussion

2.4.1 *Binding of Factor IX to Intact BAECs (Chloramine T labeled)*

The goal of the binding experiments was to determine if there was a difference in binding properties of rFIX and tFIX that ultimately might impact *in vivo* retention in the vasculature. To address this question competitive binding studies were performed to determine if there (1) was specific binding of the proteins and (2) if the binding was higher or lower on a mass basis for the two proteins. To detect this binding, the proteins were radiolabeled using the standard chloramine T method done in the laboratory of Dr. R. M. Akers at Virginia Tech. Unfortunately, the initial studies revealed technical problems that proved difficult to overcome.

For this experiment, the treatments (in triplicate wells) were added to plated cells for both the recombinant and transgenic FIX. The first treatment was ^{125}I-FIX (8ng/ml) alone in order to determine the overall (total) binding to BAECs. The second and third treatments were ^{125}I-FIX (8ng/ml) and unlabeled FIX (1600 or 800 ng/ml) in order to (1) show that the unlabeled FIX would decrease total binding of ^{125}I-FIX and to (2) determine the level of non-specific binding, and hence specific binding. The fourth treatment was the same as the first treatment to check for plating (number of cells per well) and procedural (addition of unlabeled and labeled FIX) accuracy. The concentrations of ^{125}I-FIX for each treatment were based on the assumption of no loss of protein during the labeling process.

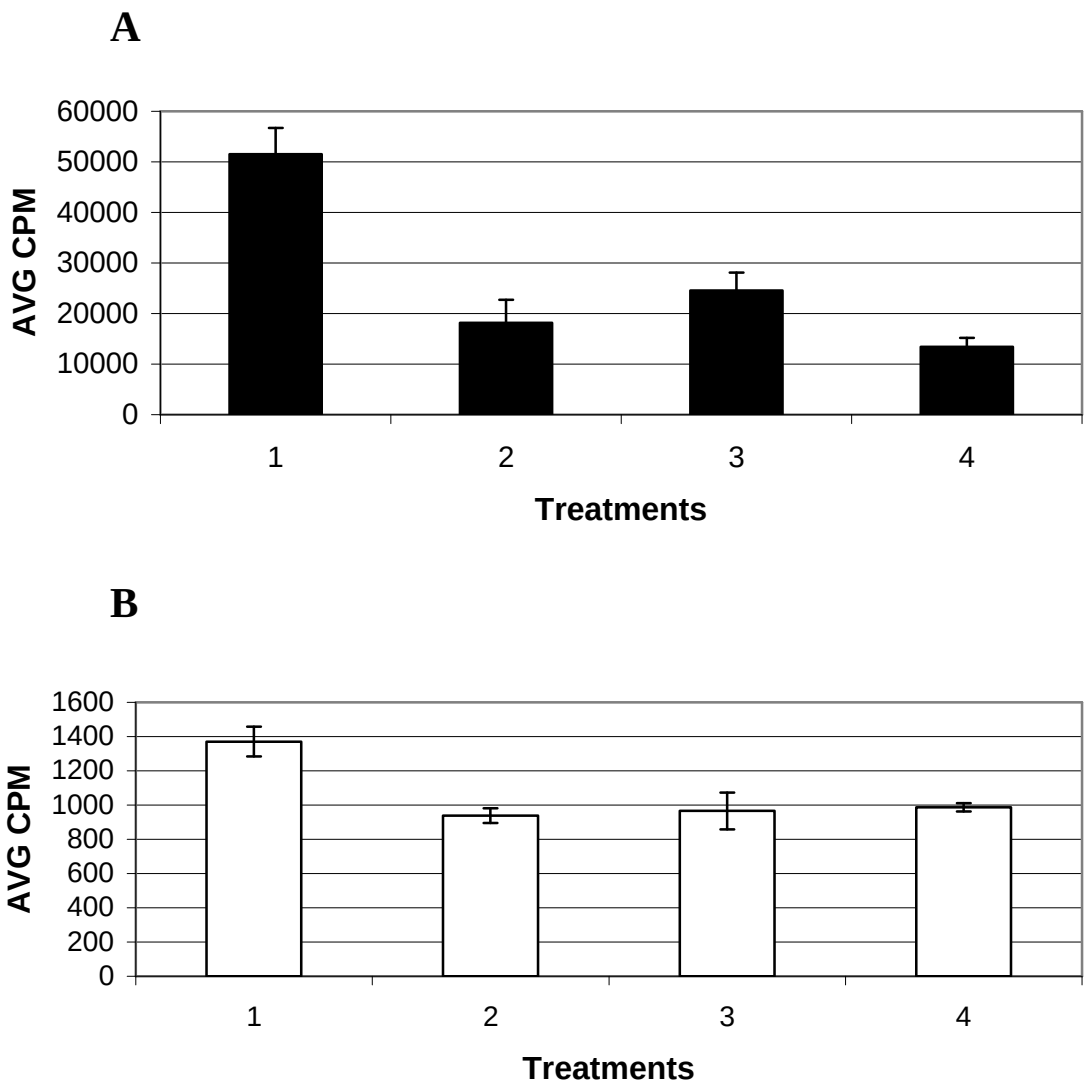

Fig. 2.4. Binding of ^{125}I-rFIX (■) and ^{125}I-tFIX (□) to intact cultured BAECs (Chloramine T). ^{125}I-FIX (8ng/ml) incubated alone (bars 1 and 4) and with FIX (1600 and 800 ng/ml respectively bars 2 and 3). Measurements (mean ± S.D., n = 3) are representative of 2 rFIX and 1 tFIX independent binding experiments.

As shown in figure 2.4, both the rFIX and tFIX bound to the intact cultured BAECs. Unfortunately large differences were found in the duplicate wells meant to check for plating and treatment accuracy (figure 2.4 bars 1 and 4). While reduction in labeled FIX binding was found in the presence of unlabeled FIX (comparing bar 1 with

bars 2 and 3 in figure 2.4) suggesting specific binding was present, the accuracy check results made conclusions suspect. However, this data does show that rFIX (0.59ng ± 0.06) had more total bound to BAECs than the tFIX (0.05ng ± 0.003). For this set of experiments, 1ng ^{125}I-rFIX corresponded to 86,824 CPM and 1ng ^{125}I-tFIX corresponded to 27,927 CPM. This explains the large difference in binding levels (y-axis CPM values) shown in figure 2.4.

2.4.2 *Biological (Clotting) Assay*

Results from the preliminary binding experiments with chloramine T radiolabeled FIX (section 2.4.1) were suspect based on poor reproducibility between duplicate sets of samples (figure 2.4). While this likely reflected issues with plating of cells due to human error, the level of specific binding also seemed quite low compared to values found in Cheung *et. al*. (1996). This suggested that there might be issues with protein activity given that Cheung *et. al*. used Iodobeads® for labeling instead of chloramine T. The biological activity of the chloramine T radiolabeled and unlabeled FIX was therefore checked using the aPTT coagulation time assay as previously described in the Methods Section (section 2.3.2). The objective of this assay was to obtain clotting times (seconds) for normal reference plasma and radiolabeled and unlabeled FIX stock material. The clotting times of the normal reference plasma (NRP) were then used to create a standard curve to compare the clotting times of the stock FIX to the clotting times of the normal reference plasma. The standard curve was used to determine (1) whether the unlabeled FIX stock material was biologically active (2) whether the radiolabeled FIX maintained its biological activity after radiolabeling and (3) the number of units of FIX added. The

number of units was used to calculate the specific activity, amount of biological activity obtained from the clotting assay, of the radiolabeled FIX.

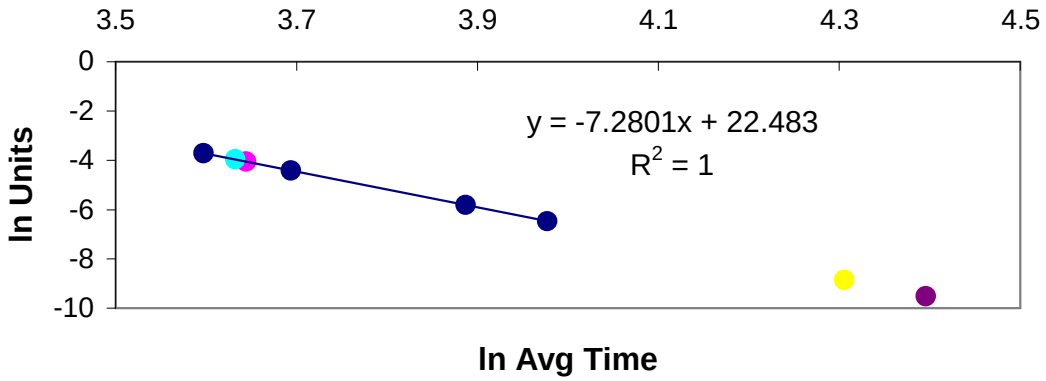

Fig. 2.5. Normal reference plasma standard curve (Chloramine T). A comparison of Chloramine T labeled proteins with unlabeled material. Shown are the clotting units vs. average clotting time (seconds) for NRP (•), unlabeled rFIX (0.5ng/µl) (•), unlabeled tFIX (0.5ng/µl) (•), labeled rFIX (0.5ng/µl) (•) and labeled tFIX (0.5ng/µl) (•) on a log-log plot (natural log). Equation shown is fitted to the standard curve. Labeled and unlabeled FIX was diluted in imidazole buffer to correspond to NRP dilutions. Measurements (mean, n = 2) are representative of 1 independent clotting assay.

As shown in figure 2.5, FIX labeled using the chloramine T method had clotting times (4.3 for the ^{125}I-rFIX and 4.4 for the ^{125}I-tFIX) that were well outside of the standard curve of NRP (times fell between 3.6 and 4.0) on a natural log scale. In contrast, unlabeled rFIX had a clotting value of 3.64 and the unlabeled tFIX had a clotting value of 3.63. This result indicated that the labeled FIX had negligible clotting activity relative to the NRP and unlabeled FIX clotting values. The protein may have been degraded or deactivated during the chloramine T radiolabeling process as chloramine T is known to be an extremely strong oxidizing reagent (35). Most likely, there was a large loss of protein during the radiolabeling process. It should be noted that

equal amounts (100ng) of labeled and unlabeled FIX were used in this assay. Labeled FIX amounts were calculated based on the amount (mass) of starting material used for radiolabeling and the volume returned after radiolabeling assuming there was no loss of material during the radiolabeling process occurred.

To circumvent this problem, FIX was then radiolabeled using Iodobeads® as in previous work (9, 10, 48) and its biological activity was checked as well using the clotting assay. The Iodobeads® radiolabeling process is a more gentle method for iodination than soluble chloramine T because there is no contact between the protein and the immobilized oxidizing agent (35). Also, simply removing beads from the reaction mixture stops the reaction; no reducing agent is necessary to terminate the reaction. For this clotting assay, the biological activity of unlabeled FIX was not checked in order to conserve limited stock material.

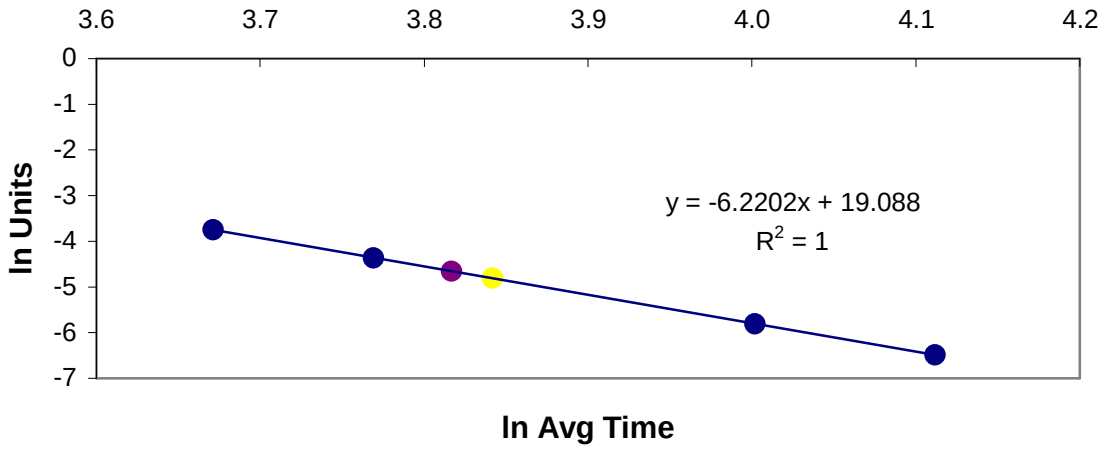

Fig. 2.6. Normal reference plasma standard curve (Iodobeads®). A comparison of Iodobeads® labeled proteins with unlabeled material. Shown are the clotting units vs. average clotting time (seconds) for NRP (•), labeled rFIX (0.5ng/µl) (•) and labeled tFIX (0.5ng/µl) (•) on a log-log plot (natural log). Equation shown is fitted to the standard curve. Labeled and unlabeled FIX was diluted in imidazole buffer to correspond to NRP dilutions. Measurements (mean, n = 2) are representative of 2 independent clotting assays.

As shown in figure 2.6, the Iodobeads® labeled FIX had clotting times that fell within the standard curve. This result showed that the FIX maintained its biological activity after radiolabeling with Iodobeads®. The specific activity, amount of biological activity per volume obtained from the clotting assay, was then calculated for both the rFIX and the tFIX.

0.0085 units in 100µl	obtained from standard curve
$\frac{100\mu l}{313\mu l} = \frac{x\ \mu l}{100\mu l}$	100µl of ^{125}I rFIX stock added to 213µl assay buffer
x = 31.9µl	
$\frac{0.0085\ units}{31.9\mu l} = 2.66e\text{-}4\ units/\mu l$	
2.66e-4 units/µl x 3.5ml = .933 units total	3.5ml is total volume of ^{125}I rFIX stock
$\frac{0.5\mu g/\mu l}{0.279\ units/\mu l} = 1790\ ng/unit$	0.5µg/µl is the cold rFIX stock conc. 0.279 units/µl came from the unlabeled rFIX value from the standard curve
1790 ng/unit x 2.66e-4 units/µl = 0.476 ng/µl	

Fig. 2.7. Sample specific activity calculation for ^{125}I-rFIX labeled by Iodobeads®.

To calculate the specific activity, the number of units of the radiolabeled and unlabeled FIX was determined from the standard curve (figure 2.6). The number of units was then divided by the volume of radiolabeled and unlabeled FIX used in the assay to obtain units/µl. The known concentration of the unlabeled FIX (µg/µl) was divided by the unlabeled FIX units/µl value to obtain µg/unit. This µg/unit value was then multiplied by the units/µl-value of the radiolabeled FIX to obtain µg/µl and converted to ng/µl. A sample set of calculations is shown in figure 2.7.

Table 2.1. Specific Activity of Radiolabeled FIX

Sample	Labeling Method		
	Chloramine T (ng/µl)	Iodobeads 1 (ng/µl)	Iodobeads 2 (ng/µl)
rFIX	no value	0.6	0.48
tFIX	no value	0.6	0.11

Chloramine-T labeled FIX had no biological activity as compared to the NRP and unlabeled FIX and therefore its specific activity was not calculated. ^{125}I-FIX maintained its biological activity after radiolabeling with Iodobeads®, however, very low yields (percentage of active FIX left after radiolabeling) of product were obtained from the labeling process. The rFIX had a yield of 42% and 34% for the first and second iodinations while the tFIX yields were 42% and 7.7% respectively. This might be due to loss of product during this labeling technique when separating the radiolabeled protein from the free ^{125}I in a chromatography column. Binding studies were further pursued with the Iodobeads® labeled FIX despite the low yields in an attempt to resolve prior technical issues that still needed to be addressed with regard to cell culture system. The goal was to try to replicate results from previous work (9, 10, 48) with the limited FIX stock available before acquiring more stock of the valuable material.

2.4.3 *Binding of Factor IX to Intact BAECs (Iodobeads® labeled)*

From the clotting assay results, it was determined that the FIX labeled with Iodobeads® maintained its biological activity (Table 2.1). The competition binding experiments to determine specific binding were repeated using Iodobeads® labeled FIX in attempts to obtain similar binding results for the rFIX from previous work by Stern *et. al*. (1983) and to measure binding differences between the recombinant and transgenic FIX.

Two treatments, in triplicate, were added to the plated cells for both the recombinant and transgenic FIX. The first treatment was ^{125}I-FIX (28ng/ml) incubated alone to determine overall binding and the second was ^{125}I-FIX (28ng/ml) and unlabeled FIX (1000ng/ml) to determine the specific binding. Higher concentrations were used in this study due to lower cpm per ng values from the Iodobeads® labeling process as compared to the chloramine T labeling method. The concentrations used here are based on the biological activity obtained from the clotting assay.

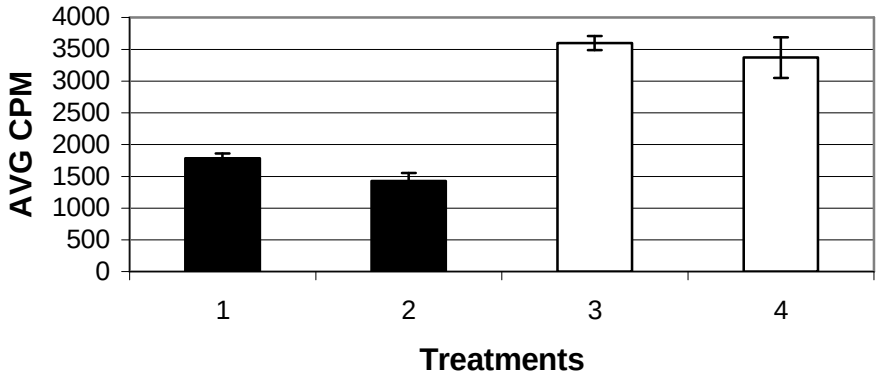

Fig. 2.8. Binding of ^{125}I-FIX to intact cultured BAECs (Iodobeads®). ^{125}I-rFIX (■) (28ng/ml) incubated alone (bar 1) and with rFIX (1000ng/ml) (bar 2). ^{125}I-tFIX (□) (28ng/ml) incubated alone (bar 3) and with tFIX (1000ng/ml) (bar 4). Measurements (mean ± S.D., n = 3) are representative of 2 rFIX and 1 tFIX independent binding experiments.

A reduction in binding in the presence of competing unlabeled FIX for both the rFIX and the tFIX was found with the Iodobeads®-labeled material (figure 2.8). This reduction in binding (comparing bars 1 and 2 and bars 3 and 4) indicates that there was some specific binding of rFIX (0.013ng ± 0.003) and tFIX (0.032ng ± 0.021) to intact BAECs. The difference between binding in the absence and presence of unlabeled FIX are

statistically significant ($p < 0.05$) for the rFIX. However, due to large standard deviation, statistical significance was not found for the tFIX ($p > 0.05$). Interestingly, there was more specific bound tFIX (64.6 ± 17.3%) than rFIX suggesting that the tFIX might be a preferential binder, but the lack of significance made this result suspect. This result seems contrary to the results obtained in the first binding experiment that suggested more specific binding occurring with rFIX than tFIX (figure 2.4). This might be a direct consequence of different radiolabeling methods used in these experiments. The chloramine T-labeled rFIX had more CPM per ng values than the chloramine T-labeled tFIX. The specific binding of rFIX (0.2 fmol) and tFIX (0.6 fmol) is low as compared to what Stern *et. al*. report (8.4 fmol) for 2×10^5 cells.

2.4.4 *Binding of Factor IX to Collagen Type IV (Cell-free assay)*

Although the Iodobeads® radiolabeled FIX maintained its biological activity, as shown by the clotting assay results, the specific binding of ^{125}I-FIX to intact BAECs in these experiments was small compared to previous work. Previous researchers (9, 44, 46) had found specific binding and it is not clear why those results were not repeatable in these experiments. BAEC are primary cells and it is possible that differences in the specific cells might be responsible for the low binding. An alternative assay was done in attempts to quantify and measure differences in ^{125}I-FIX binding removing this variable. Previous work by Cheung *et. al.* (1996) concluded that one specific binding partner for rFIX was human collagen IV. The next set of experiments was designed to quantify ^{125}I-FIX binding to human collagen IV in attempts to repeat previous work (10) and determine if tFIX also bound to human collagen IV.

In this cell free assay, competition experiments between radiolabeled and unlabeled factor IX were done to quantify the amount of binding to human collagen IV. Human collagen IV (50μg/ml) diluted in TBS was used to coat removable wells and ^{125}I-FIX (30ng/ml) was incubated with varying amounts of unlabeled FIX under different conditions. The concentrations of the ^{125}I-FIX and FIX are based on the biological activity obtained from the clotting assay. These conditions investigated incubation in the presence or absence of heparin (1μg/ml) and incubation with or without blocking solution (3% BSA in TBS). Heparin was used because Cheung ***et. al.*** (1996) reported that ^{125}I-FIX bound to human collagen IV with an affinity similar to BAECs in the presence of heparin and bound to collagen IV in the absence of heparin with a lower affinity. Blocking solution was left in some samples to determine its effect on binding.

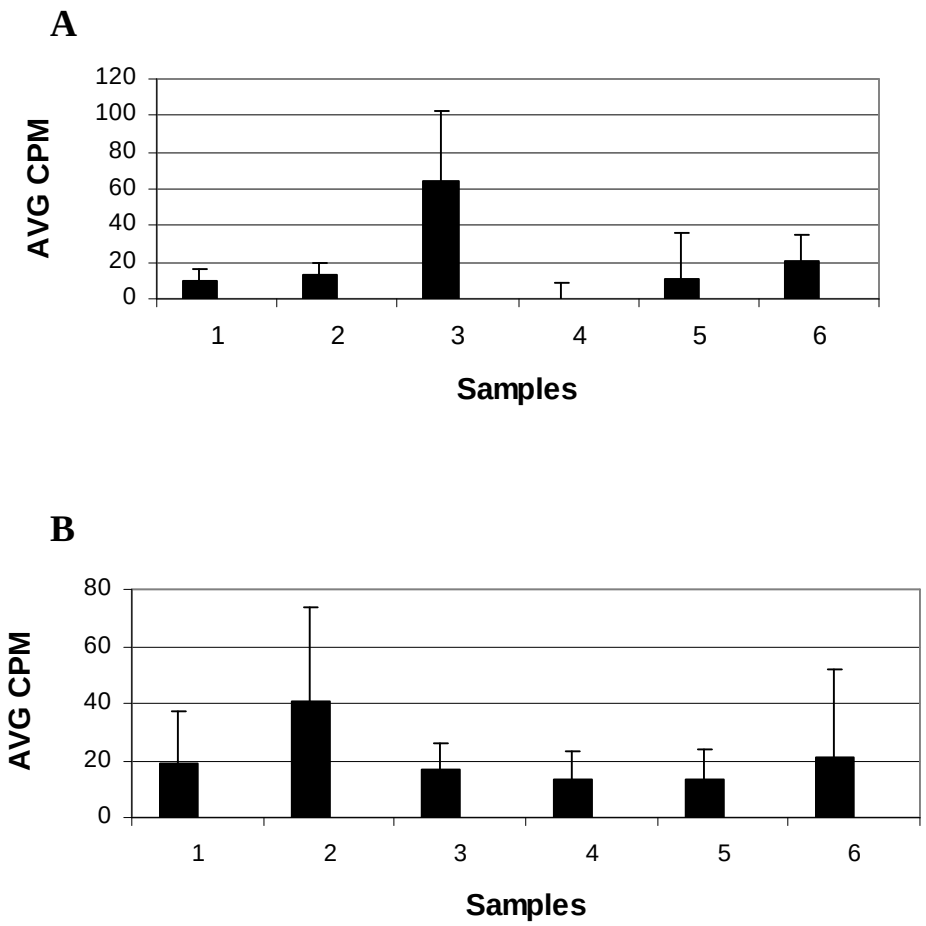

Fig. 2.9. Binding of ^{125}I-rFIX (A) and ^{125}I-tFIX (B) to human collagen IV in a cell free assay. Samples used in this experiment were ^{125}I-FIX (30ng/ml) incubated alone in TBS (bar 1), with FIX (30 and 1000ng/ml) in TBS (bars 2 and 3 respectively), with FIX in blocking solution (30ng/ml) (bar 4), with FIX (30 and 1000ng/ml) and heparin in TBS (1μg/ml) (bars 5 and 6 respectively). Background (^{125}I-FIX bound to non-coated wells) was 30 CPM and not subtracted from the CPMs in this figure. Measurements (mean ± S.D., n = 3) are representative of 4 rFIX and 1 tFIX independent binding experiments.

As shown in figure 2.9, very low CPM values (all below 100) indicated negligible binding of ^{125}I-FIX to the collagen IV coated removable wells and the standard deviation among samples was very large. This might be attributed to an unsuccessful coating process whereby, the collagen did not attach to the removable wells and washed away

before incubation with ^{125}I-FIX. Another cause might be that the coating process was successful but the ^{125}I-FIX did not bind to the collagen and washed away.

2.5 Conclusions and Future Work

Unfortunately the results from previous work (10, 45) in ^{125}I-FIX binding to intact BAECs and human collagen IV were not repeatable in this project. There were several technical issues involved in these experiments that may be the cause of these contradictory results. In the work by Cheung *et. al.* (1991), 100μg of FIX were radiolabeled using the Iodobeads® method whereas only 5μg of FIX was radiolabeled for use in the experiments of this project. Although the Iodobeads® radiolabeled FIX maintained its biological activity, as shown by the clotting assay results, the amount of FIX available for these experiments was 20 times less than used in previous work. For future studies, a larger amount of FIX should be labeled using Iodobeads® as in previous work. This should help to avoid losing a significant amount of protein during the labeling process as suggested by the clotting assay results.

The results from the cell free assay involving human collagen IV also did not agree with those from previous work (10). There was no binding of ^{125}I-FIX to the collagen coated wells present (figure 2.9). This might be a result of too little biologically active labeled FIX in the samples used, in which case labeling more FIX with Iodobeads® as previously recommended would be beneficial. Another issue may stem from the coating process of collagen IV to the removable wells. Collagen IV may have washed out of the wells or not have attached to the tissue culture plastic at all. One recommendation would be to use commercially collagen IV coated plates. Another recommendation is to use an enzyme-linked immunosorbent assay, ELISA, to help determine if the collagen IV

attached to the wells when manually coating in the laboratory (29). In this assay, the removable wells are coated with human collagen IV and a monoclonal or polyclonal antibody that is known to bind to collagen IV is incubated in the coated wells at increasing concentrations. Los *et. al*. (2004) evaluated the specificity of polyclonal goat antihuman type IV collagen antibody (SBA) by ELISA. Plates were coated with human collagen IV or fibronectin overnight. The ELISA was carried out the next day and their results showed a positive concentration dependent signal for collagen IV coated wells, more binding at increasing antibody and collagen IV concentrations. For the fibronectin-coated wells, the result was a negative concentration dependent signal. This result confirmed the specificity of the antibody under study. This group's work was not focused on detecting collagen IV attachment to removable wells; however, their ELISA technique would still be beneficial in determining collagen IV attachment. If more binding occurs with increasing concentrations of antibody at a fixed concentration of collagen IV, then this would indicate that collagen IV is attached to the tissue culture plastic.

Since it has been shown in the literature that one specific binding partner for ^{125}I-rFIX was human collagen IV (10) and the results from the cell free collagen IV assay described above contradicted these previous findings, size exclusion chromatography (SEC) was attempted as a quick and cost effective way to determine if the ^{125}I-FIX bound to collagen IV. Preliminary studies with SEC were unsuccessful due to some technical issues related to gel selection and flow issues but could be pursued further.

Other assays to determine FIX binding to collagen IV include surface plasmon resonance (SPR) and affinity coelectrophoresis. In an SPR experiment, the ligand is

covalently bound to the surface and the analyte is flowed over it (6, 12). Binding at the surface causes a change in refractive index near the sensor surface that changes the SP coupling angle (6, 43). The change in coupling angle is quantitatively detected by the system detector (6). This assay might prove convenient because the FIX would not undergo the radiolabeling process to detect binding and therefore no product is lost before performing an experiment. In affinity coelectrophoresis, both the protein and its binding partner are free to migrate during electrophoresis (26). For this assay, various concentrations of a known binding partner (collagen IV) to the protein under study are loaded into different zones, separate sections, of an agarose gel. A fixed concentration of labeled protein (^{125}I-FIX) is the introduced to the gel. Binding to its binding partner in the agarose gel slows the migration of the labeled protein through the zones. This binding produces a series of peaks on the gel from which an affinity constant may be derived. The zones containing higher concentrations of collagen IV would show a higher peak on the gel than zones containing lower concentrations of collagen IV.

Chapter 3: A Study of the Transport of Insulin-like Growth Factor-I across Cultured Mammary Epithelial and Bovine Aortic Endothelial Cell Monolayers *In Vitro*

3.1 Introduction

This chapter focuses on studies involving insulin-like growth factor-I (IGF-I) transport across *in vitro* cell layers of Mac-T, a mammary epithelial cell line, and bovine aortic endothelial cells (BAECs). First, a brief literature review followed by the purpose of this project is given. Second, the experimental procedures detailing the materials and methods used in this project are described. Next, the results of this project are presented. The chapter is closed with conclusions and recommendations for future work.

3.2 Background

3.2.1 *Epithelial Cells*

One of the primary functions of epithelial cells is to maintain a protective barrier that regulates the transport of proteins and other substances to underlying cells of the tissue (16). In general, the external and internal surfaces of organs are covered by a sheet-like layer of epithelial tissue called an epithelium (28). Cells that form the epithelium are polarized because their plasma membranes are organized into at least two discrete regions and contain different sets of proteins.

Typically the distinct surfaces of a polarized epithelial cell are called the apical and basolateral surfaces. The basolateral surfaces of most epithelia are on the side of the cell closest to the blood vessels (28). Epithelia in different body locations have characteristic morphologies and functions. Stratified (multi-layer) epithelia commonly serve as barriers and protective surfaces such as the skin. Simple (single layer) epithelia often selectively

move ions and small molecules from one side of the layer to the other such as in the stomach. The mammary epithelium proliferates, invades and has the most tumorigenic potential (55). Interaction between the epithelium and the extracellular matrix plays a major role in mammary gland branching morphogenesis. Interaction between the epithelium and the stroma is necessary for the proper patterning and function of the normal mammary gland.

3.2.2 *Mammary Epithelial Cells*

Milk synthesis occurs within clusters of differentiated mammary epithelial cells (21). Several mammary epithelial cell lines exist. These lines include the mouse COMMA-D cell line, human breast cell lines T47D, MCF-7 and HBL-100, rat mammary tumor cell lines such as TMT-081MS, MT-1-TC and Rama 25 and bovine cell lines (21). The established bovine cell lines include BMEC+H, PS-BME-7 and Mac-T.

The Mac-T cell line was produced from primary bovine mammary epithelial cells by stable transfection with SV-40 large T-antigen (56). Huynh ***et. al.*** (1991) report that SV-40 establishes primary bovine mammary epithelial cells in culture, which allows these cells to avoid senescence and crisis and have the ability to grow and survive in culture. Mac-T cells retain their ability to differentiate by synthesizing and secreting large quantities of α and β casein in response to prolactin (21, 56, 57). Zavizion ***et. al.*** (1995) suggest that the Mac-T cell line is heterogeneous and that it is comprised of at least 3 different subtypes of mammary epithelial cells which they term CU-1, CU-2 and CU-3. This group derived the 3 clonal lines by subcloning parental Mac-T cells and distinguishing them by differences in cell morphology, size, growth and cytogenetic characteristics.

The Mac-T cell line is an immortalized epithelial cell line isolated from bovine mammary tissue (4, 17, 40). It provides a useful *in vitro* model system because it retains the biochemical and morphological characteristics of primary cells *in vivo* (4, 17, 58). In addition, this cell line is widely used as an alternative to primary cells because primary cells are technically difficult, time consuming and expensive.

The Mac-T cell line is sensitive to IGF-I (4, 17, 40) and contains specific IGF-I receptors (17, 58). Zhao *et. al*. (1992) found that IGF-I exerted a positive mitogenic effect on Mac-T cells. Mac-T cells proliferate in response to IGF-I and synthesize and secrete several forms of IGF binding proteins, IGFBPs (4, 17). IGFBP-2, 3, 4 and 6 are synthesized and secreted by Mac-T cells.

3.2.3 *Insulin-like Growth Factor-I*

IGF-I is a potent mitogen for many cells and has been implicated in breast cancer cell proliferation (39) and tumor growth in lung cancer (6). It is thought that IGF-I originates from the liver and has an endocrine function (15). IGF-I is a polypeptide member of the insulin super-family that includes insulin, IGF-I and II, relaxin and insulin like-3 and 4 (6, 33, 42). IGF-I is composed of 70 amino acids and has a molecular weight of approximately 7.6 kDa. Many studies suggest that IGF-I can exert differentiative actions on a wide range of cell types (31).

Many actions of IGF-I are mediated by high affinity interactions with IGF-I receptors (IGF-IRs) and IGFBPs (6, 33). IGF-I is known to signal through IGF-IRs and IGFBPs regulate its binding. Six IGFBPs (1-6) have been identified and shown to participate in inhibiting and enhancing IGF-I actions, possibly depending on whether they are secreted or membrane associated (31). In particular, IGFBPs are responsible for

prolonging the half-life of IGF-I in circulation, limiting the bioavailability of IGF-I for interaction with IGF-IR, and affecting cellular proliferation and death (6).

3.2.4 *Purpose of Project*

Previous research at Virginia Tech quantified the binding; internalization and transport kinetics involved in trafficking IGF-I across *in vitro* monolayers of bovine aortic endothelial cells (33). Internalization and transport of IGF-I via insulin-like growth factor-I receptors was detected and binding analysis showed that insulin-like growth factor binding proteins (IGFBPs) were the main cell surface binding sites for IGF-I binding in this system. However, internalization of IGF-I by IGFBPs was not detected and binding was shown to inhibit rather than enhance transport. The purpose of this project was to determine if IGFBPs, either on the cell surface or extracellular matrix, function as a reservoir for IGF-I sequestering it for transport later through the cell by IGF-IRs. The hypothesis is that under pulse conditions there would be more IGF-I available during the chase period for transport because the IGFBPs retained IGF-I in the local environment. The result that would support this hypothesis should show that more IGF-I is transported during the chase when IGFBPs are not blocked. Figures 3.1 and 3.2 illustrate this hypothesis.

Also, specifically how varying temperature, addition of IGF binding protein-3 and challenge time of the cells with IGF-I impact IGF-I transport are addressed. The hypothesis was that at reduced temperature (4 ˚C), transport of IGF-I would be reduced compared to physiological temperature (37 ˚C) due to inhibition of cellular internalization and transport. Furthermore, with increasing IGFBP-3 concentrations, IGF-I would be bound in solution reducing transport via the cellular pathway.

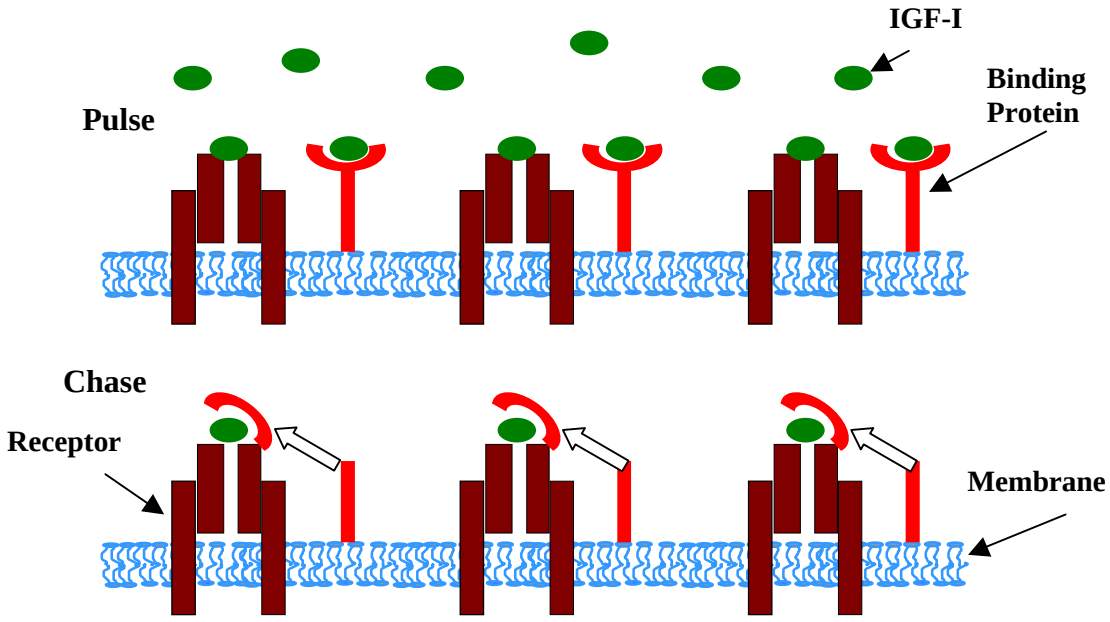

Fig. 3.1 Proposed surface binding events under pulse-chase conditions with no competitor present. During the pulse period, IGF-I binds to its surface receptors and binding proteins. IGF-I is internalized by receptor-mediated endocytosis. During the chase, when the source of IGF-I is removed, IGF-I sequestered by IGFBPs during the pulse is available for binding to IGF-IRs for internalization by receptor-mediated endocytosis.

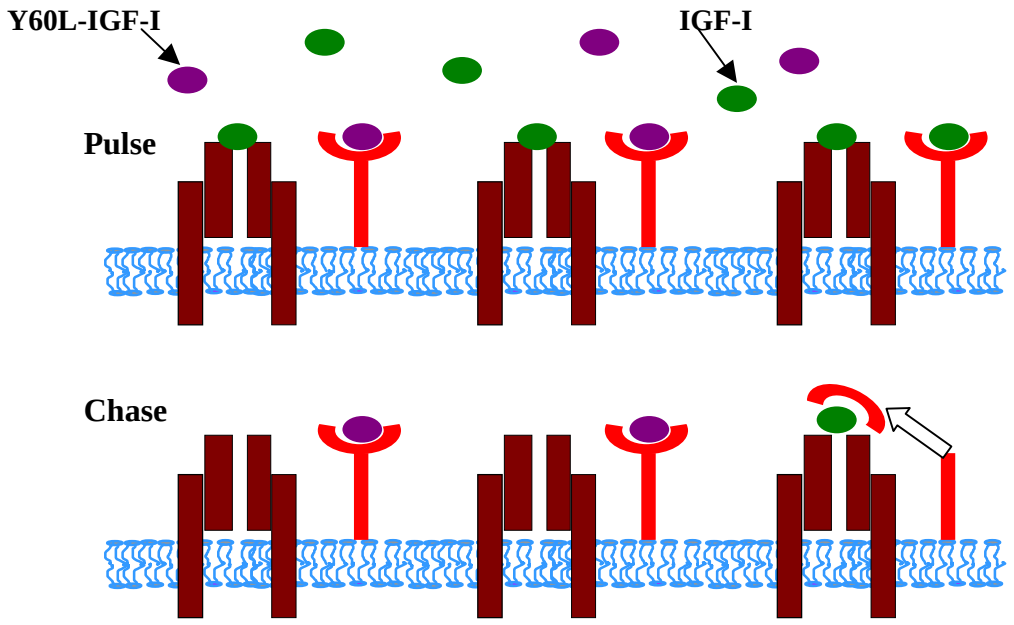

Fig. 3.2 Proposed surface binding events under pulse-chase conditions with competitor present. During the pulse period, IGF-I binds to its surface receptors and competes for binding proteins. IGF-I is internalized by receptor-mediated endocytosis. During the chase, when the source of IGF-I is removed, any remaining IGF-I bound to surface receptors is internalized by receptor-mediated endocytosis.

3.3 Experimental Procedures

3.3.1 *Materials*

Mammary epithelial cells (Mac-T cells) and ^{125}I-IGF-I were gifts from Professor R. M. Akers (Dairy Science, Virginia Tech). Bovine aortic endothelial cells were purchased from Clonetics (Walkersville, MD) and used between passage 8 and 11. Dulbecco's modified Eagle's medium (DMEM) and trypsin/EDTA were purchased from Gibco (Grand Island, NY). Dulbecco's phosphate buffered saline (DPBS), DMEM without phenol red; L-glutamine and penicillin/streptomycin were purchased from Mediatech (Herndon, VA). Bovine calf serum and fetal bovine serum was purchased from Hyclone (Logan, UT). Rat-tail collagen I was purchased from BD Biosciences Discovery Labware (Franklin Lakes, NJ). Fibronectin from human plasma was purchased from Sigma (St Louis, MO). Cell culture dishes (100mm x 20mm), 12 well and 24 well plates and Transwell® inserts (12mm, 0.4μm pore size) were purchased from Corning Costar (Cambridge, MA). IGF-I was purchased from Peprotech (Rocky Hill, NJ). IGFBP-3 and Y60L–IGF-I was purchased from Upstate Biotechnology (Lake Placid, NY).

3.3.2 *Methods*

Cell Culture – Mac-T cells were seeded at 1 x 10^6 cells/cell culture dish and grown in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. BAECs were seeded at 5 x 10^5 cells/cell culture dish and grown in DMEM containing 10% calf serum, 1% penicillin/streptomycin and 1% L-glutamine. Growth media was changed every 2-3 days after washing with DPBS and subcultured every 4-5 days when cells appeared confluent using 3ml/dish of trypsin/EDTA.

IGF-I Transport across Mac-T cell monolayers under pulse-chase conditions (figure 3.3) – Mac-T cells were seeded at 5 x 10^4 cells/cm^2 on the upper membrane of a Transwell® insert and allowed to grow in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin for eight days with media changes every 2 days. On the eighth day, the apical and basolateral chambers were switched to plain media (DMEM containing 1% penicillin/streptomycin) to initiate the study. The apical (final volume 0.5ml per insert) media received one of two treatments. Treatment one was ^{125}I-IGF-I (6ng/ml). Treatment two was ^{125}I-IGF-I (6ng/ml) and Y60L-IGF-I (2μg/ml), a non-IGF-IR binding mutant of IGF-I (3). The plate was allowed to incubate at 37 ˚C for 2 hours before collecting apical or apical and basolateral samples. After this pulse period, plain media was added to replace the collected samples. When appropriate, an additional Y60L-IGF-I (2μg/ml) was added to the apical chamber during the chase period. After 6 additional hours of incubation (chase period) at 37 ˚C, the apical and basolateral media were collected from all wells and 0.3N NaOH (0.5ml) was added to the apical chamber. The plate was then allowed to incubate overnight at 4 ˚C and the apical solutions were collected the next day. All samples and standards (serum-free media and 0.3N NaOH) were counted using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL).

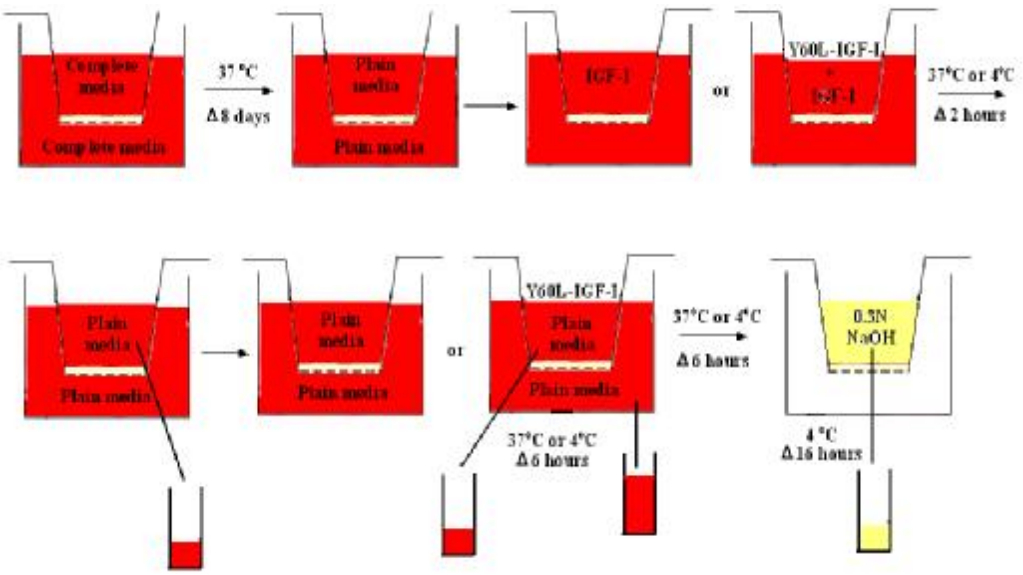

Fig. 3.3 Schematic of a Pulse Experiment (same figure as shown in the introduction chapter). Cells are seeded on the porous insert and maintained in culture for 8 days before initiating the experiment by switching to serum-free media. Radiolabeled ligand is added to the media in the apical chamber and unlabeled material, when appropriate, is added to the apical and basolateral chamber. Cells are incubated with the labeled and unlabeled material for a certain pulse time before collecting the apical and basolateral media. Fresh serum-free media is added to replace the collected material and cells are incubated again for a certain chase period before collecting apical and basolateral media. NaOH is added to the apical chamber to collect cell lysate at the completion of the study. Δ denotes the time period between steps, which were done at 4 ˚C or 37 ˚C.

Coating Transwell® Plates – Transwell® plates were coated with rat tail collagen I and fibronectin from human plasma as previously described (33) except that fibronectin instead of superfibronectin was used. The inserts were coated with both attachment factors because BAEC cultured on rat tail collagen I and fibronectin coated inserts formed the best barrier to the transport of phenol red, a measure of paracellular transport (33). Briefly, stock rat-tail collagen was diluted to 2mg/ml in 0.02N sterile acetic acid and 106µl of this solution was added to each insert. The inserts were allowed to dry in a sterile hood for 12 hours. After 12 hours, any remaining collagen was aspirated from the surface and a wash with sterile DPBS was performed. Fibronectin was then diluted to 50µg/ml with sterile DPBS and 106µl of this solution was added to each insert. The

inserts were allowed to dry in a sterile hood for 30 minutes. After 30 minutes, any remaining fibronectin was aspirated out followed by a wash with sterile DPBS (0.5ml).

IGF-I Transport across BAE cell monolayers under non-pulse conditions – Transwell® plates were coated as described above and seeded at 5 x 10^4 cells/cm^2 with BAECs. Cells were allowed to grow in DMEM containing 10% bovine calf serum, 1% L-glutamine and 1% penicillin/streptomycin for seven or nine days. Studies were initiated by switching the cells to serum-free media for 12 hours. The cells were then washed with DPBS followed by adding binding buffer, 0.5ml, (0.05% gelatin, 120mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 15mM Sodium Acetate, 25mM Hepes, 10mM Dextrose, pH 7.4) to each apical and basolateral chamber. When appropriate, unlabeled IGF-I was added to both chambers and allowed to incubate for 20 minutes at 37 ˚C. ^{125}I-IGF-I (4ng/ml) was added to each apical chamber and allowed to incubate at 4 ˚C or 37 ˚C for 12 hours. The apical and basolateral chamber contents were collected and the apical chamber was washed with binding buffer (0.5ml). After the wash step, 0.3N NaOH (0.5ml) was added to each apical chamber and allowed to incubate overnight at 4 ˚C. The apical chamber contents were collected the next day. All samples and standards (binding buffer and 0.3N NaOH) were counted using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL).

Competitive Binding Studies with BAECs – Two 24 well plates were seeded at 1 x 10^5 cells/cm^2 with BAECs. Cells were allowed to grow in DMEM containing 10% bovine calf serum, 1% L-glutamine and 1% penicillin/streptomycin for approximately 48 hours. Studies were initiated by switching the cells to serum-free media for 12 hours. The cells were then washed with DPBS followed by adding binding buffer, 0.5ml,

(0.05% gelatin, 120mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 15mM Sodium Acetate, 25mM Hepes, 10mM Dextrose, pH 7.4) to each well. Unlabeled IGFBP-3 (6ng) was added to designated wells and the plates were incubated 20 min at 37 ˚C. ^{125}I-IGF-I (1ng) was added to each well and the first plate was incubated at 4 ˚C for 12 hours and the second at 37 ˚C for 12 hours. The binding buffer was collected for counting and 0.3N NaOH (0.5ml) was added to each well. The plates incubated overnight at 4 ˚C and the next day cell lysate was collected. All samples and standards (binding buffer and 0.3N NaOH) were counted using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL).

IGF-I Transport across BAE cell monolayers under pulse conditions (figure 3.3) – Transwell® plates were coated as described above and seeded at 5 x 10^4 cells/cm^2 with BAECs. Cells were allowed to grow in DMEM containing 10% bovine calf serum, 1% L-glutamine and 1% penicillin/streptomycin for seven days. Studies were initiated by switching the cells to serum-free media for 12 hours. The cells were then washed with DPBS followed by adding binding buffer, 0.5ml, (0.05% gelatin, 120mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 15mM Sodium Acetate, 25mM Hepes, 10mM Dextrose, pH 7.4) to each apical and basolateral chamber. Y60L-IGF-I (2µg/ml) was added to the apical chamber of designated samples and allowed to incubate for 20 minutes at 37 ˚C. ^{125}I IGF-I (4ng/ml) was then added to all samples. The plate was allowed to incubate for 3 hours at 37 ˚C before the apical and basolateral contents were collected and serum-free media was added. Y60L-IGF-I (2µg/ml) was then added to the apical chamber of designated inserts. After 9 additional hours of incubation (chase period) at 37 ˚C, the apical and basolateral media was collected from all wells and 0.3N NaOH (0.5ml) was

added to the apical chamber. The plate was then allowed to incubate overnight at 4 ˚C and the apical solutions were collected the next day. All samples and standards (binding buffer and 0.3N NaOH) were counted using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL).

Degradation Analysis – Samples of basolateral media (0.4ml) were used in a Trichloroacetic acid (TCA) and Phosphotungstic acid (PTA) precipitation assay as previously described (24). Briefly, this involved adding concentrated TCA and PTA to yield final concentrations in samples of 3% and 0.3% respectively. The samples were vortexed and incubated at 4 ˚C for 30 minutes before centrifuging for 3 minutes at 10,000 x g. The supernatants and pellets were collected separately and counted using a Cobra II Auto Gamma Counter (Perkin Elmer Life Sciences Downers Grove, IL).

Phenol Red Transport across Mac-T cell monolayers – Transport of phenol red across the cell layers was assayed as described previously (33). Briefly, Mac-T cells were seeded at 5 x 10^4 cells/cm^2 and allowed to grow in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin for eight and ten days in a Transwell® plate with media changes every 2 days. On the eighth and tenth day, the apical chamber media was replaced with DMEM + (DMEM with phenol red, 1% penicillin/streptomycin and 10% FBS) and the basolateral chamber media was replaced with DMEM – (DMEM without phenol red, 1% penicillin/streptomycin and 10% FBS) after washing with DPBS. The plate was allowed to incubate at 37 ˚C for 12 hours. Samples (1ml) from the basolateral chamber were collected and analyzed using a Spectronic® Genesys™ 5 spectrophotometer (Spectronic Instruments, Inc. Rochester, NY) at 479nm.

Phenol Red Transport across BAE cell monolayers – This is the same procedure as outlined above except that Transwell® plates were first coated as described above. BAECs were allowed to grow in DMEM containing 10% bovine calf serum, 1% L-glutamine and 1% penicillin/streptomycin for eight and ten days.

Statistical Analysis – Statistical comparisons were done by two-tailed t-tests in Microsoft Excel® with significance defined as $p < 0.05$.

3.4 Results

3.4.1 *IGF-I Transport Across BAEC Monolayers at 4 ˚C and 37 ˚C*

Previous studies done in the Williams' laboratory indicated that IGF-I transport across BAE cell monolayers occurs through both a transcellular and paracellular pathway (33). This work involved competition experiments between ^{125}I-IGF-I and unlabeled IGF-I that resulted in enhanced transport of ^{125}I-IGF-I when all binding sites (IGFBPs and IGF-IRs) were blocked by unlabeled IGF-I. These experiments suggested that surface binding interactions retard ^{125}I-IGF-I transport *in vitro* at physiological temperature.

Reduced temperature is a common method to eliminate/reduce transcellular transport (25). Temperature may also have an effect on paracellular transport. These studies are an extension of previous work (33) performed in the Williams laboratory that will determine if transport is reduced by a reduction in temperature as compared to physiological temperature. To address this question, competitive binding experiments were undertaken between ^{125}I-IGF-I and IGF-I at 4 ˚C and 37 ˚C.

Another issue addressed in this study was the use of a different attachment factor for the experiments described in this thesis. Superfibronectin was used in previous work (33) and fibronectin from human plasma was used in the experiments described in this

thesis. The studies were done at different challenge times to determine if the difference in attachment factors had an effect on the transport and binding properties of BAECs.

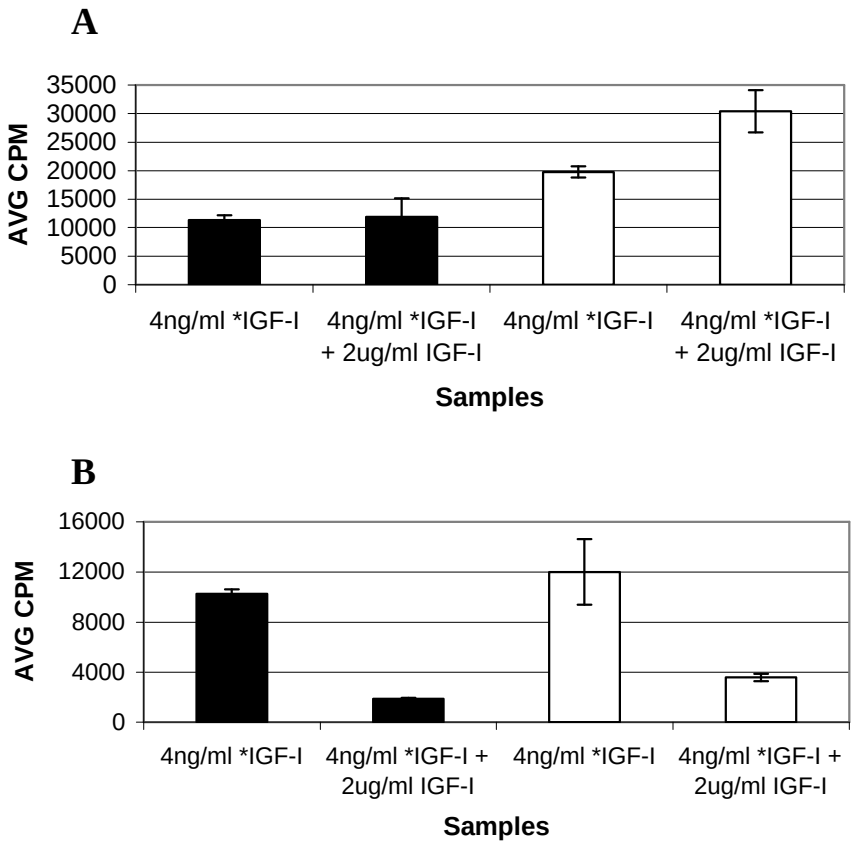

Fig. 3.4 ^{125}I-IGF-I in basolateral media (A) and in cell lysate (B) of BAE cell monolayers at 4 ˚C (■) and 37 ˚C (□). Studies were initiated by a 12-hour serum starvation period after 7 days of growth. AVG CPM represents the total counts added from each sample. Measurements (mean ± S.D., n = 3) are representative of 3 independent experiments.

As shown in figure 3.4**A**, a reduction in temperature did lead to decreased transport. Addition of unlabeled IGF-I to block surface binding lead to increased transport at 37 ˚C, but surprisingly did not lead to an increase at 4 ˚C. This difference was not reflected in any differences in cell lysate levels after 12 hours as the addition of IGF-I showed a significant ($p < 0.05$) decrease in ^{125}I-IGF-I cell lysate levels at both

temperatures and the levels were comparable (figure 3.4**B**). The transport at 4 ˚C may be a result of a difference in ligand diffusion coefficient due to the reduction in temperature, which could impact paracellular transport. According to the Stokes-Einstein equation, the diffusion coefficient (*D*), is calculated by equation 1 (19):

$$D = \frac{KT}{6pRm} \quad (1)$$

Where *K* is the Boltzmann's constant (1.38 x 10^{-16} g-cm^2/s^2-K), *T* is the temperature, m is the membrane viscosity and *R* is the radius of the diffusing protein. It follows that as temperature increases, the diffusion coefficient will also increase. Smaller diffusion coefficients might lead to decreased flux across cell monolayers.

These results show that temperature has a significant ($p < 0.05$) effect on transport after a growth period of 7 days. The transport at 37 ˚C agrees with previous work (33) that determined ^{125}I-IGF-I transport was significantly enhanced when all surface binding sites are blocked by unlabeled IGF-I. It follows that the use of fibronectin instead of superfibronectin as an attachment factor for BAECs produced results comparable to previous work (33). Also, performing these competitive binding experiments at the longer cell growth period, 9 days, had no effect on transport and binding: a reduction in temperature did lead to decreased transport (figure 3.5**A**) and addition of unlabeled IGF-I lead to increased transport at 37 ˚C (figure 3.5**A**) and showed a decrease in ^{125}I-IGF-I cell lysate levels at both temperatures (figure 3.5**B**).

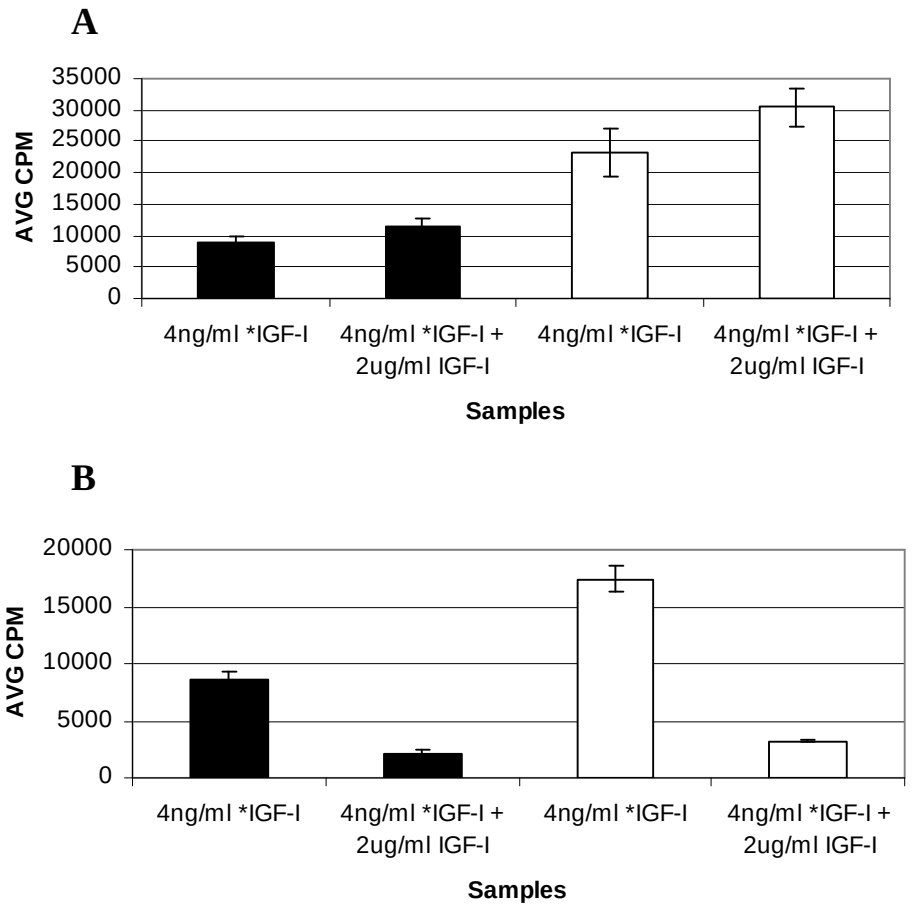

Fig. 3.5 ^{125}I-IGF-I in basolateral media (A) and in cell lysate (B) of BAE cell monolayers at 4 ˚C (■) and 37 ˚C (□). Studies were initiated by a 12-hour serum starvation period after 9 days of growth. AVG CPM represents the total counts added from each sample. Measurements (mean ± S.D., n = 3) are representative of 3 independent experiments.

3.4.2 *Phenol Red Transport Across BAE Cell Monolayers*

From the previous experiment it was determined that performing the binding assay at a longer growth period had no effect on transport and binding. A phenol red transport study was done concurrently with this previous experiment to determine any differences in paracellular transport resulting from different growth periods and attachment factors. Phenol red transport studies are desirable because the phenol red molecules do not interact with the cell so the transport obtained is only paracellular (22).

Paracellular transport is a passive transport process in which molecules pass between adjacent cells (33). This type of transport, if not minimized in the experimental setup, may alter transport results. In a phenol red transport experiment, samples from the basolateral chambers of the inserts are read on a spectrophotometer to obtain absorbance values. The absorbance values are a measure of the amount of phenol red molecules in the sample. Higher absorbance values denote larger amounts of phenol red molecules. The amount of phenol red in basolateral samples is an indicator of how much paracellular transport occurred. The blank samples (inserts maintained in culture without cells) are used in this assay to determine the total amount of phenol red transport in the absence of cells.

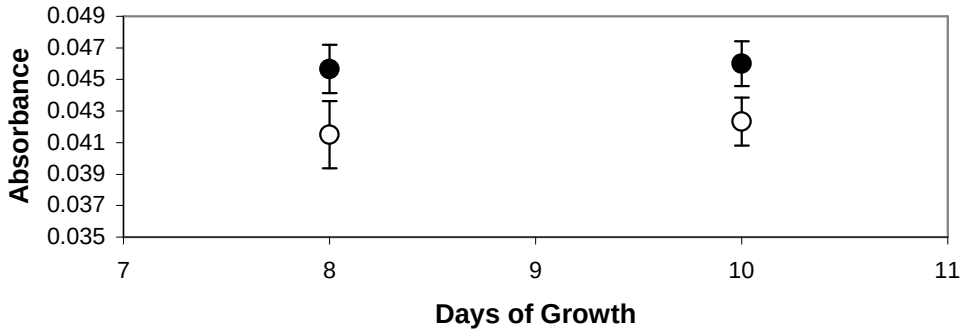

Fig. 3.6 Phenol red transport across BAEC monolayers after 8 and 10 days of growth. The absorbance values (at 479nm) were taken for cells (○) and blanks (●). Blank wells (coated Transwell® inserts with no cells maintained in culture) were used as a control. All samples were zeroed to DMEM -. Measurements (mean ± S.D., n = 3) are representative of two independent experiments.

The data in figure 3.6 show that there is a small difference in absorbance values between the blank and cell layer samples at days 8 and 10 of cell growth. However, this difference at day 8 is much less than has been reported for endothelial cells in previous work (33). Unfortunately, increased culture time (10 days of cell growth) did not lead to a greater difference in absorbance.

Phase contrast pictures taken in the Goldstein laboratory were used to qualitatively compare cell monolayers plated on fibronectin-coated inserts after 8 and 10 days of growth. These images after 8 (figure 3.7**A**) and 10 (figure 3.7**B**) days of cell growth show that BAECs cultured on rat tail collagen I and fibronectin coated Transwell® inserts covered the entire insert. There was no significant difference in cell monolayer coverage across the insert when viewed with an Olympus IX50 inverted microscope at 20x magnification. Based on the similar transport results obtained after 8 and 10 days of cell growth (figures 3.4**A** and 3.5**A**) and no greater difference in absorbance values after longer challenge times (figure 3.6), future experiments with BAECs were done after 8 days of cell growth and used fibronectin coated inserts.

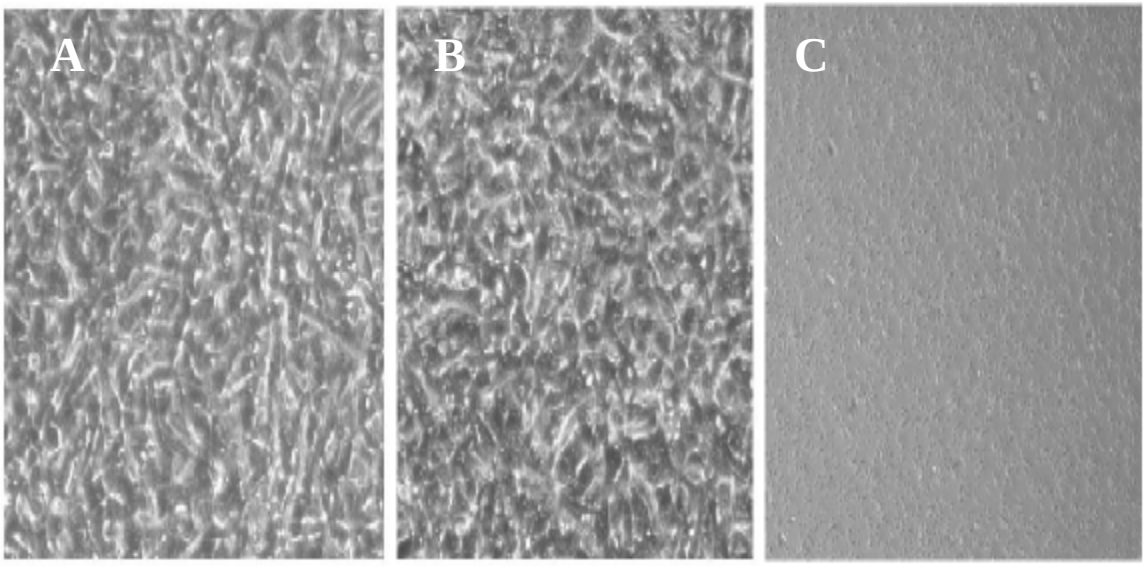

Fig. 3.7 Phase contrast images of BAE cell monolayers. Cells were maintained in culture after 8 (**A**) and 10 (**B**) days of growth and inserts maintained in culture without cells (**C**) on rat tail collagen I and fibronectin coated inserts. The 12-well plates were placed onto the motorized stage (Prior, Rockland, MA) of an Olympus IX50 inverted microscope (Opelco, Sterling, VA) and phase contrast images were acquired at 20x with a Hamamatsu cooled CCD camera (C4742-98-12NRB).

3.4.3 *IGF-I Transport under Pulse Conditions with BAECs*

A computational model developed previously (33) suggested that surface IGFBPs could function as a reservoir for IGF-I. This was a prediction and one goal of this thesis was to test that prediction. To address this, competitive binding studies between ^{125}I-IGF-I and Y60L-IGF-I, an analog of IGF-I with significantly reduced affinity for IGF-IRs and normal affinity for IGFBPs (3), were performed under pulse conditions to distinguish between IGF-IR and IGFBP mediated transport. The hypothesis is that under pulse-chase conditions, IGFBPs would be advantageous to the transport process as there would be more ^{125}I-IGF-I available via dissociation during the chase for transport. During the "pulse" period, cells are incubated with exogenous ^{125}I-IGF-I. During the "chase" period, the source of ^{125}I-IGF-I is material retained or recycled by the cells.

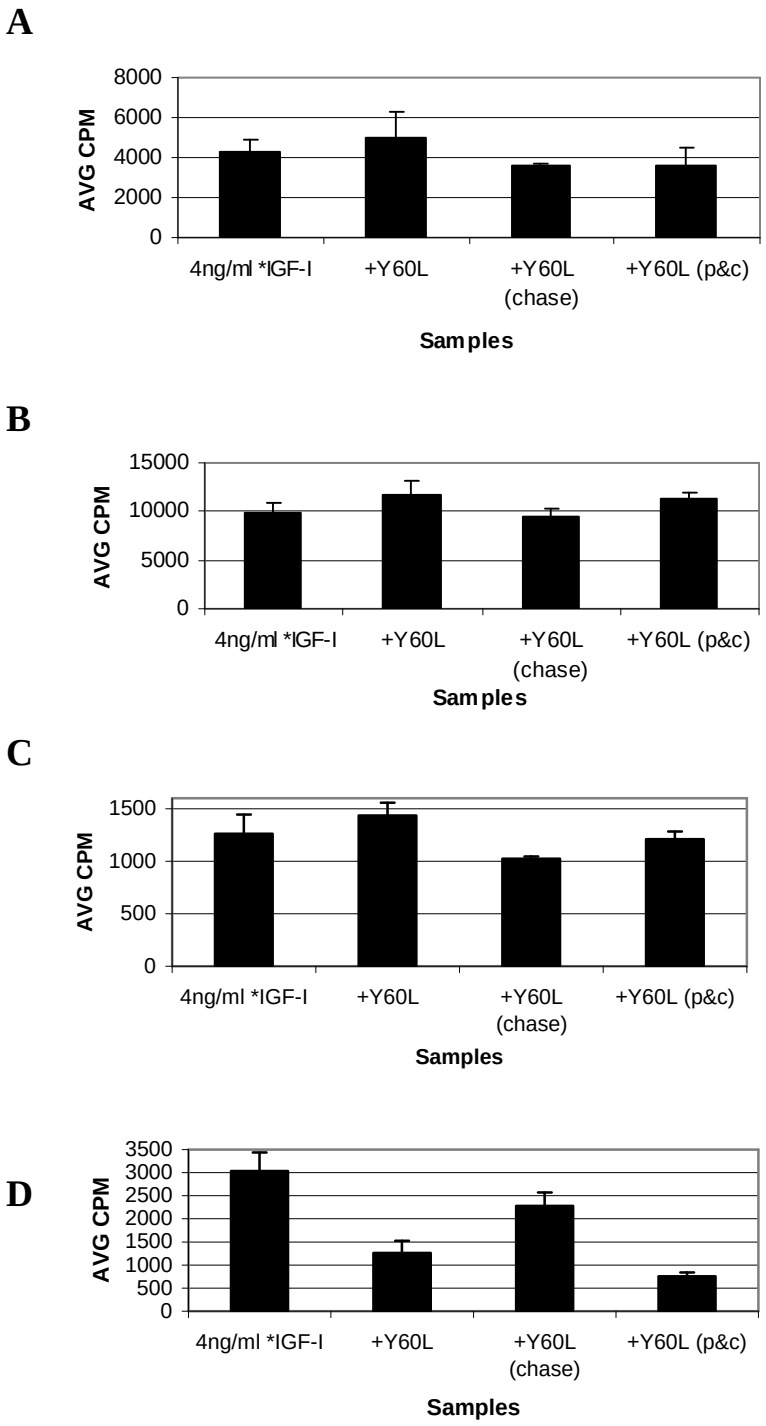

Fig. 3.8 ^{125}I-IGF-I in apical media during the chase (A), basolateral media during pulse (B) and chase (C) and in cell lysate of BAE cell monolayers (D) at 37 ˚C. Studies were initiated by a 12-hour serum starvation period after 7 days of growth. 'Chase' denotes Y60L-IGF-I added during the chase period and 'P&C' denotes Y60L-IGF-I added during the pulse and chase periods. AVG CPM represents the total counts added from each sample. Measurements (mean ± S.D., n = 3) are representative of 3 independent experiments.

^{125}I-IGF-I transport was evident during the pulse period. The addition of Y60L-IGF-I enhanced ^{125}I-IGF-I transport during the pulse period (figure 3.8**B**) similarly to the enhancement seen in the 12-hour studies. Parallel sample sets, which differed only in the treatment during the chase period, showed good agreement verifying the experimental technique. ^{125}I-IGF-I transport was also evident during the chase period. Adding Y60L-IGF-I during the pulse also resulted in a small, but not significant, increase in transport during the chase period (figure 3.8**C**). This does not agree with the computational modeling predictions in that there is no increase in transport when IGFBPs are present in the system, binding sites are not blocked by Y60L-IGF-I. The addition of Y60L-IGF-I during the pulse period decreased cell-associated ^{125}I-IGF-I (figure 3.8**D**), as one would expect if IGFBPs were blocked. Adding Y60L-IGF-I during the chase period resulted in a small decrease in transport (figure 3.8**C**) and on the amount in the cell lysate (figure 3.8**D**). Adding Y60L-IGF-I during the chase period does not enhance transport during the chase when IGFBPs were present during the pulse period. This may be due to the decrease in the amount of ^{125}I-IGF-I retained in the apical media during the chase when additional Y60L-IGF-I is added to initiate the chase period (figure 3.8**A**). The transport results obtained here do not show that the reservoir function of IGFBPs facilitates overall transport under pulse conditions. Degradation analysis was performed on the pulse and chase basolateral media samples to determine what percentage of transported ^{125}I-IGF-I was degraded, suggesting transport through the cell.

Table 3.1: Degradation of IGF-I during transport in the presence of Y60L-IGF-I TCA/PTA precipitation of transported ^{125}I-IGF-I across BAECs under pulse conditions at 37 °C. Results were calculated as the percentage of transported sample that was TCA/PTA soluble. Results indicate the mean ± S.D. and are representative of three independent experiments.

^{125}I-IGF-I	Pulse	Chase
None	82.0 ± 5.4%	61.0 ± 2.6%
Y60L-IGF-I	82.3 ± 2.1%	60.0 ± 5.2%
Y60L-IGF-I (added at chase)	84.1 ± 3.4%	60.1 ± 1.8%
Y60L-IGF-I (pulse and added at chase)	83.5 ± 2.0%	61.4 ± 1.9%

In Table 3.1, inclusion of Y60L-IGF-I during the pulse and added during the chase had no effect on the amount of ^{125}I-IGF-I internalized. These results suggest there was no change in the amount of ^{125}I-IGF-I degraded. The percentage of degraded material is higher than expected, likely indicating that a large amount of free ^{125}I label was present in the stock material used for this set of experiments. This may also be a result of a poor BAE cell monolayer, which leads to dominant paracellular transport.

3.4.4 *IGF-I Transport under Pulse Conditions with Mac-T Cells*

The pulse experiments with BAECs did not agree with computational modeling (33) predictions: there was no increase in transport during the chase period when IGFBPs were present and Y60L-IGF-I added during the chase did not facilitate transport as well. One explanation may be that the computational model needs reworking. Another could be that the experimental system using BAECs will not produce comparable results to computational modeling. Therefore, the pulse experiments were done again using Mac-T cells with the idea being that these cells should exhibit less paracellular transport and hence may be a better model for distinguishing small effects in transport and binding. Mac-T cells have been shown to form restrictive barriers on Transwell® plates without prior coating of these inserts (33).

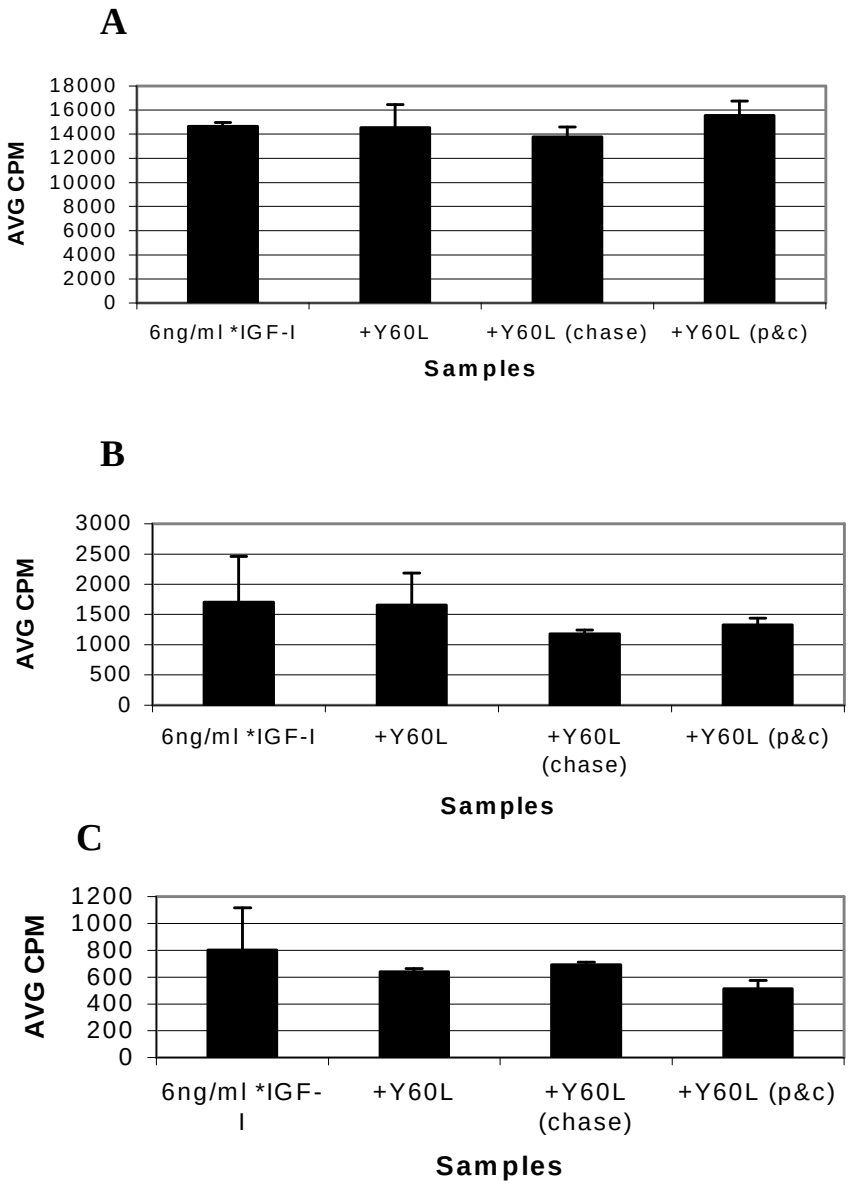

Fig. 3.9 ^{125}I-IGF-I in basolateral media during pulse (A) and chase (B) and in cell lysate of Mac-T cell monolayers (C) at 37 ˚C. AVG CPM represents the total counts added from each sample. 'Chase' denotes Y60L-IGF-I added during the chase period and 'P&C' denotes Y60L-IGF-I added during the pulse and chase periods. Measurements (mean ± S.D., n = 3) are representative of 2 independent experiments.

The addition of Y60L-IGF-I during the pulse had no effect on ^{125}I-IGF-I transport during the pulse and chase periods (figures 3.9**A** and 3.9**B**) in contrast to results from

pulse experiments with BAECs (figures 3.8**A** and 3.8**B**). Adding Y60L-IGF-I during the chase had a slight effect on transport but not a significant one (figure 3.7**B**). As for cell surface binding, there is more binding of ^{125}I-IGF-I to the cell surface when all IGFBPs are available to ^{125}I-IGF-I (figure 3.9**C**). Adding Y60L-IGF-I to initiate the chase decreased surface bound IGF-I (figure 3.9**C**).

3.4.5 *Phenol Red Transport across Mac-T Cell Monolayers*

From the pulse studies using Mac-T cells as the model system, the data suggested that Y60L-IGF-I had no effect on ^{125}I-IGF-I transport suggesting that the Mac-T cells not forming a restrictive barrier to paracellular transport or that IGFBP binding was not significant. To address this question, a phenol red transport study was then performed and used to compare barrier integrity between BAECs and Mac-T cells. Mac-T cells plated on uncoated inserts and uncoated inserts without cells (blanks) were maintained in culture for 8 and 10 days.

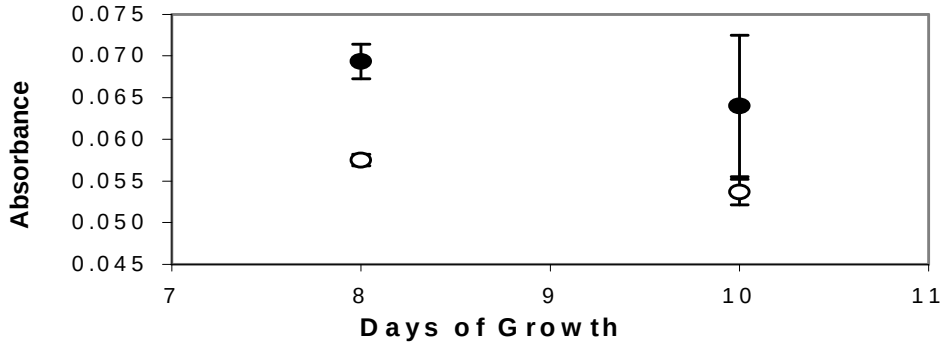

Fig. 3.10 Phenol red transport across Mac-T monolayers after 8 and 10 days of growth. The absorbance values (at 479nm) were taken for cells (○) and blanks (●). Blank wells (non-coated inserts with no cells maintained in culture) were used as a control. Samples were zeroed to DMEM -. Measurements (mean ± S.D., n = 3) are representative of 1 independent experiment.

Shown in figure 3.10, there is little difference in phenol red transport at day 8 and day 10 of cell growth. The blank samples had more phenol red transport in this study

than the other blank samples from the study done with BAECs. This might be expected if surface coating of the inserts. Surprisingly, there is more phenol red transport with the Mac-T cells than with the BAECs in contrast to previous work with this system. This result may explain why Y60L-IGF-I had no effect on ^{125}I-IGF-I transport: the Mac-T cells did not form a very restrictive barrier. More phenol red transport studies should be done with Mac-T cells to obtain more conclusive results. The data shown above is representative of 1 independent experiment.

3.4.6 *Effect of Exogenous IGFBP-3 on IGF-I Transport and Binding*

IGF-I interacts with the cell by its cell surface receptor and binding proteins (3). It is possible that IGFBP-3 added in solution might enhance IGF-I transport. According to the surface enhancement model, IGFBP-3 becomes associated with the cell surface (6). The affinity of IGF-I for the surface bound IGFBP-3 is similar to its affinity for IGF-IR. When IGF-I binds to IGFBP-3 the binding protein encourages interaction of IGF-I and its receptor.

Forsten ***et. al***. (2001) showed that, at pH 7.4, the addition of exogenous IGFBP-3 to the media decreased the binding of IGF-I to the cell surface of both Mac-T and BAE cells cultured on tissue culture plastic at 4 ˚C. The experiments described below were undertaken to determine the effect of the addition of an equal molar concentration of human IGFBP-3 on the transport of IGF-I to BAECs ***in vitro*** cultured on inserts at physiological temperature and pH.

Table 3.2 ^{125}I-IGF-I in basolateral media and cell lysate of BAEC monolayers with an equal molar concentration of IGFBP-3 after 12 hours incubation at 37 ˚C. Studies were initiated by a 12-hour serum starvation period after 7 days of growth. AVG CPM represents the total counts added from each sample. Measurements (mean ± S.D., n = 3) are representative of 3 independent experiments.

^{125}I-IGF-I	Basolateral Media (CPM)	Cell Lysate (CPM)
None	23017.3 ± 1793.7	12425.7 ± 699.8
+IGFBP-3	23293.7 ± 1083.1	9406 ± 106.1

As shown in Table 3.2, the basolateral concentration of ^{125}I-IGF-I was unaltered by the addition of IGFBP-3 to the apical and basolateral chambers after 12 hours of incubation at 37 ˚C. The addition of unlabeled IGFBP-3 did cause a reduction in the amount of ^{125}I-IGF-I in the cell lysate. The results show that an equal molar concentration of IGFBP-3 had no effect on transport. The results from this experiment suggested that a higher concentration of IGFBP-3 might be needed to see an effect. To address this question, IGFBP-3 concentrations used in this study were 6x (same concentration used in the experiment above), 60x and 600x that of ^{125}I-IGF-I. IGFBP-3 was added to the apical and basolateral chambers.

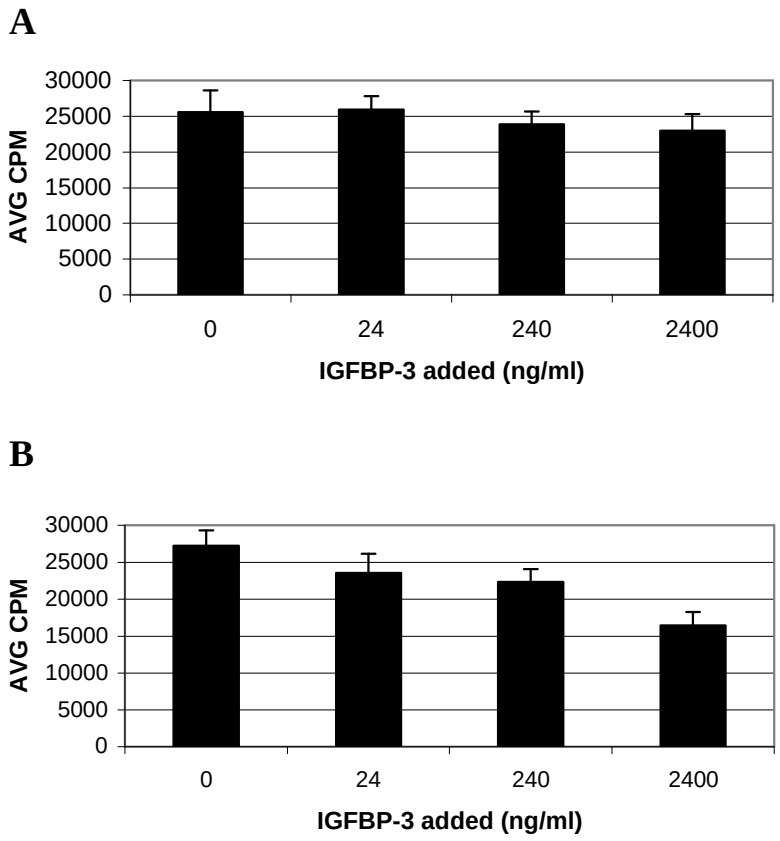

Fig. 3.11 ^{125}I-IGF-I in basolateral media (A) and cell lysate (B) of BAEC monolayers with increasing concentrations of IGFBP-3 after 12 hours incubation at 37 ˚C. Studies were initiated by a 12-hour serum starvation period after 7 days of growth. ^{125}I-IGF-I (4ng/ml) was added to all samples. AVG CPM represents the total counts added from each sample. Measurements (mean ± S.D., n = 3) are representative of 3 independent experiments.

Increasing IGFBP-3 concentration (figure 3.11**A**) had no effect on IGF-I transport ($p > 0.05$); however, it did cause a significant decrease ($p < 0.05$) in cell lysate ^{125}I-IGF-I when IGFBP-3 (2400ng/ml) is added (figure 3.11**B**).

The results from the last experiment showed that IGFBP-3 had no effect on transport and only minimal on cell association. Forsten *et. al.* (2001) indicated that IGFBP-3 has an effect on IGF-I binding at 4 ˚C. The purpose of this experiment was to

determine if IGFBP-3 inhibits cell surface association at 37 ˚C as well as it does at 4 ˚C over the 12-hour incubation period.

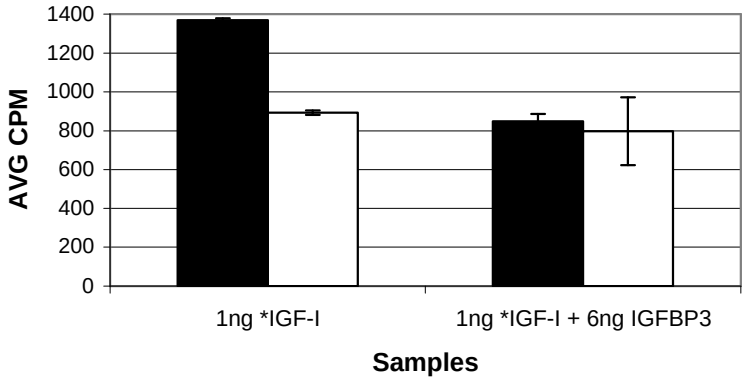

Fig. 3.12 BAE cell surface associated ^{125}I-IGF-I at 4 ˚C (■) and 37 ˚C (□). Studies were initiated by a 12-hour serum starvation period after 48 hours of cell growth. Measurements (mean ± S.D., n = 3) are representative of 3 independent experiments.

From figure 3.12, IGFBP-3 significantly ($p < 0.05$) reduced the amount of ^{125}I-IGF-I bound at 4 ˚C. However, this was not the case at 37 ˚C ($p > 0.05$). IGFBP-3 does not inhibit cell surface association at 37 ˚C as well as it does at 4 ˚C.

The results indicate that IGFBP-3 at physiological temperature has very little ($p > 0.05$) effect on the amount of cell surface associated ^{125}I-IGF-I. There is much less cell surface associated ^{125}I-IGF-I at 37 ˚C than at 4 ˚C in the absence of IGFBP-3 ($p < 0.05$). This result suggests that more material is bound and then internalized, degraded and/or expelled at 37 ˚C than at 4 ˚C. Degradation analysis was undertaken to test this hypothesis.

Table 3.3: Degradation of IGF-I in the presence of IGFBP-3 TCA/PTA precipitation of ^{125}I-IGF-I after 12 hours of incubation with BAECs at 4 °C and 37 °C. Results were calculated as the percentage of sample that was TCA/PTA soluble. Results indicate the mean ± S.D. and are representative of three independent experiments.

^{125}I-IGF-I	4 ^{o}C	37 ^{o}C
None	44.5 ± 2.0%	32.2 ± 0.8%
IGFBP-3	38.4 ± 6.0%	40.1 ± 11.5%

As shown in Table 3.3, the percentage of TCA/PTA soluble material was low at both temperatures. In the absence of IGFBP-3, on average more ^{125}I-IGF-I was TCA/PTA soluble at 4 °C than at 37 °C. This result does not agree with the hypothesis that more ^{125}I-IGF-I is internalized and degraded at 37 °C. This may be due to more association of ^{125}I-IGF-I with IGFBP-3 in solution at 4 °C, which would show up in the TCA/PTA soluble portion. Future studies should include degradation analysis of ^{125}I-IGF-I and IGFBP-3 in solution with no cells after a 12-hour incubation at both temperatures. This would help to quantify that interaction and determine if more association in solution occurs at 4 °C than at 37 °C. Another interesting result from these studies was when IGFBP-3 was present the average amount of ^{125}I-IGF-I was the same for both temperatures. In the presence of IGFBP-3, on average the same amount of ^{125}I-IGF-I was TCA/PTA soluble at 37 °C and 4 °C. These results suggest that the binding is non-specific. Future studies should include an excess amount of unlabeled IGF-I to check for non-specific binding. At 4 °C, IGFBP-3 had no effect on the amount degraded, however, there is more degraded material when IGFBP-3 was present at 37 °C.

3.5 Discussion

3.5.1 *^{125}I-IGF-I Transport across Mac-T and BAE Cell Monolayers*

IGF-I is a polypeptide member of the insulin super family with insulin-like and growth promoting activity in mammalian growth and development (52). The effects of IGF-I on cells are mediated through its specific cell surface receptors (IGF-IRs). Several groups have studied different experimental conditions that affect the interaction between IGF-I and its surface receptor. Some of these conditions are competition for binding sites between labeled and unlabeled ligand (33), addition of exogenous IGFBP (5, 34), and temperature effects (52). Vinayek *et. al.* (1994) explored the effect that temperature had on ^{125}I-IGF-I binding to human esophageal endothelial cells (HEECs). This group determined that at 37 °C, binding of ^{125}I-IGF-I to HEECs was twofold lower than binding at 23°C and fourfold lower than binding at 4°C.

The first studies in this chapter involved quantifying ^{125}I-IGF-I binding to BAECs at physiological and reduced temperatures. The specific binding of ^{125}I-IGF-I to BAECs obtained was the same at both temperatures (figure 3.4**B**), unlike results published from Vinayek *et. al.* This might be due to differences in cell types and experimental procedures. Transport across BAE cell monolayers was also studied. More transport occurred at physiological temperature than at reduced temperature (figure 3.4**A**). This observation is consistent with previous studies (25, 47) that have found that internalization of surface bound ligand appears to be drastically slowed at reduced temperatures.

Another goal of the work in this thesis was to determine if the proposed reservoir function of IGFBPs from computational modeling predictions (33) facilitated overall

transport of ^{125}I-IGF-I. This involved quantifying ^{125}I-IGF-I transport and binding across Mac-T and BAE cell monolayers under pulse conditions. The results from the competitive binding experiments in this thesis show that during the pulse and chase periods, Y60L-IGF-I added during the pulse enhances ^{125}I-IGF-I transport across BAEC monolayers (figure 3.8**A** and 3.8**B**) whereas Y60L-IGF-I had no effect on transport across Mac-T cell monolayers (figures 3.9**A** and 3.9**B**). The addition of Y60L-IGF-I to initiate the chase period resulted in a decrease in ^{125}I-IGF-I transport across BAECs (figure 3.8**B**) and Mac-T cells (figure 3.9**B**). Cell surface binding was also decreased when Y60L-IGF-I was added to initiate the chase for both BAECs (figure 3.8**C**) and Mac-T cells (figure 3.9**C**). Rao *et. al*. (2004) also investigated a cell surface reservoir effect. In their work, they hypothesized that the Interleukin 2 (IL-2) cell surface receptor on activated cells may act as a reservoir for IL-2 in the circulation leading to a prolonged persistence of IL-2 signaling. Under pulse conditions, surface associated wild-type IL-2 was negligible after 30 minutes, however mutant IL-2 with increased affinity for receptors persisted on the cell surface after 6 hours.

3.5.2 *Effect of Exogenous IGFBP-3 on ^{125}I-IGF-I Transport*

IGF-I in the bloodstream is regulated by its binding to IGFBPs, of which six have been identified (34). IGFBP-3 is the predominant IGFBP in the circulation. IGF-I can bind to IGFBP-3 to form binary complexes or with IGFBP-3 and a third protein (the acid-labile subunit) to form ternary complexes in the circulation. It is suggested that these ternary complexes are prevented from leaving the circulation due to their size, which reduces their bioavailability (5).

Payet *et. al.* (2004) studied the effect that IGFBP-3 had on ^{125}I-IGF-I transport across human umbilical vein endothelial cells (HUVEC). This group found that the

preincubation of ^{125}I-IGF-I with IGFBP-3 before adding to HUVEC monolayers did not have a significant effect on ^{125}I-IGF-I transport. The last set of experiments in this thesis also involved determining the effect of exogenous IGFBP-3 on ^{125}I-IGF-I transport. Addition of equal molar exogenous IGFBP-3 showed no effect on transport (Table 3.2) however, it significantly reduced the amount of surface bound ^{125}I-IGF-I (Table 3.2). Higher concentrations of IGFBP-3 also had no effect on transport (figure 3.11**A**), but surface bound ^{125}I-IGF-I was significantly reduced (figure 3.11**B**). These results are in agreement with Payet ***et. al.*** in that the addition of IGFBP-3 has no significant effect on transport. However, Payet ***et. al.*** found that when ^{125}I-IGF-I was preincubated with IGFBP-3 and acid-labile subunit (ALS), ^{125}I-IGF-I transport across the HUVEC monolayers was significantly reduced. This group concluded that the ternary complex with ALS and IGFBP-3 was an essential step in inhibiting ^{125}I-IGF-I transport across HUVEC monolayers.

3.6 Conclusions and Future Work

The pulse studies described in this thesis should be continued to determine why the ***in vitro*** experiments described here did not agree with the proposed reservoir function of IGFBPs. There are several possible explanations. One, this proposed function may not be correct. However, before this can be concluded, one should try to resolve a few experimental issues. First, surface coating in the BAEC experimental system may need to be revisited. In previous work (33), optimal plating and coating conditions for BAECs were found that minimized paracellular transport. The work in this thesis followed this coating and plating process, however, a different attachment factor was used in the coating process of this work. Although fibronectin was proven to be a sufficient

attachment factor for BAECs in the coating process, superfibronectin, as used in previous work, should be used in future experiments. Superfibronectin dramatically enhances (20x) the adhesive properties of fibronectin and suppresses cell migration, as noted by the manufacturer (Sigma). Phenol red transport studies show that there is a small difference in absorbance values at days 8 and 10 of cell growth (figure 3.6). However, this difference at day 8 is much less than has been reported for endothelial cells in previous work using superfibronectin (33). Increased culture time also did not lead to a greater difference in absorbance. Second, more phenol red transport studies need to be done for the Mac-T cell experimental system. As shown in figure 3.10, more phenol red transport was exhibited by Mac-T cells than by BAECs. Third, regular TCA/PTA precipitation analysis should be done on stock radiolabeled material. This will help to keep track of the radiolabel to make sure that there is not a significant amount of free ^{125}I in the stock material. Free ^{125}I can contribute to paracellular transport and skew transport results. Another recommendation would be to rework the computational model. Different pulse and chase incubation periods should be investigated to determine the optimal time when IGFBPs would display a reservoir effect.

Future studies involving IGFBP-3 should include degradation analysis of ^{125}I-IGF-I and IGFBP-3 in solution with no cells after a 12-hour incubation at both temperatures. This would help to quantify that interaction and determine if more association in solution occurs at 4 °C than at 37 °C. Another recommendation would be to preincubate ^{125}I-IGF-I with IGFBP-3 and ALS to determine if this ternary complex inhibits ^{125}I-IGF-I transport across BAEC and Mac-T cells as well as it does across HUVEC monolayers.

Chapter 4: Conclusions and Future Work

4.1 Introduction

Two important aspects of cellular events, ligand surface binding and transport processes were investigated in this work. Competitive binding experiments between ^{125}I-FIX and unlabeled FIX and ^{125}I-IGF-I and unlabeled proteins (IGF-I, IGFBP-3 and Y60L-IGF-I) were used to monitor and quantify binding and transport. BAE and Mac-T cell lines were used as model systems to investigate these cellular events. The studies presented in this masters thesis are a continuation of previous research in the Williams laboratory at Virginia Tech and the Velander and Van Cott laboratories now at the University of Nebraska-Lincoln.

This chapter summarizes the studies performed for this thesis and suggests recommendations for future work. The conclusions from the FIX study are presented first, followed by those drawn from the IGF-I transport study. The thesis is closed with lessons learned.

4.2 ^{125}I-FIX binding to cultured BAECs and Human Collagen Type IV

These experiments focused on FIX interactions with BAECs and extracellular proteins. To quantify this interaction, FIX was radiolabeled using the standard chloramine T method. The preliminary binding studies with chloramine T-labeled FIX suggested specific binding of FIX to BAECs, however, the accuracy check with duplicate sample sets showed large differences in binding, making the results suspect. It was suspected from these preliminary studies that there might be issues with protein activity. The biological activity of unlabeled and labeled FIX was checked in a coagulation time assay. Results from this assay indicated no clotting activity for the labeled FIX

suggesting that the FIX was degraded or deactivated during the radiolabeling process. Most likely some of the starting material was lost during the radiolabeling process. More FIX was labeled using Iodobeads® as done in previous work by Cheung *et. al*. (1991). The biological activity of the Iodobeads®-labeled FIX was checked and results indicated that the FIX maintained its activity. Competitive binding studies were resumed using the newly labeled FIX to try and obtain similar results as in previous work by Stern *et. al*. (1983). Results indicated some specific binding, but it was much lower than published results from previous work. Cheung *et. al*. (1996) reported that one specific binding partner for rFIX was human collagen IV. Binding studies were then pursued in a cell free assay using human collagen IV coated removable wells. Results from this experiment showed no binding of FIX to the collagen IV coated wells. Size exclusion chromatography (SEC) was attempted as a quick and cost effective way to determine if the ^{125}I-FIX bound to collagen IV. Preliminary studies with SEC were unsuccessful due to some technical issues related to gel selection and flow issues.

Unfortunately the results from previous work (10, 45) were not repeatable in this project. There were several technical issues involved in these experiments that may be the cause of these undesirable results. In the work by Cheung *et. al*. (1991), 100µg of FIX were radiolabeled using the Iodobeads® method whereas only 5µg of FIX was radiolabeled for use in the experiments of this project. For future studies, this amount of FIX should be labeled using Iodobeads®. This should help to avoid losing a significant amount of protein during the labeling process. Concerning the cell free assay using collagen IV coated plates; the wells should be checked for attachment of the collagen. An ELISA would help in detecting collagen IV attachment to the tissue culture plastic.

Another assay to quantify FIX binding to collagen IV is Surface Plasmon Resonance. This assay might prove convenient because the FIX would not undergo the radiolabeling process and therefore no product is lost before performing an experiment.

4.3 Transport of IGF-I across cultured Mac-T and BAE Cell Monolayers

These experiments focused on cell surface binding and transport processes of IGF-I. Previous research at Virginia Tech quantified the binding, internalization and transport kinetics involved in trafficking IGF-I across *in vitro* monolayers of BAECs. The studies described in this thesis are a continuation of that work. Preliminary studies involved competitive binding experiments at reduced and physiological temperature to determine the effect of temperature on IGF-I transport and to repeat results from previous work (33) at 37 ˚C. Results indicated that a reduction in temperature led to decreased transport. Also, addition of unlabeled IGF-I lead to increased transport at 37 ˚C as it did in previous work. This result suggested that the different attachment factor, fibronectin instead of superfibronectin, had no adverse effect on binding and transport. A phenol red study was undertaken concurrently with the binding experiment to determine the paracellular transport resulting from fibronectin-coated inserts at different growth periods. Results from this study indicated a small difference in absorbance values between blank and cell monolayer samples for both days of cell growth. It was determined that longer periods of cell growth were not advantageous due to similar absorbance values as shorter growth periods. After confirming that the different attachment factor did not affect transport and binding, competitive binding experiments between ^{125}I-IGF-I and its analog Y60L-IGF-I under pulse conditions were performed. The goal was to determine if experiments could be designed, based on computational

predictions (33), to show enhanced overall transport of IGF-I due to the presence of IGFBPs. Results from these experiments indicated that removing IGFBPs enhanced overall IGF-I transport through BAE cell monolayers. This result was contrary to computational predictions, so the pulse-chase experiments were performed using Mac-T cells as the model system. The idea behind this was that Mac-T cells should exhibit less paracellular transport and hence be a better model for distinguishing small effects in transport and binding. Surprisingly, the results from these studies indicated that the removal of IGFBPs during the pulse period had no effect on transport during the chase. A phenol red study done with the Mac-T cells indicated more paracellular transport for these cells than the BAECs in contrast to earlier work suggesting cell culture problems existed. The last set of experiments in this study looked at the effect of exogenous IGFBP-3 on IGF-I transport and binding to BAECs. Results indicated that an equal molar amount of IGFBP-3 had no effect on transport and a minimal effect on surface associated IGF-I. Furthermore, increasing the concentration of IGFBP-3 had no effect on transport but a higher concentration (2400ng/ml) significantly reduced the amount of surface associated IGF-I. Competitive binding studies at 4 ˚C and 37 ˚C were done to determine if IGFBP-3 inhibits surface binding of IGF-I at 37 ˚C as well as it does at 4 ˚C. Results indicated that at 4 ˚C, IGFBP-3 significantly reduced surface bound IGF-I, however, at 37 ˚C, IGFBP-3 does not inhibit binding.

The pulse studies described in this thesis should be continued to determine why the *in vitro* experiments described here did not agree with the proposed reservoir function of IGFBPs. There are several possible explanations. One, this proposed function may not be correct. However, before this can be concluded, one should try to resolve a few

experimental issues. First, surface coating in the BAEC experimental system may need to be revisited. In previous work (33), optimal plating and coating conditions for BAECs were found that minimized paracellular transport. The work in this thesis followed this coating and plating process, however, a different attachment factor was used in the coating process of this work. Although fibronectin was proven to be a sufficient attachment factor for BAECs in the coating process, superfibronectin, as used in previous work, should be used in future experiments. Superfibronectin dramatically enhances (20x) the adhesive properties of fibronectin and suppresses cell migration, as noted by the manufacturer (Sigma). Phenol red transport studies show that there is a small difference in absorbance values at days 8 and 10 of cell growth (figure 3.6). However, this difference at day 8 is much less than has been reported for endothelial cells in previous work using superfibronectin (33). Increased culture time also did not lead to a greater difference in absorbance. Second, more phenol red transport studies need to be done for the Mac-T cell experimental system. As shown in figure 3.10, more phenol red transport was exhibited by Mac-T cells than by BAECs. Third, regular TCA/PTA precipitation analysis should be done on stock radiolabeled material. This will help to keep track of the radiolabel to make sure that there is not a significant amount of free ^{125}I in the stock material. Free ^{125}I can contribute to paracellular transport and skew transport results. Another recommendation would be to rework the computational model. Different pulse and chase incubation periods should be investigated to determine the optimal time when IGFBPs would display a reservoir effect.

Regarding future studies with exogenous IGFBP-3, they should include degradation analysis of ^{125}I-IGF-I and IGFBP-3 in solution with no cells after a 12-hour

incubation at both temperatures. This would help to quantify that interaction and determine if more association in solution occurs at 4 °C than at 37 °C. Another recommendation would be to preincubate ^{125}I-IGF-I with IGFBP-3 and ALS to determine if this ternary complex inhibits ^{125}I-IGF-I transport across BAEC and Mac-T cells as well as it does across HUVEC monolayers.

I have learned a great deal while working on this masters thesis. I've learned that technical issues will arise with any type of research and to not let that discourage and hinder your progress. I've also learned that there is more to doing an experiment besides working in the lab and crunching numbers. There is a lot of preparation that goes into each experiment such as reading through current literature to keep abreast of the latest techniques and discoveries. Learning the theory and logic behind each experiment is important. Envisioning each step of the procedure, knowing its purpose before hand and making sure that everything makes sense. When analyzing data I've learned to sit and think about what I am analyzing, what it tells me about the experiment and how the data will help me to improve the experiment and my technique.

It is unfortunate that I had to face so many issues with my results, but that is the nature of scientific research. I hope that the next person who takes on this project will learn from this thesis as I have done with so many others and improve upon what I have done thus far. I hope that the recommendations that I gave in this chapter will help the next person find success with these experiments.

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VITA

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Upon graduation from Kettering University, Amanda began her graduate studies in the fall of 2002 at Virginia Polytechnic Institute and State University in Blacksburg, VA. Upon completion of her Masters of Science degree in Chemical Engineering May 2005, she plans to pursue a PhD in Chemical Engineering at the University of Nebraska – Lincoln.