Characterization of Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) interaction with the Bovine Aortic Endothelial (BAE) cell surface : Examination of the Role of Heparan Sulfate Proteoglycans (HSPG).

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(ABSTRACT)

Insulin-like growth factor binding proteins (IGFBPs) are known to be important modulators of the insulin-like growth factor (IGF-I). However, their precise role is as yet unclear. Further, recent studies have indicated that IGFBP-3 has a receptor mediated growth inhibitory response of its own. In the present study, we quantified the binding characteristics of IGFBP-3 to bovine aortic endothelial (BAE) cells. Binding studies at 4 ^{o}C were conducted and a specific binding curve for IGFBP-3 was obtained. IGFBP-3 was found to bind with an equilibrium dissociation constant (K_D) value of 3.1 x 10^{-10} M. The role of heparan sulfate proteoglycans (HSPG) in the IGFBP-3 binding mechanism was also examined. It was seen that inactivation of the cell surface HSPGs with 75 mM sodium chlorate did not affect IGFBP-3 binding. Further, there have been reports of inhibition of IGFBP-3 binding by heparin in the media. Hence, the most probable interaction of HSPG with IGFBP-3 occurs in the extracellular region, with soluble HSPGs acting as receptors for IGFBP-3 and decreasing the net cell associated ligand receptor interaction. This is likely, since IGFBP-3 is known to possess a heparin binding domain. Simultaneous introduction of IGF-I and IGFBP-3 into the extracellular media decreased IGFBP-3 binding to the cell surface, which might imply that IGF-I and IGFBP-3 regulate each other's action.

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Chapter I : Introduction

1.1 Biotechnology : an interdisciplinary science :

Biotechnology concerns applied bioscience and technology and involves the practical application of biological organisms, or their subcellular components to manufacture and service industries and to environmental management. Successful application of biotechnology can only be achieved through the integration of several scientific disciplines including biochemistry, microbiology, genetics, molecular biology and chemical and process engineering (J. E. Smith, 1985).

Biotechnology is an ancient science: microorganisms have been used to produce a variety of products (eg ethanol) for over 8 millennia. After starting out primarily as an industry oriented field, the science of biotechnology has been impacted greatly by the rapid advancements in biology. With the help of techniques from molecular biology, the amount of a particular product generated by a microorganism can be altered. Changes can also be affected in various other properties such as adhesion to the reaction vessel and motility of the organism. The development of a powerful biological tool, recombinant DNA technology, has helped give rise to the new field of molecular biotechnology (Primrose, 1991). Genetic information can now be exchanged between cells of different origins. This for example, made possible the production of valuable commercial products in simple microbes such as bacteria and algae. An immediate application of this nascent field was in the health care industry. Therapeutic proteins such as insulin, factor VIII, and human growth hormone are being manufactured by recombinant DNA technology. Gene therapy is being used to treat patients with enzyme deficiencies, cancer, and other diseases (Kamely and Klumer, 1985). The last few years have seen a rapid evolution of this specialized biotechnology in two distinct areas of effort. The primary focus has been in the post development, industrial application oriented pharmaceutical industry (Moses and

Cape, 1991), where lab ideas are tested by way of clinical trials, or pilot plant productions. The second area of effort has been the development of therapeutic agents.

The primary function of a drug is to elicit a certain favorable reaction from the human body by interacting with it at the molecular level. Very often, the interaction is via cell surface binding between a transmembrane protein and the drug. It is precisely this interaction of a molecule (ligand) with other cell surface entities (receptors) that we are interested in as researchers in this field. Figure 1.1 shows a typical interaction between a ligand and a cell surface receptor. Insulin-like growth factor-I (IGF-I) is one such ligand for which the overexpression in mammals can lead to unchecked malignant cell growth (Werner and LeRoith, 1997). We chose to investigate the conditions in the extracellular environment which could regulate the binding of this growth factor.

After administration, a drug should reach a target area and bind to the corresponding cells in such a way as to block an existing response or elicit a new one. A rational method for the design of such agents is dependent partly on whether the corresponding binding sites on the cell surface are known (Dean, 1987). If no prior knowledge of the binding site structure exists, a recourse to mathematical deductions has to be made. The behavior of similar compounds is examined, and statistical predictions as to the potency of the artificial molecule are made. In cases when the receptor and the corresponding molecular structure are known, drug design is primarily focused on structural characteristics required for the ligand to fit the known binding site. Thereafter, this complex can be characterized with respect to parameters such as interaction energy, binding affinity, and kinetic association and dissociation rates.

Several engineering considerations must be kept in mind for optimal drug delivery. As with natural ligand receptor interactions, artificial compounds may not always act in isolation (Lauffenburger and Lindermann, 1993). There may be entities present on the cell surface or in the media, which regulate the binding of a drug to a receptor. An alteration in the level of these compounds may in turn affect the response elicited by the artificial

ligand. In fact, reasonably good control of drug response can be achieved by a two fold approach - (a) an accurate quantification of the concentration profiles for each of these known secondary regulators, and (b) an analysis of the effect on these profiles of a variation of physical and chemical conditions such as pH and temperature.

Transport phenomena such as diffusion and mass transfer must also be taken into consideration. For instance, in the case where the drug may interact with a soluble receptor present in the extracellular environment, the diffusional effects and the overall kinetics of ligand-surface receptor interaction will be affected. A differential mass balance would need to be constructed, which, based on certain assumptions, may yield an analytical form of the ligand concentration profile (Goldstein *et al.*, 1989). The amount of soluble receptor can then be varied as a parameter, and the resulting impact on ligand concentration profile and kinetics can be determined. Thus, simple engineering analysis can lead to an accurate quantification of purely biological phenomena, an approach which may be pivotal in understanding cellular processes as a whole.

One of the most powerful and elegant approaches to drug design is to model an artificial compound after a natural ligand (Lauffenburger and Lindermann, 1993). Receptor ligand interactions are very specific. However, this specificity is usually restricted to certain areas of the molecule which are deemed “active” for that reaction. Ligands which cause undesirable effects on the body have this same characteristic. An effective blockage of the binding site can be achieved if somehow a molecule is constructed, which bears structural similarity with the natural ligand. However, it must have a modified set of “active sites” so that it is not able to elicit the same response. Hence, the undesired ligand can be “competed off” by introducing an excess quantity of this engineered molecule. These highly specific modifications are usually achieved by having available a database of structurally similar molecules which serve as chemical starting points, and specific reactions applicable to them (Kenekin, 1993). A sophisticated computer program can then be employed which picks out the reactions required and their sequence to affect the modification of choice.

Understanding how the extracellular environment affects IGF-I activity may lead to the design of therapeutic agents which would help to modulate the cellular response to IGF-I. Clearly, various techniques, ranging from basic molecular biology methods, concepts from endocrinology and biochemistry, to mathematical analysis and engineering design as discussed above would need to be employed. Thus, it is important for the investigator to have a diverse background in order to be effective in this interdisciplinary field.

1.2 Cellular Signaling - basic information :

Many higher order organisms develop organized tissues to allow specialized functions to be performed. How the growth of these tissues is regulated, and what the underlying mechanisms are by which the constitutive cell type responds to changes in their immediate environment are important questions. Most of these processes are governed by extracellular signals and certain specific ligand-receptor interactions which may be dependent on the surroundings.

Recent research in cellular signaling has lead to several such mechanisms being uncovered in a variety of cellular systems, such as at the membrane surrounding the human fetus (Han *et al.*, 1996) and the rat brain (Walter *et al.*, 1997). Moreover, it has been seen that as far as mammalian based systems are concerned, these processes broadly involve certain common features of the cell and have similar modes of operation (D'Ercole, 1996, Binoux, 1996, Oh, 1998). The most important of these features are detailed below.

The plasma membrane of a mammalian cell is a semi-permeable barrier enclosing the subcellular organelles and cytoplasm. Specifically, it is a bilayer of phospholipid molecules. The lipids are arranged in the form of a double leaflet, with the hydrophilic ends facing outside. Thus, the hydrophobic interior of the bilayer helps to partition the

extracellular medium and the cytosol, which are both aqueous in nature. The phospholipids are mobile and, within the same sublayer, can freely diffuse (Lodish *et al.*, 1993). This phenomena, as we shall see later, proves to be very helpful in the regulation of the levels of the binding sites of receptors on the cell surface (Jacobsson *et al.*, 1990, Lauffenburger and Lindermann, 1993, Robertis, 1987). Another important feature of the membrane is that, depending on the cell type, different parts are specialized for performing different tasks. For example, the epithelial cells lining the intestine, have specific ion channels on the surface to absorb particular nutrients. The membrane is critical for regulating the crossflow of nutrients, and can serve numerous other functions such as: maintaining cell shape via anchoring of actin filaments and microtubules, catalyzing vital biochemical reactions occurring inside the cell and regulating the pH within the cell (Lodish *et al.*, 1993). The most relevant feature to our work is the presence of large transmembrane molecules, known as receptors, which can act as binding sites for extracellular signaling molecules.

1.2.1 Receptors

A receptor is defined as a protein on the surface of the target cell or in the nucleus or cytoplasm, which binds with high affinity to a particular signaling substance and elicits a biological response (Lodish *et al.*,1993). Figure 1.1 depicts a typical interaction between a signaling molecule and a receptor. There may be found a specific set of receptors which respond to their own particular ligand. When the ligand binds to the receptor, dimerization may occur (D'Ercole, 1996), or the receptor may be activated via comformational change (Yvonne *et al.*, 1996). Signal transduction and response generation via a sequence of secondary messengers may follow (Lauffenberger and Lindermann, 1993). It has generally been seen that most fat soluble (lypophilic) ligands are able to permeate through the plasma membrane and signal by binding to proteins within the nucleus or cytosol (Picard, 1998). Water soluble or hydrophilic ligand, on the other hand, are seen to bind to receptors present on the surface of the cell, owing to the

fact that they are unable to diffuse through the hydrophobic region inside the plasma membrane (Lodish *et al.* ,1993).

Surface receptors fall into distinct classes, depending on their mode of action, with ion channels and protein kinase receptors being the primary types (Lodish *et al.*,1993). With ion channels, as shown in figure 1-6, the ligand binding induces a change in the conformation of the receptor, causing it to open a specific channel in the protein. With protein kinase receptors (figure 1-7), binding causes the receptor to either phosphorylate a substrate protein thereby altering the activity of that protein, or even autophosphorylate the receptor itself. A particular subclass of relevance are the protein tyrosine kinase receptors, where a tyrosine residue of the substrate protein (or the receptor itself), is phosphorylated (Lauffenberger and Lindermann, 1993). This substrate protein often acts as a secondary messenger. Signal transduction may also occur via guanine nucleotide binding proteins, or G-proteins (Lauffenberger and Lindermann, 1993). Coupling of a quiescent G-protein with a bound receptor leads to release, following GTP binding to the ternary complex, of a form of G-protein which is capable of regulating particular intracellular proteins.

The number of receptors on the cell surface is variable (Haggis, 1987). In order to elicit the right magnitude of response in the presence of a large concentration of the signaling ligand in the extracellular environment, the receptors may be upregulated or downregulated (Lodish *et al.*,1993). Downregulation most commonly takes place by the process of receptor mediated endocytosis (Lauffenberger and Lindermann, 1993). After the ligand and receptor bind, a group of these complexes aggregate at a particular location of the cell surface, and are internalized via a coated or smooth pit formation . The coating refers to a lattice made of the protein clathrin in the cytoplasmic side of the membrane at the location of the pit structures (Cooper, 1997). This clathrin coat, if present, is removed upon internalization, and the complexes aggregate in organelles called endosomes. Subsequently, the receptor may be separated from the signaling molecule and is then either degraded in the lysosome (a cellular organelle), recycled back to the cell surface, or is

stored in vesicles for future up-regulation processes (Lauffenberger and Lindermann, 1993). An alternate method for regulation could involve either introducing a conformational change so that the receptor is unable to bind to the ligand, or allowing a receptor-ligand complex to form but not allowing the complex to elicit the desired cellular response.

1.2.2 Ligands

Ligands are primarily soluble elements within the extracellular fluid, which initiate a cell signal (Haggis, 1987). Binding is extremely sensitive, with ligand concentrations on the order of .01-1 nM able to initiate the signal process. The method of signaling by hydrophilic ligands falls into four major classes : endocrine (figure 1-2), autocrine (figure 1-5), paracrine (figure 1-3) and juxtacrine (figure 1-4). Endocrine signaling, is when the ligand is released into the blood stream by a cell which is very distant from the target tissue (Norman, 1987). The ligand, or hormone as it is often called in this case, can be bound to certain other molecules which may help protect it from enzymatic or other forms of degradation in the blood stream. In contrast, autocrine signaling involves a generation of signaling molecules by the target cell itself (Robertis, 1987). Autocrine binding generally occurs extracellularly although intracellular binding is a possibility. This type of signaling is thought to play a role in both normal and malignant signaling (Keiss *et al.*, 1997). The idea that a cell may secrete its own signaling compounds underlines the importance of understanding signaling process. The third type is paracrine signaling. Paracrine signaling occurs when the source and target cells are in close proximity to each other. This is relevant during embryo development (Han *et al.*, 1996). Juxtacrine is the fourth type where the two cells are directly adjacent to each other (Lauffenburger and Lindermann, 1993). This type of signaling does not involve soluble ligand but an interaction between two surface molecules.

The regulation of ligand concentration levels is achieved via a complex feedback system, which may also affect other growth factors (Dunaiski, 1997). This is important since, in

the most general case, growth and differentiation may be regulated by a group of ligands, as opposed to regulation by a single molecule. For example, changes in the level of growth hormone regulate the expression of IGF-I.

1.2.3 Ligand Regulators :

In addition to the basic components of a general signaling system discussed above, there are other molecules which play an important role in this process. For instance, a growth factor may not always function in isolation but may be assisted or hindered by other extracellular molecules. This may be achieved by direct interaction with the ligand and transportation to the receptor binding site where dissociation from the complex and potential receptor binding could occur. There may also be a more complicated interaction where an extracellular moiety helps to disrupt the binding between the growth factor and a previously bound protein by causing either conformational changes, or by binding to one of the two molecules directly. Intense research continues in this area and numerous binding proteins are being discovered at a rapid rate (Oh *et al.,* 1996). Some such proteins have also been observed to elicit cellular responses on their own, as seen in the IGF-1 system (Oh *et al*, 1993, Oh, 1997, Oh, 1998). An exciting area of research is the analysis of such responses and the attempt to isolate the receptors which mediate them.

Proteoglycans (PG) are another important type of ligand regulator (Arai *et al.*, 1994, Kjellen and Lindahl, 1991). These versatile glycoproteins are ubiquitous and are found in the extracellular matrix and the cell surface of all mammalian cells (Jolles, 1994). They were initially thought to provide simple support and definition for tissues, but over the last two decades, have been shown to be mediators of several important cellular events. Their basic structure, as shown in figure 1-8 and 1-9, consists of a core protein backbone to which at least one polysaccharide chain called a glycosaminoglycan (GAG) is connected. These chains are repeats of disaccharides consisting usually of uronic acid and n-acetylglucosamine. How their length is determined is still unknown (Lodish *et al.,* 1993). An important feature of these molecules is their large negative charge (Wight and

Mecham, 1987), which is due to the fact that the sugars on the GAG chains tend to be sulfated. Most functions of the PGs are related to this negative charge. In particular, cell surface PGs help anchor cells to the extracellular matrix by binding to collagen or other matrix molecules via charge interactions (Wight and Mecham, 1987). The primary GAGs are keratan sulfate, dermatan sulfate, chondroitin sulfate, and heparin sulfate (Jolles, 1994). Proteoglycans are classed by the type of the disaccharide repeat unit present in the GAG chains, but the name is related to the protein core. The study of proteoglycans forms a separate branch of research, and novel functions for these molecules are being uncovered in increasingly diverse systems.

1.3 Specific information on the IGF-I system :

Insulin-like growth factor (IGF-I) is a powerful mitogen for cellular proliferation and differentiation and has been implicated as a factor in some types of malignancies (Schofield, 1992). It is member of a larger family of insulin-related peptides which includes insulin, IGF-I, IGF-II, and relaxin (Spencer, 1991). It is likely these molecules evolved from a common ancestor, and that differences in sequence have resulted in unique functional roles. Structurally, IGF-I is a single chain polypeptide consisting of 70 amino acid residues with a predicted molecular weight of 7600, and an isoelectric point of 8.8 (Schofield, 1992). IGF-I was originally thought to function primarily as an endocrine signaling factor, but it has recently been observed that most target tissues could also act as sources for IGF-I. In fact, a recent speculation is that the IGFs seem to have a functional similarity with other growth factors such as the transforming growth factor-β (TGF-β) and the platelet derived growth factors, which possess both autocrine and endocrine modes of action (Spencer, 1991).

IGF-I is primarily found *in vivo* in the serum circulating as a ternary complex comprised of a binding protein, insulin-like growth factor binding protein-3 (IGFBP-3), and an acid labile subunit (ALS) (Clemmons, 1997), which is analogous to other endocrine hormones (Spencer, 1991, Delafontaine *et al.*, 1996, Conover, 1996). On reaching the target tissue,

the growth factor, by some unknown mechanism, dissociates from the complex. The IGF-I initiates a signal by binding to its receptor (IGFR-I).

IGFR-I, the receptor for IGF-I, is a transmembrane tyrosine kinase which exists as a tetramer of two 135 KDa alpha subunits and two 95 KDa beta subunits. Upon ligand binding, two such receptors dimerize and substrate secondary messengers, such as cAMP, (Cyclic Adenosine Monophosphate) Ca^{+2} and phosphate metabolites within the cytoplasm, are phosphorylated (Li *et al.,* 1997). The insulin-like growth factor binding proteins (IGFBP) provide important regulation of IGF-I. IGFBP is a family of at least 6 binding proteins which collectively serve a variety of functions to regulate IGF-I activity. IGFBP-3 is the member we focused on. It is glycosylated with a size of 47 KDa. Evidence indicates that IGFBP-3 can inhibit (Oh *et al.,* 1993, Oh, 1997, Oh, 1998) and potentiate (Yvonne *et al.,* 1996) IGF-I regulated cellular responses. Recent studies have proposed that IGFBP-3 activity is governed by the extracellular environment (Yvonne *et al.,* 1996). The situation is further complicated by the observation that IGFBP-3 elicits a growth inhibitory response independent of IGF-I in certain cell lines (Oh *et al.,* 1993, Oh, 1998). An obvious implication of this is the question whether this event is receptor mediated and, if so, what is the putative receptor.

Heparan sulfate proteoglycans (HSPG) have been implicated as the surface receptors for IGFBP-3 (Yvonne *et al.,* 1996). Though HSPGs have been shown to be an important factor in IGFBP-3 binding, their role as receptors has not been proven. There has also been a recent claim that type-V TGF-β receptors may be putative receptors for IGFBP-3 (Leal 1997). Here, recombinant human IGFBP-3 was shown to inhibit TGF-β binding to type-V TGF-β receptor in mink lung epithelial cells. Further, the approximately 400 KDa ^{125}I-IGFBP-3 affinity labeled putative IGFBP-3 receptor was immunoprecipitated by specific antiserum to the type V TGF-β receptor. Finally, the affinity labeled putative receptor could only be detected in cells expressing the type V TGF-β receptor, but not in cells lacking the type V TGF-β receptor. These results are strong evidence that IGFBP-3 is a functional ligand for the type V TGF-β receptor. However, this finding is restricted to

only the mink lung epithelial cell line ; there has been no evidence to date of similar results in a different system. Further, a recent study by Mohseni-Zadeh and Binoux (1997) has shown that in competitive binding experiments, IGFBP-3 was as potent as unlabelled IGF-I in displacing its truncated analogue, ^{125}I-des(1-3)IGF-I, which has weak affinity for IGFBPs, from its binding to the cell surface. This may imply that IGFR-I may function as a dual receptor for IGF-I and IGFBP-3. Thus, the question of the putative receptor for IGFBP-3 is still an open one, and awaits further research.

Although the IGF-I system is complex, insight gained from its study will be useful in not only increasing our understanding of cellular proliferation and differentiation, but also will be applicable to health care situations. A better understanding of how this growth factor works may lead to the design of therapeutic agents, which could be tested by way of clinical trials.

1.4 The Bovine Aortic Endothelial Cell system :

In the case of endocrine signaling, after a growth factor is released into the blood stream, the protein must somehow traverse through the wall of the blood vessel if it is to reach a particular target tissue. Of the several layers of cells in a blood vessel, the endothelial cell layer lines the lumen. Growth factors may bind to receptors on the surface of the endothelium (the layer of endothelial cells), and be internalized. They may undergo the process of transcytosis and be transported through the cytoplasm to the underlying tissue (Haggis, 1987). During malignancy, presence of these proteins could cause excessive cellular proliferation in the target tissue. Clearly, an effective strategy to control this unchecked cell growth is to try and limit the transcytosis of the growth factors through the endothelium. In order to do this, a working knowledge of the molecular interactions taking place at the endothelial cell surface are imperative. To develop such an understanding of the IGF-I system, we chose the bovine aortic endothelial cell system.

This was preferred over a human based system because of availability, ease of culture, and reduced cost in maintaining the cells in culture.

1.5 Specific Aims :

Our basic hypothesis was that IGF-I regulation by IGFBP-3 is influenced by HSPGs, either on the surface or as soluble molecules in the extracellular environment. Using both cell binding and cell free experiments, we wanted to examine the influence of HSPGs on IGF-I and IGFBP-3 binding characteristics. It has been seen that IGFBP-3 can elicit a response of its own and that this signal is receptor mediated (Oh *et al.*, 1993, Oh *et al.*, 1995, Oh, 1997, Oh, 1998). We studied the interaction of IGFBP-3 with BAE cells and attempted to quantify the interaction. Following this, our hypothesis of the role of HSPGs as regulators of IGFBP-3 activity was tested by use of chloration studies.

The text is organized as follows. Chapter II discusses the various materials and methods used in the experimental procedures. A detailed analysis of the cell based assays used, and a discussion of the corresponding results is given in Chapter III. Chapter IV describes the different cell-free experiments conducted. Finally, a summary of the research done and a glimpse of future directions are given in Chapter V.

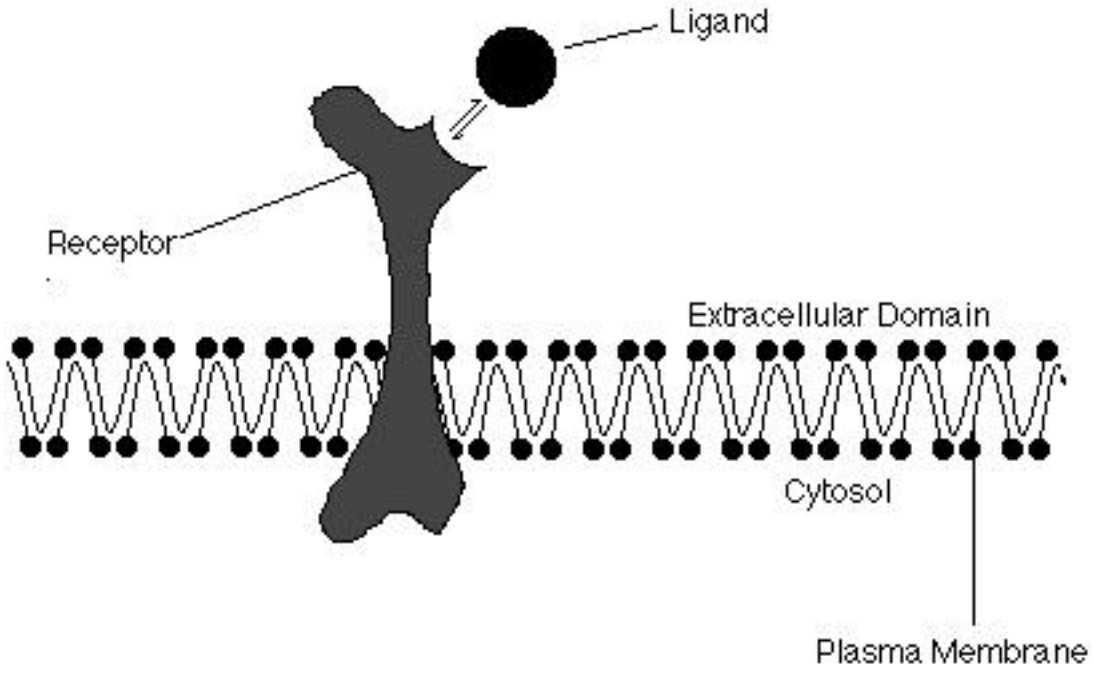

Fig 1.1 A typical ligand receptor interaction. (Adapted from Lauffenberger and Lindermann, 1994).

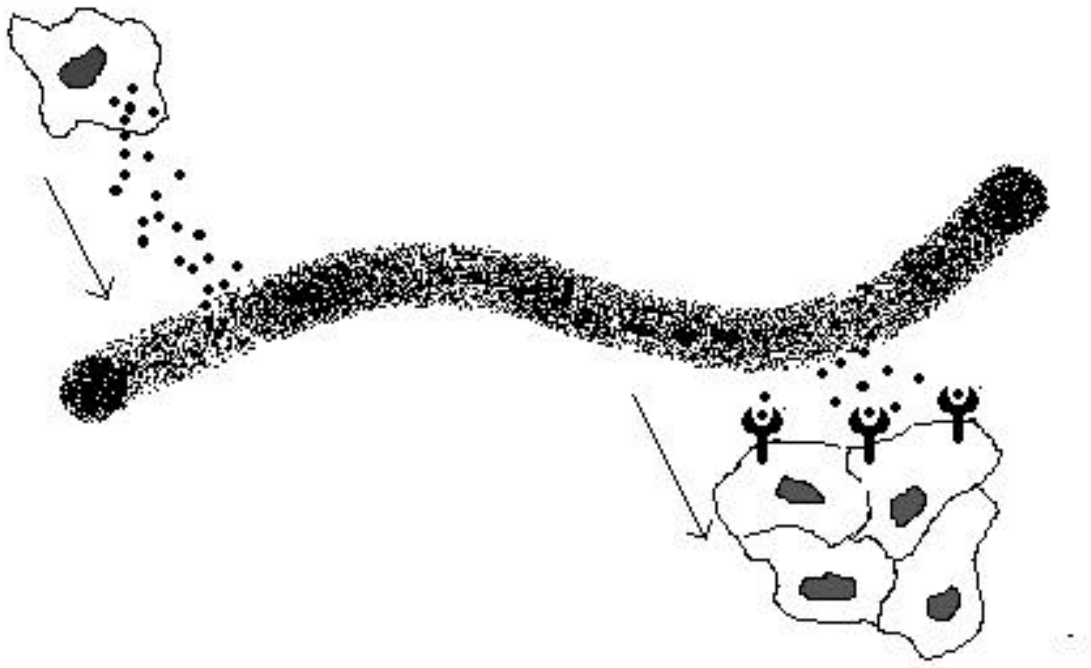

Fig 1.2 : Endocrine Signaling. (Adapted from Lodish et al., 1997).

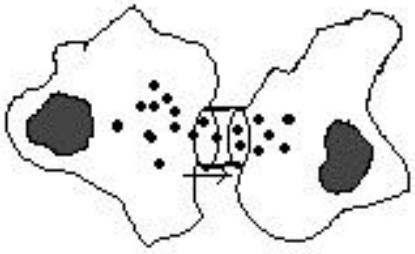

Figure 1.3 Juxtacrine signaling

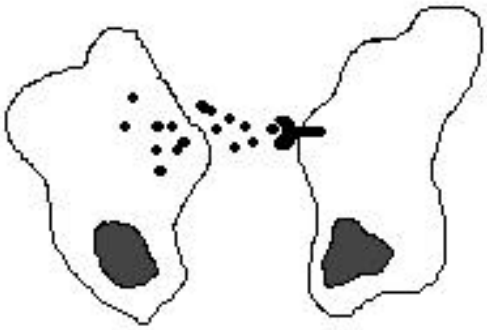

Figure 1.4 : Paracrine signaling. (Adapted from Lodish t al., 1997)

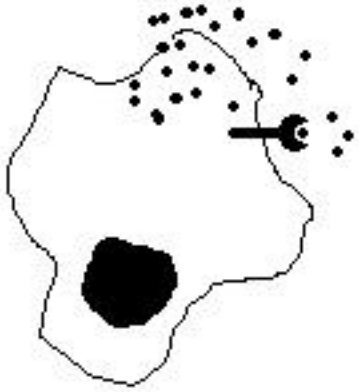

Figure 1.5 : Autocrine signaling. (Adapted from Lodish et al., 1997)

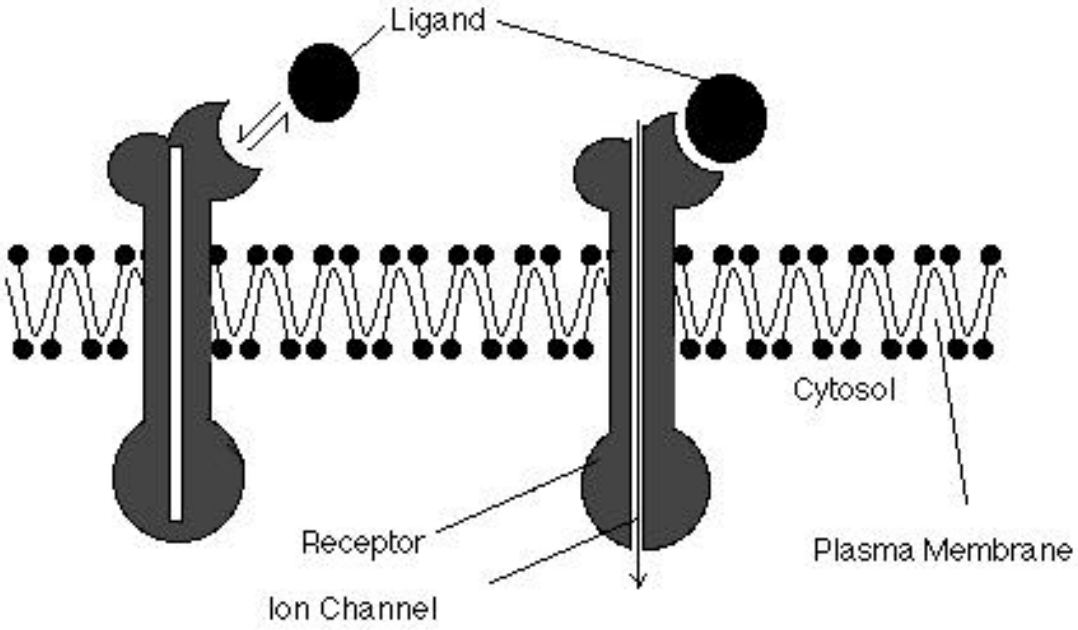

Fig 1.6 : Ligand activated ion channel (Adapted from Lodish et al., 1997)

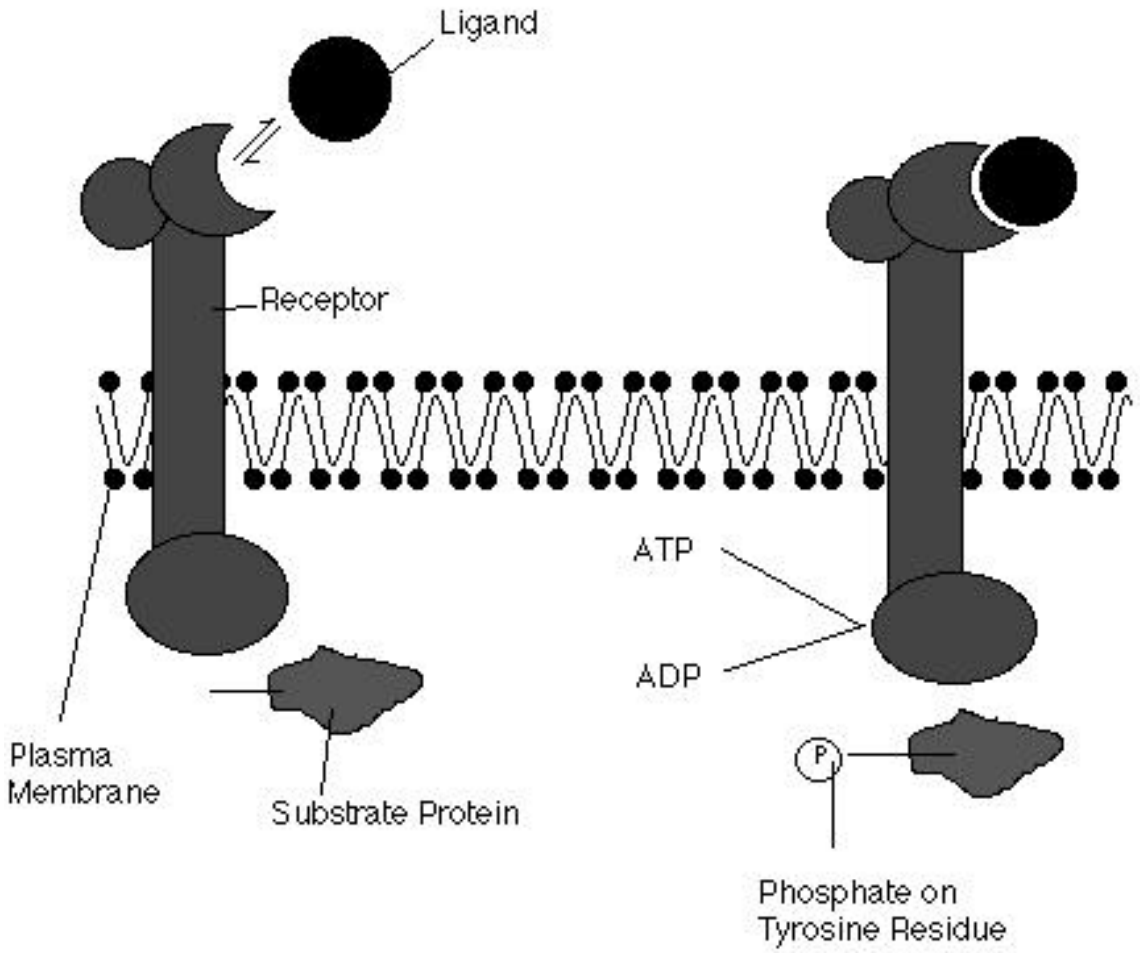

Figure 1.7 : Receptor with tyrosine kinase activity. (Adapted from Lodish et al., 1997)

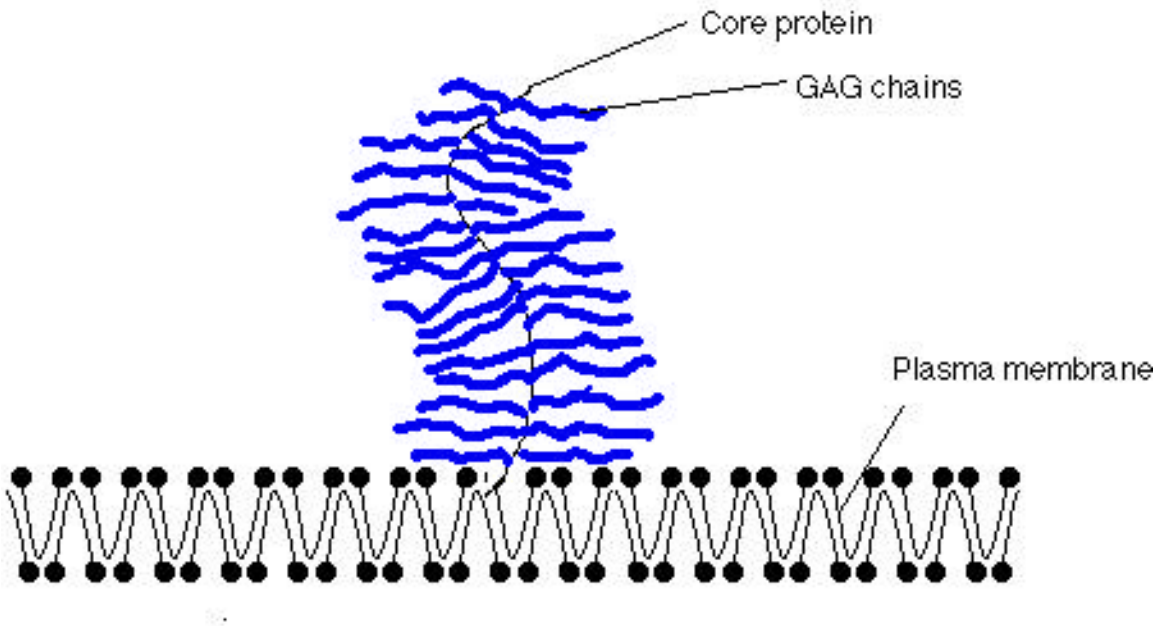

Figure 1.8 : Sketch of a typical proteoglycan

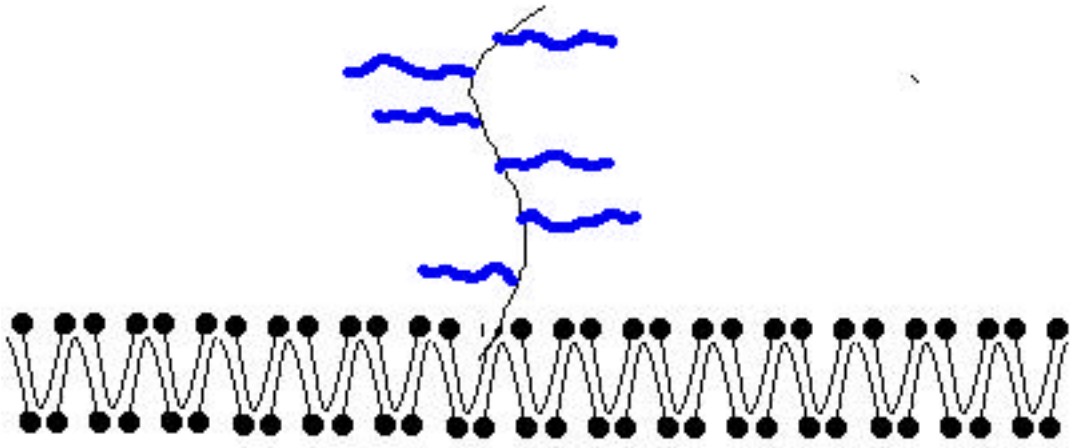

Figure 1.9 Illustration of a cell surface proteoglycan. (Adapted from Lodish et al., 1997).

Chapter II : Materials and methods

2.1 Cell based assays

2.1.1Cell cultures :

Bovine aortic endothelial (BAE) cells were used to conduct cell based binding studies. The cells were primary cultures, that is, they were not an immortalized cell line. The basic procedure for cell culture consisted of 2 steps : (a) establishing the primary culture by plating in 100 mm dishes, and (b) plating 24 well plates for binding studies. The method of plating varied as per the requirements of each study. The endothelial cells displayed a typical cobblestone appearance, their growth being restricted to a monolayer. The cells were maintained at a temperature of 37 ^{o}C, and in an environment with constant source of CO_2 (5 %) in air and humidity.

2.1.2 Passaging of cells :

BAE cells were obtained from both Dr. M. A. Nugent, (Boston University School of Medicine) and Clonetech Inc. (Louisville, KY) and used between passage 5 and 16. These were subsequently thawed and grown for experimental use until a maximum passage of 16. The media used was Dulbecco's Modified Eagles Medium, (DMEM) (GIBCO BRL, Gaithersburg, MD) supplemented with 10% Bovine calf serum (Hyclone Logan, UT), Penicillin (100 μg/ml), Streptomycin (100 μg/ml) and Glutamine (2 mM) (Cellgro Herendon, VA), henceforth called complete DMEM. The cells were split and replated when confluence was observed. This was done by washing the cell layer with 1 X Phosphate Buffered Saline (PBS), followed by addition of 2 mls of trypsin (Cellgro, Herendon, VA, USA) to dislodge the cells from the plate. Re-plating was then done by dilution to a 1:10 ratio of cells to fresh media and the passage number was increased by one.

2.1.3 Cell plating for experiments :

Twenty four well plates were primarily used for experiments. These were deemed to have the optimum number of wells since they were the minimum number required which could afford triplicates for each set of data. However, for certain studies, such as determination of efficiency of chloration, 12 well plates were used since they required a lesser number of samples. Also, 12 well plates yielded a higher amount of material, hence, a decrease in binding (for example, from 50,000 cpm to 500 cpm) could easily be measured. During passaging, a portion of the cell suspension was set aside for carrying out binding studies. An aliquot of 0.2 ml was taken from this stock cell suspension in order to determine the total number of cells. This was done by adding the aliquot to 10 mls of Isotone II (Coulter Electronics Inc., Miami, Fl, USA) and using the Coulter Counter, Model Z1 (Coulter Electronics Inc., Miami, Fl , USA) to determine the cell number. Based on the cell density to be used for plating the wells (this varied as per experimental protocol), an appropriate dilution was done with complete DMEM. In general, the plating density was 1 x 10^5 cells/ml. One ml of this suspension was then added to each of the 24 wells (2 mls for 6 well plates). The cell culture was then incubated for a period of 3 to 4 days.

2.1.4 Purification of Proteoglycans :

In cell culture, proteoglycans (PGs) are found in three primary areas : (a) the extracellular matrix, (b) on the surface of the cells themselves, and (c) in the growth (figure 3.11). Since PGs are regulators of several extracellular events, the cells release these molecules into the surrounding matrix environment (Heinegard and Sommarin, 1987).

For the purpose of experimentation, it is necessary to purify them from the media and concentrate. The basic steps involved in this process are : (1) preparation of conditioned media, (2) use of anionic exchange chromatography to purify the PGs from this media, (3)

dialysis of the resulting extract to eliminate previously added urea, and in the case of radioactive PG, free ^{35}S sulfate (SO_4^{-2}) ions, and (4), generation of calibration curve and determination of the concentration of the final PG extract.

2.1.5 Conditioned Media Preparation .

Conditioned media preparation was done as previously described. (Forsten *et al.*, 1997). 100 dishes of size 100mm were plated with BAE cells in complete DMEM. The passage number was 9. After incubation for a period of 4 days, the plates appeared 95 % confluent by visual inspection. They were then washed with 10 ml each of plain DMEM. This was followed by incubation in plain DMEM without serum for 1 hour. The media was then replaced with fresh DMEM in 95 plates (10 mls per dish). To the other 5 dishes, ^{35}S sulfate (NEN life sciences**,** specific activity of 1 mCi/ml) supplemented DMEM was added. The radioactive sulfate ions would be incorporated into the GAG of the proteoglycans, with the final purified extract being ^{35}S PG.

Following a 24 hour incubation period, the conditioned media was collected and pipeted into 50 ml centrifuge tubes. Centrifugation was then done at 750 x g for 30 min. The supernatant was collected in separate tubes, and stored for future use.

2.1.6 Anionic Exchange Chromatography :

To extract cell surface proteoglycans from the conditioned media, anionic exchange chromatography was used as previously described (Forsten *et al.*, 1997). Briefly, 950 mls of unlabelled media and 50 mls of radioactive conditioned media were obtained. A Q Sepharose column (Sigma) was used to carry out the purification. The beads were obtained in a pre swollen form, in 20% ethanol (Sigma). A slurry of these beads was prepared, which

was at least double the volume required, i.e., 13 mls for 6 ml beads. This was done by pouring 13 mls of slurry in a 25 ml graduated cylinder and topping up with TBS, 1 M urea. The cylinder was then covered with paraffin and rocked gently in order to resuspend the beads. This suspension is then allowed to settle. The TBS, 1 M urea is added in order to remove fine bits of gel which might interfere with the column. These remain in suspension. This top layer is then slowly decanted. The washing step is then repeated. The appropriate column is then set up, and TBS, 1 M urea is allowed to run through one full column. The washed gel is then made up to 14 mls with distilled water, and the entire amount is then poured onto the column. For the radioactive material, a column of volume 1 ml (1.5 x 3.5 cm) was used, and a 5 ml column was employed for the unlabelled conditioned media.

Prior to purification, 1 M urea was added to the media and stored for 24 hours at 4 ^{o}C. This was done so that the proteoglycans dissociate from aggregate form and any bound proteins are released. The column was then equilibrated with at least four column volumes of Tris buffered saline (TBS, 50 mM Tris, pH 8, 150 mM NaCl, 1M urea). The conditioned media was then loaded onto the column at a flow rate of 1 ml/min. Following the loading, a wash step was conducted with TBS, 50 mM Tris, 300 mM NaCl, 1 M urea at a flow rate of 1 ml/min for 1 hour. This was done in order to remove any the anionic proteins and glycoproteins. The fraction containing the desired proteoglycans was eluted with TBS 50 mM Tris, 1.5 M NaCl, 1 M Urea at a flow rate of 1 ml/min. In order to determine the concentration of the purified extract, a standard curve with bovine kidney Heparan Sulfate (Sigma) was generated, using a Dimethylmethylene blue (DMB) assay, as described below. A sample of the BAE PG was then compared to this curve.

2.1.7 Size Exclusion Chromatography :

Gel filtration, also called size exclusion chromatography, separates molecules based on size. Different gels have pores with different ranges of sizes, and act as molecular sieves to separate molecules. The gel matrix contains numerous porous beads with an eluant in the interstitial spaces. The separation is affected when the larger molecules do not to pass through the pores, but between them, and are eluted first. Smaller molecules have access to the pores and are retarded in the gel, and thus elute after the larger molecules.

The gel used in the process was Sepharose CL, a cross linked agarose derivative of Sepharose commercially available with : 2%, 4% or 6% agarose, designated as 2B, 4B and 6B respectively. Agarose has two functions : it increases the matrix rigidity, while decreasing its porosity. In this study, a CL-6B column was used to determine the fraction of BAE PG labeled with ^{35}S sulfate. A total of 80,000 CPM of ^{35}S BAE PG was used to conduct a chromatography experiment as discussed above. The fractions were counted on a Packard Tricarb 2100TR Scintillation Counter (Mededin, CT). The results are shown in figure 2-1. Roughly 75% of the total eluted radioactivity is incorporated into the proteoglycans.

2.1.8 DMB Assay :

Concentration was determined based on the DMB assay (Farnsdale, 1986) using a bovine kidney heparan sulfate standard. A range of concentrations of the standard heparan sulfate were prepared. The range was kept between 0-5 μg protein, since the dye (DMB) gives linear results only within this range. TBS was used as a buffer to bring each sample up to the same volume. 1 ml of DMB was then added, and a spectrophotometer was used (HITACHI® U-2000 Spectrophotometer, Hitachi Instruments, Inc.) to measure the absorbance at 525 nm wavelength. The reading obtained with 0 μg protein was treated as the zero point, i.e., the spectrophotometer was initialized to this value.

2.1.9 Binding Studies :

After the 24 well plate of cells reach confluence, the medium was changed to unsupplemented DMEM, followed by incubation for 72 hours. When the extracellular environment is devoid of calf serum, there are fewer growth factors available to the cell. Consequently, cell surface receptors increase in number, since the absence of signal initiation causes minimal internalization and receptor downregulation. After serum starvation, three wells were collected in order to determine the average cell number per well. Two major types of assays were conducted : co- and pre-incubation. Pre incubation studies were carried out in order to determine the interaction of a reagent bound to the cell surface with another reagent in the medium. For this, the cells were incubated with a selected concentration of a reagent at 4 ^{o}C for 2.5 hours prior to addition of the second reagent. Co-incubation involved simultaneous addition. The binding study was carried out at 4 ^{o}C. This ensured that the metabolic activity of the cell was slowed to such an extent that, for the time frame of the experiment, minimal receptor downregulation or internalization occurred. After two initial washes with binding buffer (DMEM, 25 mM HEPES, 0.5% Gelatin), the cells were allowed to equilibrate in 0.5 mls/well of the same binding buffer. The appropriate reagents were added and the cells were then treated depending on the type of assay, (co- or pre-incubation), for a total period of 2.5 or 5 hours. After this, two rapid washes with binding buffer were done. The cells were incubated with 1 M NaOH (0.25 ml/well/wash) and then a second rapid wash followed. The NaOH washes were combined for each well and the samples were counted for total radioactivity in a Beckman 5500 Gamma Counter (Irvine, CA). For each experiment at least one reagent was radiolabeled.

2.1.10 Chloration Studies :

One of the primary issues we focused on was the role played by cell surface heparan sulfate proteoglycans in IGFBP-3 binding to the surface of BAE cells. HSPGs, as discussed earlier, consist of a core backbone protein to which several glycosaminoglycan (GAG) chains are covalently attached. These GAG chains have a high density of negative charge, owing to the presence of sulfate ions. This charge might affect the tertiary structure of these molecules. To test the role of HSPGs in IGFBP-3 binding, a possible method could be the disruption of the charge density on the GAG chains, hence altering the structure of the molecules. Since it is known that biological macromolecular interaction has a very high degree of specificity in binding events, such a conformational change would likely affect the binding characteristics of the proteoglycans. To test this idea, sodium chlorate was introduced into the extracellular media, allowing the sulfate ions to be replaced by chlorate ions. This is a standard procedure done to inactivate PGs (Fannon and Nugent, 1996).

The method employed to carry out this study consisted of three steps : (a) determination of optimum conditions for chloration, (b) evaluation of the efficiency of chloration, and (c) a binding study with chlorated and non-chlorated cells to determine the change in IGFBP-3 binding characteristics.

2.1.10a Effect of chloration on cell viability: Determination of optimum time and concentration levels.

Two 6 well plates were plated at a cell density of 1.43×10^6 cells per ml, 2 mls per well.s The plating procedure was largely the same as discussed previously. However, one important change was made. Usually, after trypsin is used to dislodge the cells from the plate, calf serum is added to neutralize the trypsin activity. Addition of calf serum, which is a rich source of sulfate, is undesirable, since we are trying to inactivate cell surface HSPGs. Hence, the use of calf serum was replaced by centrifugation of the cell extract at 351 x g for 5 min. The pellet was resuspended in DMEM supplemented with glutamine (100U/ml). The

cells were then allowed to settle and attach for 5 hours. The media was then changed to DMEM supplemented with 2% dialyzed calf serum and glutamine. Sodium chlorate 0-150 mM was then added to half of the wells. After an incubation period of 72 hours, the cells were harvested at regular intervals and counted in a Coulter counter.

2.1.10b Evaluation of efficiency of Chloration :

This method involved (a) cell plating, (b) chlorate treatment, (c) incorporation of ^{35}S labeled sodium sulfate into the media, and (d) extraction, and (e) evaluation of the distribution of the radioactivity added. The first two steps were done as described above.

2.1.10c Sulfate incorporation :

Labeling medium was prepared, which consisted of DMEM supplemented with 10% dialyzed calf serum and ^{35}S sulfate to a final concentration of 60 μCi/well. The media was removed from the chlorated cells, and a wash with 2 mls/well of PBS was conducted. The same volume of labeling medium was then added, and the cells were incubated for 4 hours. Standard precautions for handling of radioactivity were taken, which primarily consisted of the following steps :

- Lining the hood with radioactivity absorbing paper
- Wearing of gloves, coat, and goggles
- Keeping a radioactive trash container close at hand
- Lining of the sample trays with aluminum foil
- Keeping a small container of charcoal in the incubation area to absorb any airborne radioactivity.

Next, the following reagents were prepared :

PMSF Stock (120 ml):	500 mM PMSF.
Buffer 1 (30 mls) :	0.15 M NaCl, 25 mM Tris HCl (pH 7.4)
Scraping Buffer (15 mls) :	1 M Urea, 1 mM DTT, 10 mM Tris HCl (pH 7.4), 10 mM EDTA, 2 mM PMSF.
TUT (15 mls)	10 mM Tris HCl, 8 M Urea, 0.1 X triton X-100, 1 mM Sodium Sulfate.

After incubation, the media from each well was collected and transferred to a 50 ml centrifuge tube. To this, 48 µl of PMSF stock were added, making a final concentration of 2 mM. Since the PMSF did not dissolve at room temperature, the suspension was heated till a clear solution was obtained. The tubes were put on ice for 5 min, and the samples were then transferred to 24 1.5 ml centrifuge tubes. The cell layer was then washed with 2 mls/well of ice cold Buffer 1. 1.5 mls of cold (refrigerated) Scraping Buffer was then added to each well. A disposable cell scraper (one per dish) was then used to immediately scrape up the monolayer of cells. The cell suspension from each well was then transferred to 1.5 ml centrifuge tubes. These tubes were then incubated on ice for 10 min. The previously aliquoted extracellular media and the above cell suspension were then centrifuged at 12000 x g for 10 min. The supernatants were collected and transferred to fresh 1.5 ml centrifuge tubes and the pellets were discarded. The supernatants were the Conditioned Media and the Extracellular matrix (ECM) extracts. The ECM pellet was resuspended in 500 µl of TUT, and was put on ice for 10 min. This was followed by centrifugation at 12000 x g for 10 min. The supernatant was collected as the Cell Extract.

2.1.10d Analysis if distribution of radioactivity : Efficiency of chloration.

After obtaining the cell, ECM and media extracts, a Zeta Probe (Bio-Rad, Hercules, CA) microfiltration assay was carried out to determine the amount of sulfate incorporated in each of these fractions. The study was largely carried out as per protocol detailed below, however, there were a few modifications. Bovine serum albumin was not used, and the samples were instantaneously passed through the membrane with no incubation time. In order to obtain optimum results (Rapraeger and Yeaman, 1989), TUT is used as the wash fluid. This was done and 95% efficiency reading was obtained (Forsten, private communication). This fraction was evaluated by comparing sulfate incorporation with and without chloration for each of the cell, ECM, and conditioned extracts.

2.2 Cell free Assays :

Studies were conducted which circumvented the use of live cells. The primary reason was to gain greater specificity in the molecular interactions, since the presence of numerous other surface and secreted entities in a cell based system make it difficult to derive definitive conclusions. This was attempted in two ways: (a) precipitation of a proteoglycans by binding with cetylpiridnium chloride (CPC), and(b) separation based on retention of proteoglycan containing complexes on a charged membrane.

2.2.1 Cetyl Piridnium Chloride Precipitation :

CPC is known to bind to negatively charged molecules such as proteoglycans (Roden *et al.*, 1972). Here, this property of CPC was used for precipitation experiments where binding of a radiolabelled reagent to a PG was determined by the amount of radioactivity in the precipitate (Ausubel, 1989). Desired concentrations (0-10ng/µl) and volumes (0-0.5 ml) of reagents were combined in 1.5 ml centrifuge tubes, and incubated for 5 minutes at 37 ^{o}C. This was followed by addition of 10 % CPC (stock, v/v). The amount varied as per volume

used. Care was taken that the reaction volumes were equal, for this, a makeup amount of TBS was added. This is because the reaction volume density may affect the precipitation. The solutions were then incubated for 30 minutes at 37 ^{o}C after which they were centrifuged for 10 minutes at 1500 x g. The supernatant was removed with the help of a micropipet, and transferred to a scintillation vial. The portion of the centrifuge tube containing the pellet was also transferred to a separate vial.

2.2.2 CAFAS Assay:

This assay is based on separation by charge, and has been developed in our laboratory (Wang, submitted). The Zeta-Probe blotting membrane (Bio Rad Hercules CA) is used to retain negatively charged molecules. This is a quaternary derivatized nylon membrane. Owing to the positive charge on the Zeta-Probe membrane, large negatively charged molecules are retained on it, whereas other reagents pass through. This concept is used to conduct binding studies where a radioactive label is incorporated into a positively charged reagent and then incubated with a negatively charged entity such as PG. The efficiency of binding is gauged by monitoring the amount of radioactivity retained on the membrane.

The membrane is cut to the appropriate size (4' x 5') and soaked in TBS, 0.15 M NaCl, 1 mg/ml BSA buffer for at least 30 minutes. Care is taken to ensure minimal bubble formation on the membranes as this could hinder the filtration process. The membrane is then installed on the Bio-Dot Microfiltration apparatus (Bio-Rad Hercules, CA). Since the apparatus has 96 wells (each of 3mm diameter), several different binding assays can be carried out during one experiment. Connections are made to a vacuum pump via an air trap. An initial wetting of the membrane is done by adding 0.2 ml of TBS, 0.15 M NaCl, 1 mg/ml BSA buffer to each well and pulling through by vacuum. The solutions were originally incubated in the wells themselves, however, it was found (Wang, M. S. Thesis) that the degree of nonspecific binding can be significantly reduced by conducting the incubation in

600 µl vials. No incubation was carried out for investigating single reagent interactions. The solutions are then transferred to the wells and pulled through by vacuum. Further reduction of nonspecific binding is achieved with 2 washes with 0.2 ml TBS, 0.15 M NaCl, 1 mg/ml BSA buffer. The wet membrane is then removed and allowed to dry on paper towel for 1 hour. The areas of filtration were then cut out as per predetermined layout and transferred to vials for measuring the radioactivity in a scintillation counter (Tri-Carb 2100TR, Packard, Downers Grove, IL). The results reflect an average of triplicates.

2.3 Materials Used :

^{125}I IGF-I, prepared by the Chloramine T procedure, was a generous gift of Dr. R. M. Akers (Department of Dairy Science, Virginia Tech). The concentration was 0.5 ng/ml, with a specific activity of 0.02 µCi/ml. IGFBP-3 was purchased from Upstate (Lake Placid, NY) and also labeled by Dr. R. M. Akers. It had a concentration of 0.5 ng/ml with a specific activity of 0.043 µCi/ml. IGFBP-3 and IGF-I were purchased from Upstate (Lake Placid, NY). Sodium chlorate, gelatin, hepes, Sepharose Gels (Q and CL-6B) were obtained from Sigma Chemicals, (St Louis, MO). Trypan blue, Tris base, Tris HCl, PBS and DMEM were purchased from Gibco BRL (Gaithersburg, MD). BSA was from Fisher Biotech (Fair Lakes, NJ). Bovine Aortic Endothelial cells were a generous gift from Dr. M. A. Nugent, Boston University School of Medicine and purchased from Clonetics (Louville , KY).

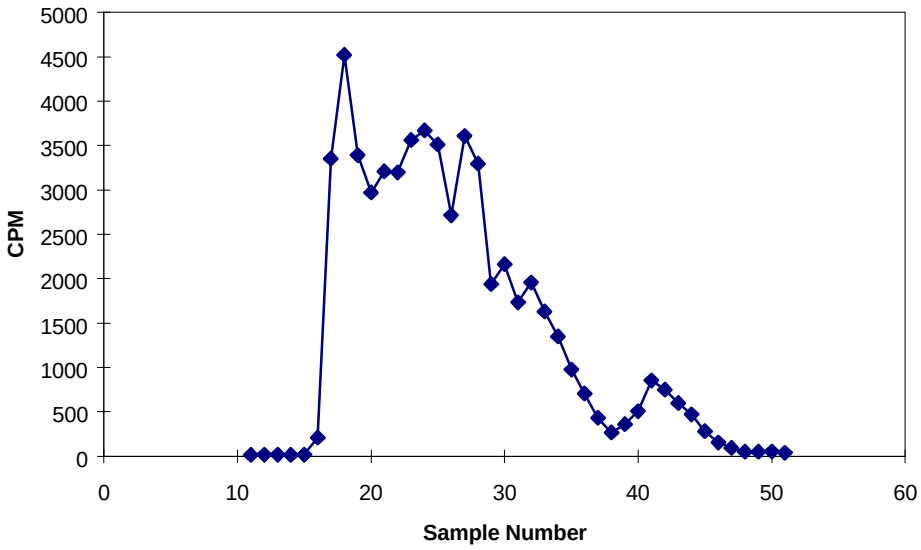

Figure 2.1 : **Size exclusion chromatography study of ^{35}S BAE PG**. ^{35}S BAE PG (80, 000 CPM) was run at 1 ml/min on a Sepharose CL-6B column and the resulting elution profile was plotted as shown above.

Chapter III : Cell based assays

3.1 Introduction

With the endocrine mode of action, in order to elicit a response on the target tissue, IGF-I must first be transferred across the blood vessel wall. In mammals, the lumenal layer directly in contact with the blood stream is called the endothelium and is composed of a layer of endothelial cells. IGF-I and IGFBP-3 could interact and bind with the cell surface molecules on the endothelial cells and undergo the process of receptor-mediated transcytosis to cross the vascular wall. Regulation of exogenous IGF-I accessibility to the target tissue may benefit from an increased understanding of how this process occurs. We chose to do this in a bovine aortic endothelial model cell system. Our hope is that understanding how a generic mammalian endothelial monolayer functions will help predict the response in the human body. However, just as for the human system, the presence of numerous other cell surface entities which may be playing a role in the binding process make precise evaluation difficult.

Within the IGF-I system, our primary focus was on the analysis of IGFBP-3. First, our hypothesis of the role of HSPGs on the cell surface or in the media in IGFBP-3 binding was examined. The effect of competitive addition of IGF-I on IGFBP-3 binding was then evaluated. The binding of a ligand to the cell surface may not only be to its high affinity receptor, but also to numerous other low affinity or non specific binding sites. In order to obtain values for specific binding of IGFBP-3, calf serum, which has an abundance of IGFBP-3, was used to occupy all the possible binding sites for IGFBP-3. The specific binding sites are saturated, while the nonspecific sites, which are much larger in number, are not saturated (within the range of excess ligand used). Thus, if a given quantity of labeled IGFBP-3 and calf serum is introduced, the amount of bound radioactivity should yield the magnitude of nonspecific binding occurring. This can then be subtracted from the total binding level to yield the specific binding of IGFBP-3. An initial set of

experiments was conducted to find the optimum amount of calf serum needed to determine the non specific binding. An experimental specific binding curve for IGFBP-3 was then obtained.

The next set of experiments examined the role of surface HSPGs in IGFBP-3 binding. Sodium chlorate, was used to inactivate the GAG chains on the surface HSPGs. The optimum duration and level of chloration was first quantified and, the effect of chloration on IGFBP-3 binding to BAE cells was studied.

3.2 Effect of P9 on IGFBP-3 binding :

This study was done to shed light on the role of media HSPGs in the binding of IGFBP-3 to the BAE cell surface. A previous study was done to ensure that IGFBP-3 binds to the cell surface (data not shown). However, no study has been done to characterize the binding of P9 HS to the BAE cell surface.

Pre-incubation with P9 did not cause a change in IGFBP-3 binding to BAE cells (figure 3.1). Briefly, BAE cells were grown to confluence on a 24 well plate and incubated in plain DMEM for 3 days. The cells were then incubated with P9 HS (6-300 ng/ml) for 2.5 hours at 4 ^{o}C (see Materials and Methods : Binding Studies). This was followed by two washes and incubation with 1 or 2 ng/well of ^{125}I-IGFBP-3 for 2.5 hours. The cells were then harvested with NaOH and retained radioactivity was measured on a gamma counter.

This result could imply that the interaction of P9 HS with the cell surface is independent of IGFBP-3 binding to its receptor. However, since P9 binding was only assumed, it is possible that the P9 may not have any interaction with the cell surface, or it may bind only in a nonspecific manner. In the latter case, the bound P9 may not have been retained during the washes. This could have accounted for the unaffected IGFBP-3 binding though this nonspecific binding may still allow IGFBP-3 binding to P9.

The error bars for all the experimental results were calculated according to the following formula :

(1) $$\text{STD ERROR} = \frac{\text{STDEV(C1, C2, C3)}}{\sqrt{3}}$$

where

C1, C2, C3 = The triplicate samples used.

STDERROR = The error calculated.

STDEV = The standard deviation of the triplicate sample. The standard deviation is calculated using the "nonbiased" or "n-1" method, and uses the following formula :

(2) $$\sqrt{\{[3(C1^2, C2^2, C3^2) - (C1 + C2 + C3)^2]/6\}}$$

The above formula is specialized for a triplicate set of data.

3.3 Effect of IGF-I on IGFBP-3 binding :

The aim of this experiment was to evaluate the effect of IGF-I in the media on IGFBP-3 binding to the BAE cell surface. Mohseni-Zadeh and Binoux (1997) have demonstrated through competitive binding experiments that IGFBP-3 was as potent as unlabelled IGF-I in displacing from the cell surface its truncated analogue, D-des(1-3)IGF-I, which has weak affinity for IGFBPs. Also, Oh *et al.* (1993) have shown that there occurs a significant diminution of the IGFBP-3 inhibitory effects on monolayer growth by co-incubation with native IGFs, but not by co-incubation with IGF analogs with decreased affinity for IGFBP-3. This implies that the binding of IGFBP-3 to Hs578T human breast cancer cells (the cell system used) is affected by IGF-I. Hence we wanted to analyze the effect of IGF-I on IGFBP-3 binding in the BAE cell system.

The significance of the experimental results was evaluated by conducting a Students t-test. In this method, probability values (P-values) for pairs of distributions are calculated in order to determine whether the corresponding means are significantly different. Both the distributions are assumed to be Student's t-distributions with n - 1 degrees of freedom. For each evaluation, a probability value is returned, which is compared to a pre-determined significance level. A significance level of 0.1 was chosen for our studies. P-values lower than this implied a significant difference, higher values meant that the mean values being compared are not significantly different.

Co-incubation with IGF-I reduces IGFBP-3 binding (Figure 3.2). The P-value for the 0 ng/well and 5 ng/well IGF-I additions was 0.026, which was much lower than the chosen significance level of 0.1. This meant that it is highly improbable that these binding measurements came from the same population and have the same mean. In other words the difference between them was significant. Results for the 5 and 10 ng/well additions were also significantly different, with a P-value of 0.077. This implied that there was a significant reduction in IGFBP-3 binding. Briefly, confluent BAE cells were incubated with IGF-I (0-10 ng/well) and ^{125}I-IGFBP-3 (1 ng/well) for 2.5 hours at 4 ^{o}C in binding buffer (see Materials and Methods : Binding Studies). The results are scaled so that 100% is the binding of IGFBP-3 in the absence of IGF-I, which retains 3.5% of the total radioactivity added per well. A steady decrease is observed, with the addition of 10 ng/well of IGF-I yielding the lowest level of IGFBP-3 binding. This suggest that IGF-I binds to IGFBP-3 and prevents it from binding to the cell surface. However, the results are based on a single study, more experiments would need to be done in order to yield reproducible values. There has been recent evidence that IGFBP-3 might function to regulate IGF-I accessibility to the cell surface, when present *in vivo* (Mohseni-Zadeh and Binoux, 1997). This result re-enforces that idea, lending credibility to the hypothesis that IGF-I and IGFBP-3 remain in a stable complex form even when near the target cell system. A possible follow up to this study might be an analysis of the binding characteristics of the binary complex of IGFBP-3/IGF-I to the BAE cells, details of which are discussed in Chapter V.

3.4 Effect of P9 HS on IGF-I binding

In keeping with our basic hypothesis, we wanted to examine the role of heparan sulfate proteoglycans on the IGFBP-3 regulation of IGF-I binding to BAE cells. An important first step is to analyze the effect of media HSPGs on IGF-I binding.

A set of co- and pre-incubation studies were done in order to examine the role of heparan sulfate proteoglycans in IGF-I binding to BAE cells. The results are shown in figure 3.3. The primary aim of this experiment was to see whether there is a difference in binding characteristics of IGF-I. P9 HS (0-30 ng/well) was added to confluent BAE cells either simultaneously with ^{125}I-IGF-I, or *following a 2.5 hour incubation of the cell with* ^{125}*I-IGF-I*. Co-incubation yielded a P9 HS dose-dependent increase in IGF-I binding, whereas there was no significant change affected in IGF-I binding on pre-incubation.

For co-incubation, the P-value associated with the 2.5 ng/well and 12.5 ng/well additions of P9 HS was 0.21, and with 12.5 ng/well and 30 ng/well points was 0.07. This meant that though the first data pair was not significantly different, the increase in IGF-I binding from 12.5 ng/well to 30 ng/well P9 HS addition was significant. The corresponding values for pre-incubation were 0.14 and 0.28, neither of which are significant. Hence, these results may imply that P9 HS facilitates the binding of IGF-I to the cell surface.

Co-incubation with a broader range of P9 HS concentrations was also tested (see figure 3.4), which essentially shows no change in ^{125}I-IGF-I binding. The results were not in accordance with previous examinations (see figure 3.3). A possible reason for error could be a cell gradient problem : during the plating of 24 well plates, care must be taken to ensure proper mixing of the stock solution, otherwise the wells may receive an unequal number of cells. This could have lead to incorrect readings. Also, these readings are based on the total binding of IGF-I to the cells, hence the nonspecific binding may cause a variation in the experimental results.

Hence, a definitive conclusion cannot be drawn owing to the errors as discussed above. In order to accurately determine the effect of P9 HS on IGF-I binding then, an experiment should be conducted similar to the one above, but with evaluation of the specific binding of IGF-I to the cell surface. This would yield more reproducible and reliable results.

3.5 : Preparation studies for using CS as a nonspecific inhibitor.

3.5.1 Investigation of optimum time, method of stain incubation, and amount of CS added on efficiency of trypan blue method.

Trypan blue staining is a standard method to determine the total number of live cells in any given cell sample (see Chapter II : Trypan Blue Method). The Coulter counter method counts both live and dead cells and also can unfortunately count small bubbles and debris that may be present in the cell suspension. Hence, trypan blue staining was used primarily to validate the counts obtained from the Coulter counter. During initial studies with the trypan blue staining method, the resulting cell counts were found to be inordinately lower than expected. Studies by co-workers using similar plating densities yielded cell counts twice higher in orders of magnitude. A possible explanation could be the lysing of cells by the time they were examined under the microscope. Clearly, an optimization of the various steps in the procedure was required.

Incubation carried out on ice has a favorable effect on the cell numbers, as seen in figure 3.5 (a) and (b). This is probably due to the fact that trypsin, though partially neutralized by the addition of calf serum, might still be active. Since enzymes such as trypsin usually function optimally at temperatures closer to those found in mammals, incubation at 0 ^{o}C may drastically reduce the rate of proteolytic action. Different amounts of calf serum (50 and 100 µl) were also tested. 100 µl serum proved to be better than 50 µl, probably because the trypsin inactivation was greater. Also, since a steady decrease was observed

in almost all the cases, the optimum time for incubation is really as short as possible, preferably between 5 and 10 minutes.

3.5.2 Determination of calf serum level required for specific IGFBP-3 binding

One of our objectives was to characterize the interaction of IGFBP-3 with the BAE cell surface. Hence, in order to determine the specific binding of IGFBP-3, calf serum, with its abundance of IGFBP-3 was chosen to inhibit the nonspecific binding. The following is a description of a study done to determine the optimum amount of calf serum required for this purpose.

Optimum amount of calf serum was determined to be 20 % (v/v). Briefly, confluent BAE cells were incubated with ^{125}I-IGFBP-3, and calf serum (0%-20%) for 2.5 hours at 4 ^{0}C in binding buffer (see Materials and Methods : Binding Studies). After incubation , the cells were harvested with NaOH, and the bound ^{125}I-IGFBP-3 was determined using a gamma counter.

Initially, a study of IGFBP-3 binding with 5% calf serum (CS) was conducted. The expected dose dependent response was observed, however, no observable saturation of the curve was noticed. The concentration range used was 0.25-10 ng/well IGFBP-3. Also, the decrease in binding due to the addition of CS, within the limits of error, was also not observed to be saturable. The concentration of CS was therefore increased to 20%, and a *difference* between the total and nonspecific binding was observed to be saturable**,** as shown in figure 3.6. The concentration range was increased to 0.5-25 ng/well IGFBP-3. In order to confirm these results, a different approach was taken. 1 ng/well of IGFBP-3 was incubated in the presence of increasing amounts of CS (0-20%) (figure 3.7). Besides observing a definite decrease in binding of IGFBP-3 in presence of CS. This result was verified by calculating the P-values for the data. The P-value associated with the 0 µl and 50 µl addition of CS was 0.0006, implying that the decrease was very significant. Further,

the P-value for the 50 µl and 100 µl CS additions was 0.095, which meant the difference (with a significance level of 0.1) was just about significant. Hence, these values suggest a saturation in IGFBP-3 binding. 100 µl of CS was chosen as the value to be used for future binding studies.

3.6 IGFBP-3 Binding studies : Determination of binding curve :

In keeping with our objective of characterizing the nature of IGFBP-3 binding to the BAE cell surface, we constructed a specific binding curve for this molecule. Using previously obtained data, 20% calf serum was used to determine the specific binding curve of IGFBP-3. Figure 3.8 shows the results. Briefly, confluent BAE cells were incubated with IGFBP-3 (1-25ng/well) with or without 20% calf serum for 2.5 hours, at 4 ^{o}C in binding buffer (see Materials and Methods : Binding Studies). Following, the cells were harvested with NaOH, and the bound ^{125}I-IGFBP-3 was determined with a gamma counter. The total non and specific binding determined were plotted as a function of amount of IGFBP-3 added, and the calculated specific binding curve is also shown. The binding exhibited a typical dose dependency as expected, and the total and nonspecific curves, within the limit of error, showed a trend towards saturation. A possible source of error could be the use of excess CS instead of unlabeled ligand (IGFBP-3). This introduced numerous other entities into the extracellular region which may be involved in the binding process. A regression analysis of the specific binding curve yielded a dissociation constant (K_D) of 3.1 x 10^{-10} M, assuming no ligand depletion. Assumption of ligand depletion gave the same value for K_D (Details in Appendix A). This implies that the assumption of no ligand depletion is valid in this case. Further, these values are 3 orders of magnitude different from that obtained by Cortizo and Gagliardino (1995), which was 4.0 x 10^{-7} M. A possible reason could be an inaccurate evaluation of the non specific binding, since calf serum was used instead of excess unlabeled ligand. The availability of only 4 data points may have further lead to an inaccurate K_D value. Also, the difference in cell lines (Swiss 3T3 fibroblasts were used by the above authors) may also be responsible. However, since the K_D value obtained is lower than the literature value, it is possible that IGFBP-3 binding

to BAE cells is of a higher affinity than to Swiss 3T3 fibroblast cells considered by Cortizo and Gagliardino (1995).

In order to circumvent the error introduced by use of calf serum, a separate analysis was conducted where the total IGFBP-3 binding to the cell surface was modeled as a function of the amount of ligand added. This yielded a K_D value of 0.032×10^{-9} M (0.033×10^{-9} M assuming no ligand depletion), and a nonspecific association equilibrium constant (Kn) of 0.93×10^{-13} M. The K_D values obtained above and those calculated form specific binding analysis differ by an order of magnitude. This implies that calf serum may not be used as a substitute for excess unlabeled ligand in quantification of binding characteristics of IGFBP-3 to BAE cells.

3.7 Effect of chloration on cell viability :

An important aspect of our research was to examine the role of PGs on the cell surface and in the extracellular media, and to examine their interactions with the IGF-I system. A possible role of surface HSPGs is as a receptor for IGFBP-3. In order to test this notion, we used the process of chloration. HSPGs consist of a core protein, and at least one GAG chain. These chains have a large number of sulfate ions, which play an important role in determining the structure of the proteoglycan. When sodium chlorate is incorporated into the media, chlorate ions would replace the sodium ion on the GAG. Since the two ions differ in charge and size, the replacement may cause changes in the conformation of the HSPGs, which renders them incapable of binding to ligand. This has been shown to be effective with bFGF (Fannon and Nugent, 1996). In order to ensure that most of the sulfate ions are replaced, an assay to determine the efficiency of chloration was conducted, as described in section 3.8.

Prior to testing the binding of IGFBP-3 under conditions of chloration, however, the optimum duration of chloration has to be determined (figure 3.9). Briefly, this was done

by treating a portion of the cells with 75 mM chlorate, followed by a 48 hour incubation period (The zero time point in the figure represents the post incubation value). Cell counts were then taken at regular time intervals (0, 24, 48, 72). A steady decrease in cell viability is seen during the entire time period for both types, however, the chlorated cells have a lower cell number. At 48 hours, the trade off between cell counts and level of chloration seemed to be at an optimal level : a 72 hour point is equally valid, if the significantly lower cell counts are not an issue. Besides the duration of chloration, the optimum concentration of sodium chlorate to be used also has to be determined (figure 3.10). Briefly, cells were incubated with sodium chlorate (0-150 mM) for 12 hours at 37 ^{0}C, 5% CO_2. They were then harvested and cell numbers determined by counting in a Coulter counter. The optimum chlorate concentration was close to 25 mM. However, even with a 25% drop in cell counts, 75mM of chlorate was chosen to be more appropriate. This was because a higher concentration of chlorate ions may yield a better efficiency of chloration.

3.8 Determination of efficiency of chloration :

The efficiency of chloration was found to be 95% when 75 mM sodium chlorate was used (Dr. K. E. Forsten, private communication). Initial experiments, however, yielded an efficiency of roughly 75 %, as shown in figure 3.11. Briefly (see Chapter II : Chloration Studies), 12 well plates were cultured to confluence, and half of them were chlorated with 75 mM sodium chlorate for 48 hours. ^{35}S sodium sulfate was then added to the media and followed by incubation for 4 hours. This labeling media had been supplemented with 10% dialyzed calf serum. CS, besides containing a abundance of numerous growth factors, also has a significant amount of sulfate in it. This sulfate, if not removed, may be incorporated along with the labeled sulfate ions, and lead to erroneous results. Hence, dialysed CS was used, which has a negligible amount of free sulfate. Cell extract (CE), Extracellular matrix (ECM) and Conditioned Media (CM) extracts were then prepared, and the modified CAFAS assay was then used to determine the radioactivity retained. This

step essentially separated the bound radioactivity from the free ^{35}S sulfate ions. This also might disrupt aggregates of PGs which may have been formed.

A high value of efficiency is required for the following reasons. Since GAG chains are highly charged, even a small amount of GAG containing surface PGs could actively bind to a corresponding ligand. Hence, in order to rule out surface PGs as receptors in a given binding event, a very high fraction of them should be inactivated. This is why binding studies with chlorated cells are conducted with almost all the GAG chains on the surface PGs being chlorated. Again, the implicit assumption here is that only the GAGs are affected by the chloration process, which may not be true. Hence, lower the efficiency, larger is the uncertainty in the binding experiment. The ^{35}S sulfate incorporation into the conditioned medium and the ECM are also shown. The ECM and CM are monitored since they also contain PGs which may be binding to the ligand.

3.9 Effect of chloration on IGFBP-3 binding.

The aim of this study was to compare IGFBP-3 binding to the BAE cell surface with or without chloration. Since the GAGs on the chlorated cells would be inactivated, any changes in the binding characteristics of IGFBP-3 may implicate surface HSPGs as playing a role in the process.

Chloration of BAE cells did not affect the level of IGFBP-3 binding. Results are shown on figure 3.12. Briefly, cells were incubated in supplemented DMEM (see materials and methods : plating of cells) for 5 hrs before being switched to DMEM with 2% dialyzed calf serum for 5 hours. 75 mM sodium chlorate was then added wells and incubated for 48 hrs.

The binding study was conducted using ^{125}I-IGFBP-3 (0.5-15 ng/well). Cells were incubated with this reagent for 2.5 hrs at 4 ^{o}C in binding buffer and followed by harvesting

with NaOH. Bound radioactivity was measured using a gamma counter. The results were scaled to account for the difference in cell numbers in the chlorated and non-chlorated cells. The results after scaling are almost same for both, except at 15 ng/well IGFBP-3, where the non chlorated cells yield a higher value of binding. This indicates that cell surface PGs are not likely to be the receptor for IGFBP-3. Chloration results in the replacement of the sulfate ion on the GAG chains with a chlorate ion and likely renders incapable of binding to IGFBP-3. Hence one can conclude from this that the surface HSPGs, may not play a role in IGFBP-3 binding to the BAE cell surface. However, Smith *et al* (1994) have found that in a Rat Sertoli cell system, pretreatment of the cells with sodium chlorate retarded the decrease in recombinant IGFBP-3 from the media. However, these studies were different from ours in that the reduction in IGFBP-3 from the conditioned media was measured by ^{125}I-IGF-I ligand blot analysis and Western blot analysis using an antiserum specific for human IGFBP-3. We quantified the amount of IGFBP-3 binding to the cell surface by radioactive labeling of IGFBP-3, and measuring the bound label with a scintillation counter. Further, the difference in cell systems (Rat Sertoli cells were used by the above authors) may to some extent account for the difference in results. Also, Smith *et al* (1994) used non glycosylated IGFBP-3 where as our study was conducted with glycosylated IGFBP-3. Nevertheless, these findings conflict with our results, and keeps the question of the receptor for IGFBP-3 still in debate.

3.10 Summary :

In this chapter, various aspects pertaining to the IGF-I system were studied. The effect of pre-incubation with P9 HS on IGFBP-3 binding was first studied. P9 HS, when added prior to ^{125} I-IGFBP-3, seemed to have no effect on IGFBP-3 binding. Though the corresponding co-incubation studies were not conducted, there is evidence that HS in the media inhibits IGFBP-3 binding (Booth *et al.*, 1996, Koedam *et al.*, 1997). A possible reason could be the conformational change undergone by the proteoglycan on complexing with its receptor on the cell surface. A further complication is the presence of cell surface

HSPGs. These have been implicated to be a receptor for IGFBP-3, also for heparin in the media (Yvonne *et al.*, 1996). The latter is unlikely, since negative charge on both HSPGs and heparin molecules would make complexing energetically unfavorable. Studies have also been carried out by the same author on the role of surface HSPGs in IGFBP-3 binding. Complete digestion of the surface HSPGs with heparatinase did not significantly decrease IGFBP-3 binding (Yvonne *et al.*, 1996). These results support our own findings based on chloration studies. It was seen that chloration and hence inactivation of surface GAG chains had a minimal effect on IGFBP-3 binding. Further credibility to this result can be lent owing to the fact that similar conclusions have been drawn from studies with disparate cells : BAE cells (ours) and C6 rat glioma cells (Yvonne *et al.*, 1996). Hence, the most probable interaction of P9 HS with IGFBP-3 occurs in the extracellular region, soluble HSPGs acting as receptors for IGFBP-3, and decreasing the net cell associated ligand receptor interaction. This is likely, since IGFBP-3 is known to possess heparin binding domains (Fowlkes *et al.*, 1996).

Co-incubation with IGF-I was shown to decrease IGFBP-3 binding. The characteristics of IGF-I binding were then looked at, with an analysis of the effect of P9 HS. Co-incubation with P9 HS yielded a increase in IGF-I binding, while pre-incubation caused an decrease in IGF-I binding. A specific binding curve for IGFBP-3 was also obtained. Finally, standardization of the trypan blue technique was done with respect to time, amount of calf serum, and temperature of incubation. Hence, these studies shed light on some of the characteristics of IGFBP-3 and IGF-I binding to the BAE cell surface, and represent the start of a series of studies which can be conducted to gain a better insight into the IGF-I system.

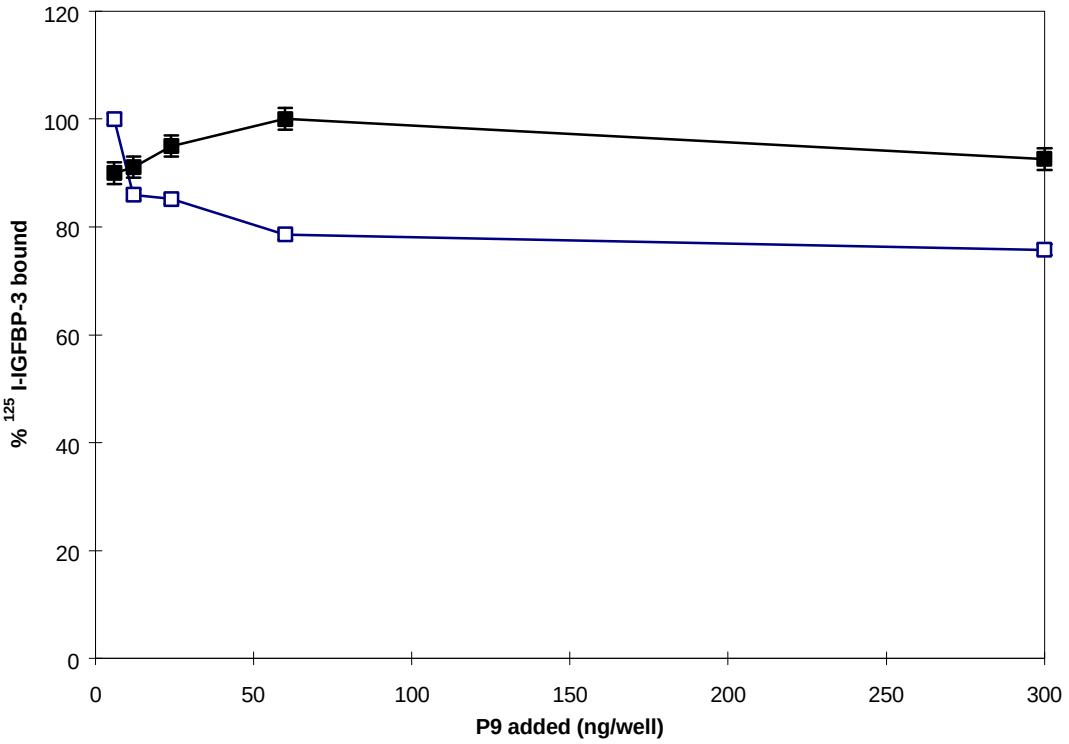

Figure 3.1 : **Effect of pre-incubation of BAE-P9 on ^{125}I-IGBP-3 binding**. BAE P9 (0-50 µl/ml, 6 ng/µl) was pre-incubated with BAE cells (2.9 x 10^5 cells/well, passage 14) for 2.5 hours at 4 ^{o}C. Two washes followed. Then IGFBP-3 (1ng : ■, 2 ng : ❑) was added. The results are averages for triplicates, and are scaled to the point where the highest binding of IGFBP-3 occurs.

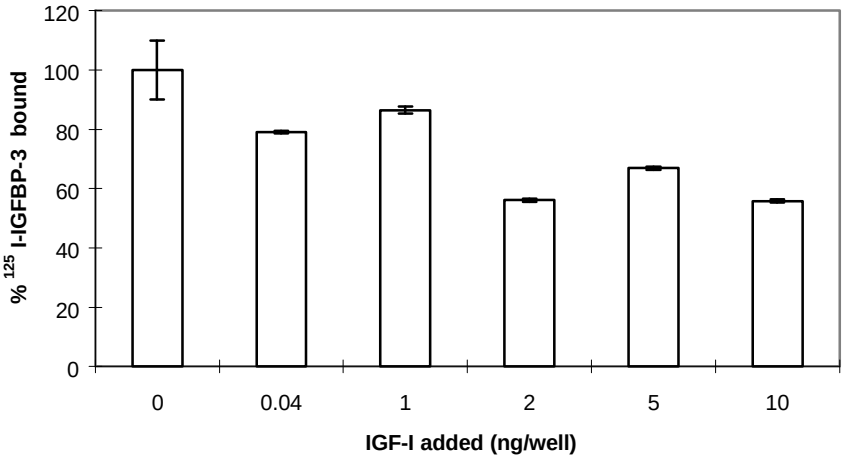

Figure 3.2 : **Effect of co-incubation with IGF-I on IGFBP-3 binding to BAE cells**. IGF-I (0-10 ng/well) was co-incubated with IGFBP-3 (1 ng/well) with BAE cells (2.3 x 10^5 cells/well, passage 15) for 2.5 hours at 4 ^{o}C. Results are averaged over triplicates and scaled to the highest binding value, which occurred at 0 ng/well IGF-I addition. Standard error is shown.

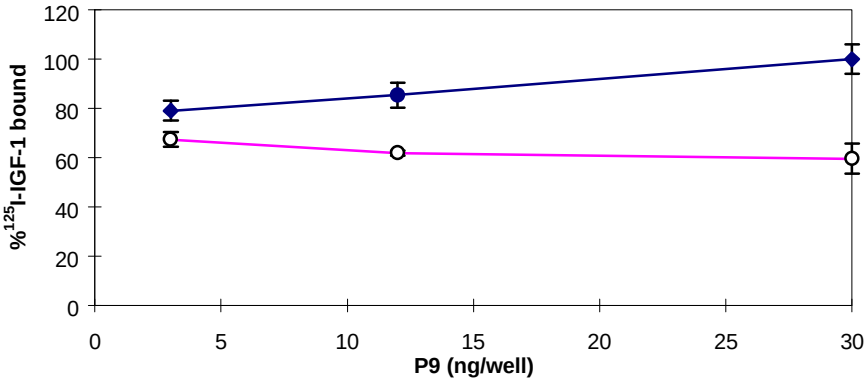

Figure 3.3 : **Effect of P9 HS on ^{125}I IGF-I binding.** BAE P9 (0-30 ng/well) was co- (●) or pre-incubated (❍) with ^{125}I IGF-I (1 ng/well) with BAE cells (2.9×10^5, passage 13) for 2.5 hours at 4 ^{o}C. Results are scaled to the highest binding value which occurred on co-incubation with 30 ng/well of P9 HS. Results are averaged over triplicates. Standard error is shown.

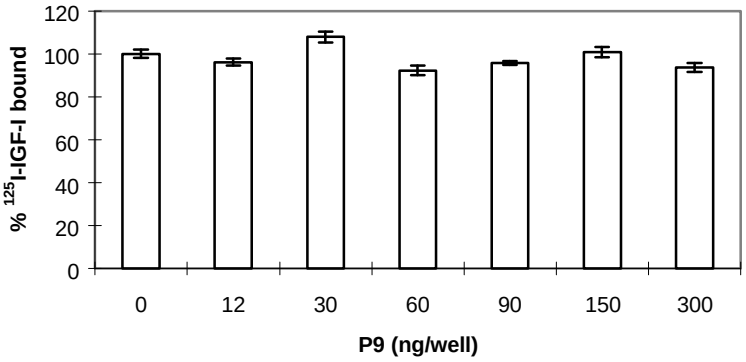

Figure 3.4 : **Effect of co-incubation of BAE P9 HS on ^{125}I-IGF-I binding**. BAE P9 HS (0-300 ng/well) was co-incubated with ^{125} I-IGF-I (1 ng/well) with BAE cells (2.6 x 10^5 cells/well, passage 14). Results are scaled to the highest binding value, which occurs on addition of 30 ng/well of BAE P9. Standard error is shown.

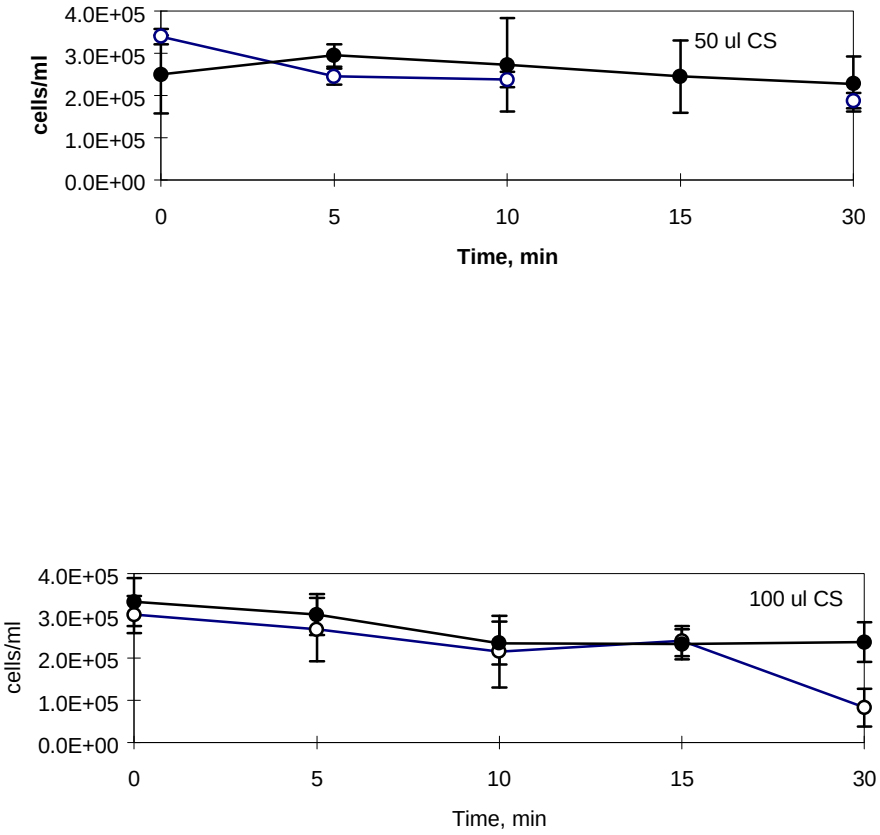

Figure 3.5 : **Determination of optimum conditions for the trypan blue method**. (a) 50 µl (12.5%, v/v) CS and (b) 100 µl (20%, v/v) CS was used with BAE cells (passage 16) for 0-30 minutes. Incubation on ice (●), and 37 ^{0}C incubation (❍) are shown. Results were averaged over triplicates. Standard error is shown.

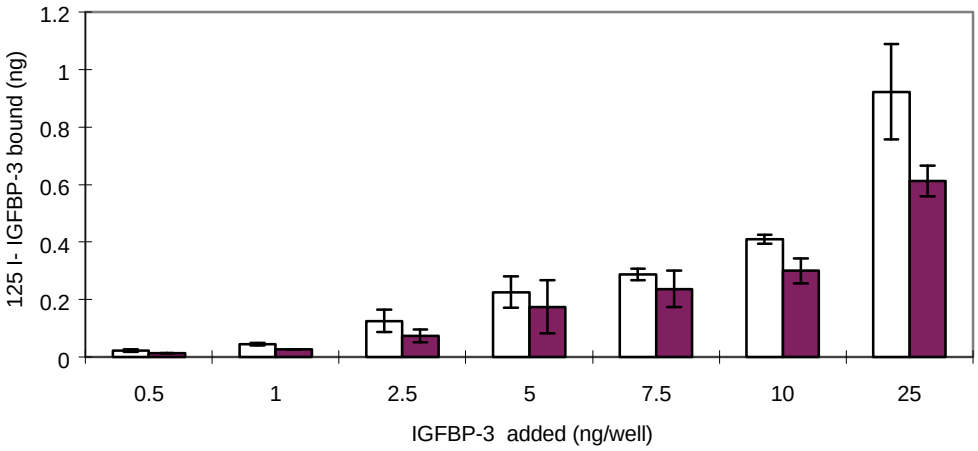

Figure 3.6 : **Effect of calf serum on IGFBP-3 binding**. Calf serum (20%, v/v) was co-incubated with IGFBP-3 (0.5 to 25 ng/well)with BAE cells (2.9 x 10^5, 2.5 x 10^5 cells/well, passage 13) for 2.5 hours at 4 ^oC. The dark columns denote the presence of serum. Results are averaged over triplicates. Standard error is shown.

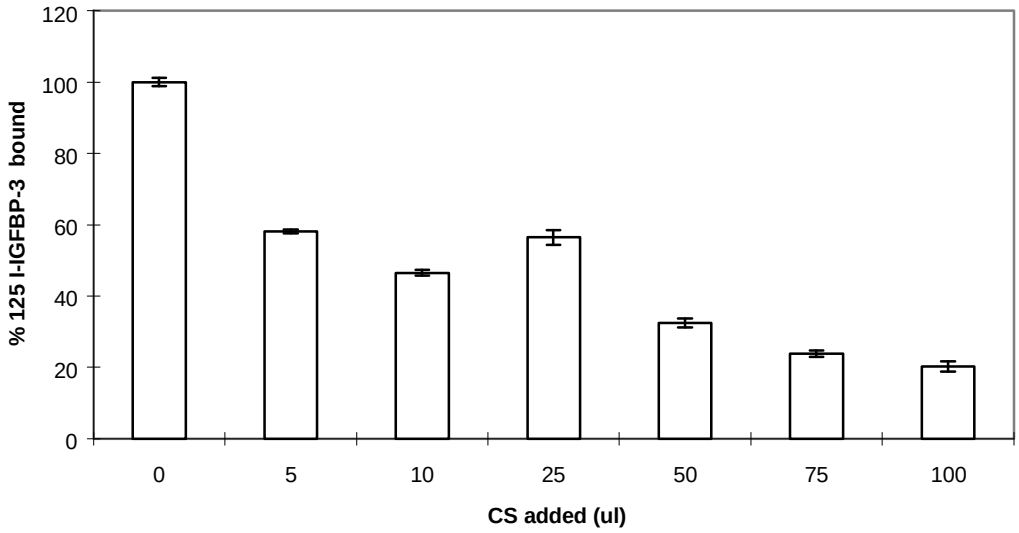

Figure 3.7 :**Determination of optimum level of calf serum required**. Calf serum (0- 20%, v/v) was co-incubated with IGFBP-3 (1 ng/well) with BAE cells (3.9 x 10^5, passage 12)) for 2.5 hours at 4 ^oC. Results are scaled to the highest binding value, which occurred when no calf serum was added. Results are averaged over triplicates. Error bars are calculated as discussed.

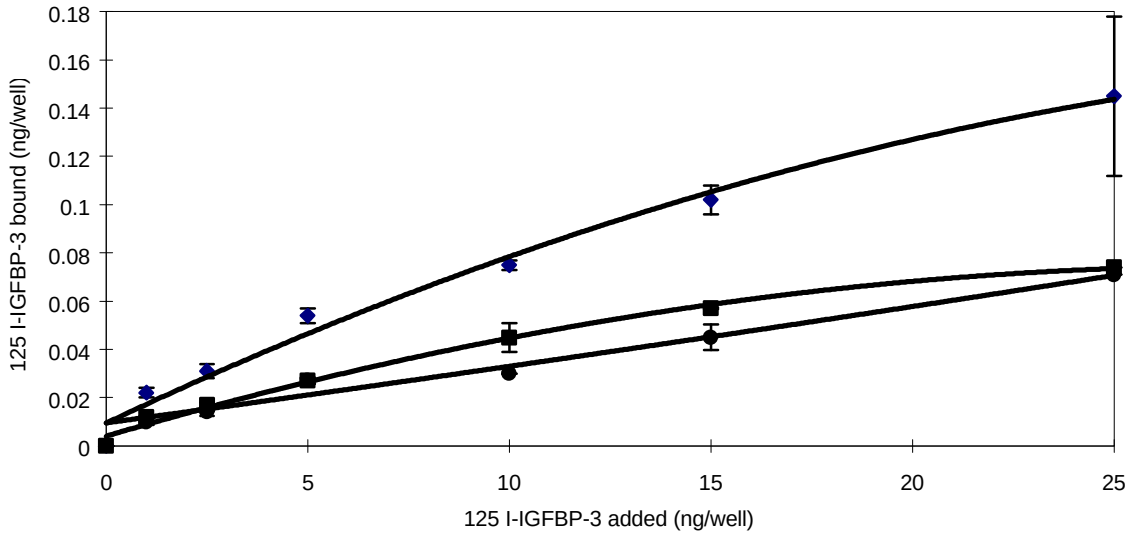

Figure 3.8 : **Binding curves for IGFBP-3**. IGFBP-3 (0-25 ng/well) was incubated with/without calf serum (20%, v/v) with BAE cells (4.6 x 10^5 cells/well, passage 9)) for 2.5 hours at 4 ^oC. Total binding (◆), non specific binding (■), and specific binding (●) are shown. Calf serum was used for the middle and lower curves. Results are averaged over triplicates. Standard error is shown.

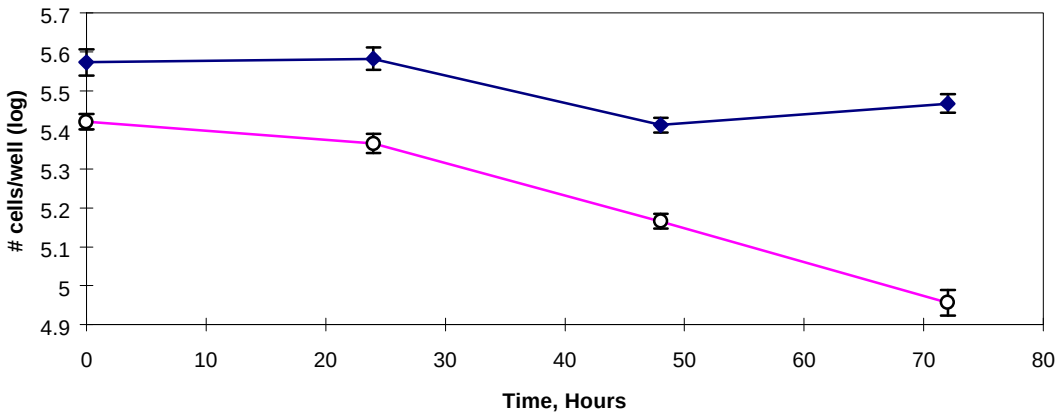

Figure 3.9 : **Determination of acceptable duration of chloration**. BAE cells (passage 8) were incubated with(❍) /without (●) sodium chlorate (75 mM) for 0, 24, 48 or 72 hours. The t = 0 reading was taken before addition of chlorate. The difference in initial cell numbers is due to different plates used. Standard error is shown.

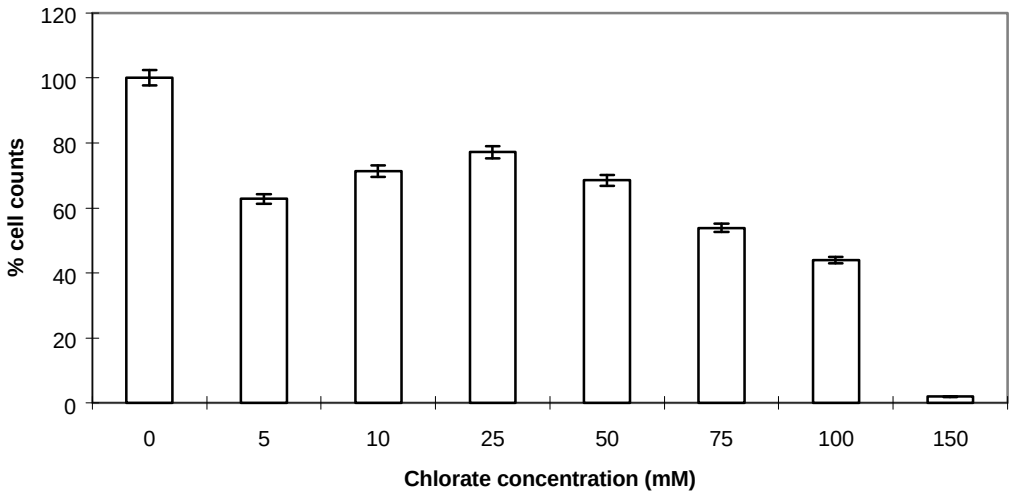

Figure 3.10 : **Determination of acceptable chlorate concentration**. BAE cells (passage 10) were incubated with sodium chlorate (0-150 mM) and cells were counted after a 12 hour incubation. Results are scaled to the highest cell counts, which occurred when no chlorate was added. Results are averaged over triplicate measurements with coulter counter. Standard error is shown (note that the 150 mM Chlorate reading has no standard error bar, owing to the negligible error associated with it).

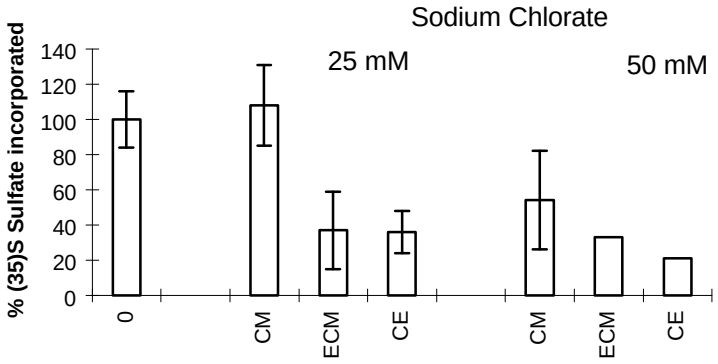

Figure 3.11 : **Determination of the efficiency of chloration**. BAE cells (2.6 10^5 cells/well, passage 11) were treated with sodium chlorate (25 or 50 mM) for 72 hours and then incubated with 50 μCi/ml ^{35}S sodium sulfate for 4 hours. CM stands for conditioned media, ECM for extracellular matrix and CE for cell extract. The 0 bar was generated by treating as 100% the CE, ECM, and CM values when no chlorate was added. The difference in the heights of the 0 and CE bars yielded the efficiency values. The mM values refer to the amount of chlorate added. Results are scaled to the value obtained on no addition of chlorate. Results were averaged over triplicates. Standard error is shown.

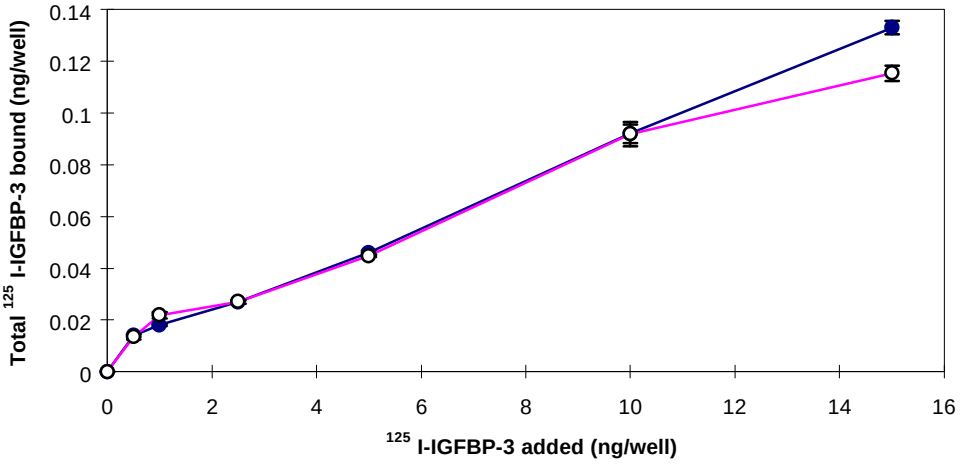

Figure 3.12 : **Effect of chloration on ^{125}I-IGFBP-3 binding**. After a 5 hour serum starvation**,** sodium chlorate (75 mM) was incubated with BAE cells for 48 hours at 37 ^{o}C (passage 8) prior to addition of ^{125}I-IGFBP-3 (0-15 ng/well). Chlorated (●) and non chlorated (❍) cells are shown. The difference in cell densities was taken into account by multiplying the chlorated values with the ratio of chlorated to non-chlorated cell numbers. Results are averaged over triplicates. Standard error is shown. This experiment is representative of one of three experiments.

Chapter IV : Cell free assays

4.1 Introduction

In the previous chapter, experiments based on binding to cells in culture were discussed. Though these systems have the advantage of being close parallels to the interactions in the human body, they have a major drawback. Definitive conclusions are difficult to draw since the presence of other molecules on the cell surface introduces a factor of uncertainty with respect to specificity of interaction. Even in the extracellular environment, numerous secreted peptides and enzymes may affect the binding event being studied. The ambiguity caused by these extraneous factors is particularly significant when precise quantitative information is being sought in order to model a particular phenomena. For example, the efficiency of chloration is determined by measuring the ^{35}S sulfate incorporated into the cells. The resulting effect is likely specific to the HSPGs, but other entities on the surface may also be susceptible to chloration and sulfation procedures. The technique however has been used successfully with other cell types (Fannon and Nugent, 1997).

In order to circumvent this problem and achieve greater specificity, cell-free assays were tested, where only the reagents being studied are allowed to interact. A range of separation processes, from precipitation to filtration are used to glean a quantitative insight into the binding phenomena.

This chapter focuses on the two primary cell free techniques employed : cetlypiridnium chloride (CPC) precipitation and the CAFAS assay. First, the CPC method is discussed, along with an analysis of IGFBP-3 and IGF-I binding to P9 HS. The interaction of IGFBP-3 and IGF-I with the Zeta Probe membrane is then discussed. The relative stickiness of both these reagents is also examined by measuring binding to incubation vials, membrane, and vessel wall. Finally, the retention of IGFBP-3 and IGF-I on the Zeta Probe membrane is determined.

4.2 CPC precipitation Studies :

As discussed in chapter II, the CPC method was conducted with several different modifications in order to improve the efficiency of precipitation. CPC binds to negatively charged molecules such as proteoglycans (Roden *et al.*, 1972). The purpose of these studies was to determine the amount of IGF-I or IGFBP-3 bound to P9 HS by precipitating P9 HS from a suspension of the reagents. However, a major problem encountered was the low level of radioactivity associated with the ^{35}S labeled P9 HS. Hence, it was often difficult to discern binding events from background radiation. Also, this protocol requires a minimum concentration of 0.1 mg/ml of proteoglycan for reliability (Ausubel, 1989). This level is usually achieved by the addition of a carrier proteoglycan such as chondroitin sulfate dermatan sulfate. However, this step could not be taken in our studies, since these proteoglycans would interfere with the binding of the target reagents. Nevertheless, some meaningful results were obtained, which are discussed below.

4.2.1 CPC precipitation of ^{35} S P9 HS : Determination of efficiency :

CPC treatment caused 83.5% of the ^{35}S sulfate label on the P9 HS to be precipitated. Briefly, 0.2 ml of ^{35}S-P9 HS (6 ng/µl) was incubated with 0.04 ml of 10% (v/v, stock) CPC solution, for 30 minutes at 37 ^{o}C. This concentration was chosen primarily to ensure that the resulting precipitate has a radioactive count well above background. Centrifugation followed (1500 x g for 10 min), and the supernatant and pellet were counted for radioactivity on a liquid scintillation counter. Since the GAG chains of the HSPG bind to CPC and are precipitated, this might imply that a fraction of the PG are inactive towards CPC. There could be four possibilities in this regard : (1) Some of the PGs may have lost the ^{35}S sulfate ion labels, (2) The PGs may have been broken into smaller sugars and peptide fragment and no longer possessing the same binding characteristics to CPC, (3) They may have stuck to the wall of the centrifuge tube used for the assay, (4) The mixture was overloaded with excess of P9 HS. Of these, (1) seems

more likely, since loss of label from a reagent is a frequent occurrence. Thus, this study showed that CPC does precipitate P9 HS.

4.2.2 CPC precipitation of IGF-I, IGFBP-3, IGF-I/P9, and IGFBP-3/P9 :

The purpose of this experiment was to determine whether IGF-I or IGFBP-3 is precipitated by CPC, and whether this is affected by the addition of P9 HS. An increase in precipitate would indicate binding of P9 HS with IGF-I or IGFBP-3.

An initial experiment was conducted following the predetermined procedure. The percentage of IGFBP-3 being precipitated in the absence of P9 was 9.8%, and in the presence of P9, was 9.0%. The corresponding numbers for IGF-I were 1.3% and 1.0%. There was no major change seen in the binding characteristics of both IGFBP-3 and IGF-I. The above binding levels were much lower than those obtained with only P9 HS, which yielded an of 83.5% precipitation. A possible reason for the low binding levels could be that there is a significant time in precipitating with CPC, during which the P9-IGFBP-3 or P9 IGF-I complexes may be dissociating. Hence, a second experiment was done where the incubation time with CPC was reduced to 2 minutes, and centrifugation was done immediately following this step. Also, a higher centrifugation force (20,000 x g) for a longer time period (20 minutes) was used. A doubling of the amount of complex precipitated with the addition of P9 HS was seen (IGFBP-3 : 6.1% and 12.3% , IGF-I 0.42% and 0.82%, Table 4.1). A follow up experiment in order to reproduce this result was done. However, this time IGF-I showed no change in binding (the precipitation with and without P9 HS was 1%) and IGFBP-3 binding decreased from 26% to 19%.

Hence, the CPC assay did not yield consistent results, and was subsequently discontinued.

4.3 CAFAS Assay :

4.3.1 Determination of IGFBP-3 and IGF-I binding to the Zeta-Probe membrane, without transferring material across the membrane.

In order to conduct binding studies using the Bio-Dot Microfiltration assay, it is important to know the amount of nonspecific binding exhibited by the reagents used. In keeping with this aim, the present study aimed to characterize the interaction of IGF-I and IGFBP-3 with the Zeta-Probe membrane.

IGF-I and IGFBP-3 both showed an increase in binding to the membrane with time. Results are shown in figures 4.1 and 4.2. Briefly, l ng/well IGFBP-3 and 1 ng/well of IGF-I were added to each well of the Bio-Dot apparatus as per predetermined layout. This was followed by incubation in the wells for up to 2.5 hours, where aspiration of the liquid occurred at a given time. Following, the membrane was dried, the radioactive areas were cut out and measured in a liquid scintillation counter. Wang (1997) observed that incubation of reagents carried out the in wells yielded an undesirable degree of nonspecific binding. IGFBP-3 seems to bind a greater quantity to the membrane compared to IGF-I, with saturation occurring at 1 hour after addition. IGF-I, in contrast, shows a binding of 80 % of amount added at 1 hour. This implies that IGFBP-3 seems to be a stickier molecule than IGF-I. A possible source of error could be the time taken to draw out the samples, which was not factored in, and could cause inaccurate time scales.

The above results are binding values for the membrane. A study was also done to determine the amount of radioactivity retained on the walls of the wells. The total radioactivity added was recorded, and the amount retained on the wall was obtained by subtracting the amount stuck on the membrane. As shown in figure 4.3, IGFBP-3, as expected from previous results, showed a wall retention of 10%, whereas IGF-I had no significant binding to the wall.

4.3.2 : Determination of IGFBP-3 retention in incubation vials, and on Zeta-Probe membrane with no incubation time.

Our primary aim is to determine the conditions under which IGFBP-3 and IGF-I would have a minimal sticking to the membrane and walls. This is important in order to reduce the error during binding studies. It is important that the molecules remain in solution during the study, otherwise only a fraction of the added reagents would actually be interacting, the rest would be stuck to the well surface, and sterically hindered from binding to other molecules. Hence, a different approach was attempted. The incubation step was carried out in 0.6 ml centrifuge tubes, for 1 hour. The solutions were then added to the wells, and immediately removed and counted. The results are as shown in figure 4.4, for both 0.5 ng/well and 1 ng/well of each reagent. Again, in accordance with previous results, IGFBP-3 displayed a higher stickiness. Also, the retention of radioactivity in the incubation vials for IGFBP-3 was 9.0%, where as for IGF-I it was only 2.5%. The membrane retention was 32% for IGF-I, and 43% for IGFBP-3.

4.3.3 Determination of IGF-I and IGFBP-3 retention on Zeta Probe membrane, by pulling through across membrane.

The above results have established that it is not feasible to carry out incubation of IGFBP-3 or IGF-I in the Bio-Dot apparatus well, owing to their large retention values on the Zeta Probe membrane. A binding study with P9 would consist of *pulling through the incubated mixture across the membrane*, and measuring the retained radioactivity. This is based on work by Wang *et al.*, (submitted). For this, it is important to determine whether the retention is low enough without the P9, otherwise the high background would obscure the readings. This value was 10 % for IGF-I, and 23 % for IGFBP-3, as shown in figure 4.5 and the percentage retention was similar for both 0.5 ng/well and 1 ng/well for each reagent.

Hence, with a retention of 10 %, IGF-I, the CAFAS assay could be used for analysis of P9 interaction with IGF-I. This has been done by Wang *et al.*, (submitted) and has been shown to be negligible. However, the high stickiness of IGFBP-3 (23 % retention) makes it an unsuitable candidate for this assay.

4.4 Conclusions :

In this chapter, CPC precipitation and the CAFAS assay were examined as possible methods for conducting cell free experiments. CPC precipitated a large fraction of the ^{35}S P9 HS suspension (83.5%). However, conflicting results were obtained with IGF-I and IGFBP-3 (see Table I). Hence, this method was not deemed suitable for carrying out binding studies.

A preliminary study of the binding characteristics of IGF-I and IGFBP-3 to the Zeta-Probe membrane was also done. A transient binding study revealed that IGFBP-3 was a much stickier molecule than IGF-I (figures 4.1 and 4.2). It was also found that the degree of nonspecific binding to the walls of the Bio-Dot apparatus can be significantly reduced by carrying out the incubation in separate vials (see figures 4.3 and 4.4). Finally, the retention (after pulling the sample suspension across the membrane by vacuum) of IGF-I on the Zeta-Probe membrane (10%) was seen to be less than half that of IGFBP-3 (23%). Hence, the use of this method for binding studies is feasible only with IGF-I, and not IGFBP-3.

Table 4.1

CPC Precipitation of IGF-I, IGFBP-3, IGF-I/P9 and IGFBP-3/P9.

Assay conditions	IGF-I (%)	IGF-I + P9 (%)	IGFBP-3 (%)	IGFBP-3 + P9 (%)
1500 x g, 10 min	1.3	1.0	9.8	9.0
20, 000 x g, 20 min	0.42	0.81	6.1	12.3
20, 000 x g, 20 min	1.0	1.0	26.0	19.0

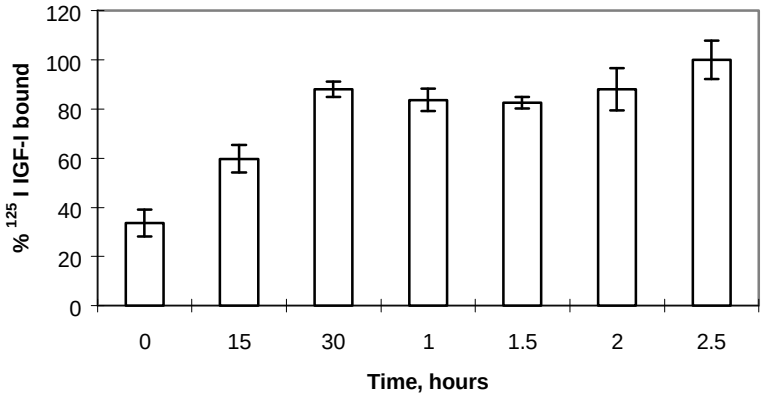

Figure 4.1 : **^{125}I-IGF-I binding to the Zeta Probe membrane**. ^{125}I-IGF-I (1 ng/well), was incubated in the Bio-Dot microfiltration apparatus over 0-2.5 hour span. The results shown are average of triplicate sets of data, and are scaled to the highest binding value, which occurred at 2.5 hours. Refer to text for further details.

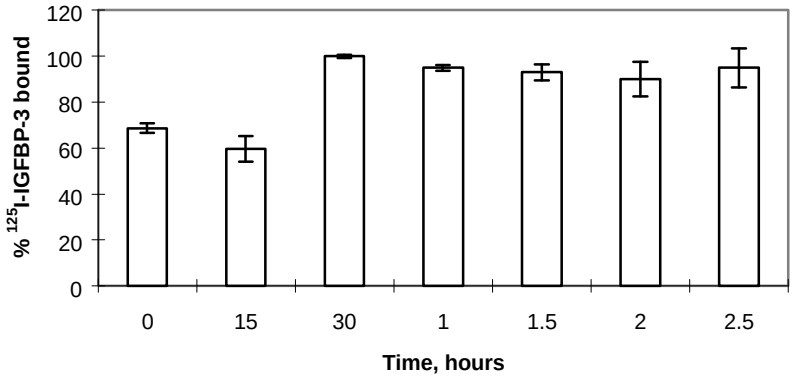

Figure 4.2 : **^{125}I-IGFBP-3 binding to Zeta Probe membrane**. ^{125}I-IGFBP-3 (1 ng/well) was incubated in the Bio-Dot Microfiltration apparatus over a 0-2.5 time span. Results are averages of triplicates, and are scaled to the highest binding value, which occurred at 30 minutes. Standard error is shown.

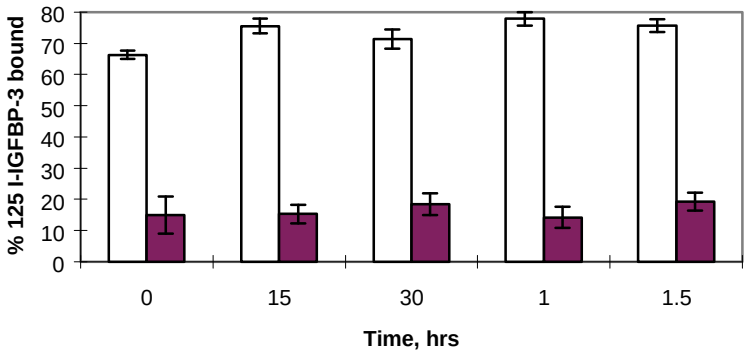

Figure 4.3 : **^{125}I-IGFBP-3 binding to Bio-Dot apparatus**. ^{125}I-IGFBP-3 (1 ng/well) was incubated in the Bio-Dot apparatus for 2.5 hours, and the material bound to the wall and membrane was measured. Open columns represent membrane binding, and filled columns represent wall binding. Results are averages of triplicates. Standard error is shown.

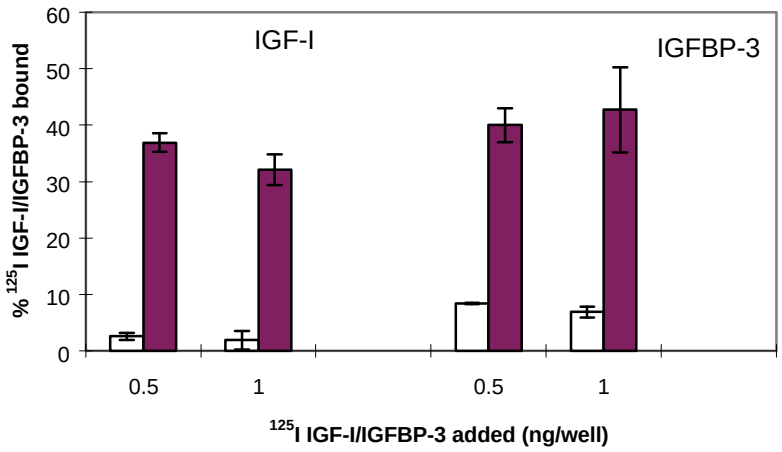

Figure 4.4 : **Retention of ^{125} I-IGF-I and ^{125} I-IGFBP-3 on membrane and incubation vials**. ^{125}I- IGF-I (0.5 ng/well, 1 ng/well) and ^{125}I-IGFBP-3 (0.5 ng/well, 1 ng/well) were incubated 0.6 ml centrifuge vials for 2.5 hours. Thereafter they were transferred to the Bio-Dot apparatus for a brief exposure. The darker columns represent membrane retention, lighter ones represent vial retention. Results are scaled to the total radioactivity added in each case. Standard error is shown.

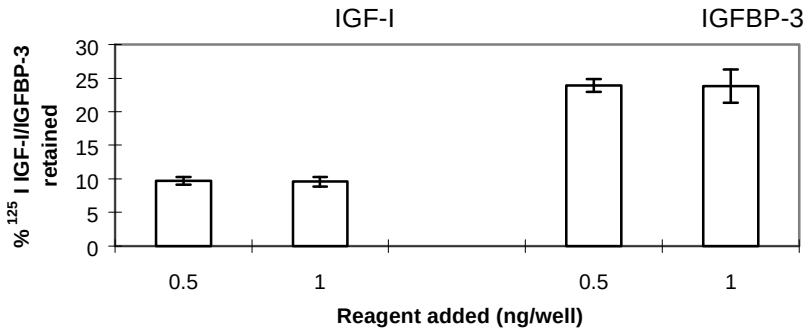

Figure 4.5 : **Retention of ^{125}I-IGF-I and ^{125}I-IGFBP-3 on Zeta Probe membrane**. ^{125}I-IGF-I (0.5 ng/well, 1.0 ng/well) and ^{125}I-IGFBP-3 (0.5 ng/well, 1.0 ng/well) were incubated in 0.6 ml tubes, and transferred to the Bio-Dot apparatus wells and pulled through by vacuum. Results are scaled to the total radioactivity added in each case. Standard error is shown. .

Chapter V : Future directions

5.1 Introduction

As research by numerous investigators has shown, IGFBP-3 shows varied characteristics with different cell lines, and new facets of this molecule are being unearthed at a rapid rate (Clemmons, 1992, 1993, 1997, Conover *et al.*, 1998, Oh 1997, 1998, Oh *et al.*, 1993, 1995). Our work was primarily focused on two aspects : (a) The characterization of IGFBP-3 interaction with BAE cells, and (b) The role of HSPGs, both in the media and on the surface, in this process. Several useful results were obtained, however, a significant amount of work still remains to be done. This chapter discusses some of the possible studies that could be undertaken, and the modifications that could be affected to existing methods which could yield more reproducible conclusions.

5.2: Cell based assays

5.2.1 P9 binding to BAE cells :

Pre-incubation of cells with P9 affords an opportunity to examine whether P9 HS interaction with the cell surface, if at all present, has an effect on the reagent in question (IGF-I or IGFBP-3). However, quantification of P9 HS binding to the cells must first be done. ^{35}S sulfate labeled BAE P9 HS can be incubated with BAE cells, as per the standard binding study protocol (see Chapter II : Binding Study). However, the low level of radioactivity associated with the labeled Sulfate may yield counts indistinguishable from background readings. The level of specific binding can then be judged to see if appropriate for future pre-incubation studies.

5.2.2 Effect of co-incubation of P9 with IGFBP-3

Pre-incubation of P9 with BAE cells and its effect on IGFBP-3 binding was studied (see Chapter III : Cell Based Assays, Section 3.1). It has been seen that addition of exogenous HSPG in the media had no effect on the binding of IGFBP-3 to the cell surface. Co-incubation of these two molecules would yield further information on this interaction, and this assay could serve as a starting point for further investigation. .

5.2.3: Co and pre-incubation of IGF-I with IGFBP-3 : Effect on IGFBP-3 binding

Co-incubation of IGF-I with IGFBP-3 was studied in Chapter III, and was shown to have reduced IGFBP-3 binding. Oh *et al.* (1993) have also reported a decrease in the inhibitory effects of IGFBP-3 on co-incubation with IGF-I. However, as mentioned in Section 5.1.2, competitive binding to the cell surface and IGF-I-IGFBP-3 binding may be occurring together, and our study has not taken that into account. The above authors determined that inter-ligand binding was the case. This was done by co-incubation of IGFBP-3 with analogs of IGF-I which have a reduced affinity for IGFBP-3 but an unchanged affinity for the cell surface. No change in IGFBP-3 induced cell response was seen. A similar study could be carried out on BAE cells. If the presence of IGF-I analogs affect IGFBP-3 cell binding, IGF-I may have a common surface receptor with IGFBP-3, or at least an interaction with IGFBP-3 at the cell surface. If not, then the ligands may be binding in the media and adversely affecting IGFBP-3 binding.

5.2.4 P9 HS and IGFBP-3/IGF-I complex interaction

IGF-I is present in the blood stream in the form if a ternary complex also consisting of IGFBP-3, and an acid labile subunit (ALS) (McCusker *et al.*, 1997). This increases the halflife of IGF-I, which may otherwise undergo digestion by the numerous enzymes in the plasma. Such blood borne growth factors must traverse the capillary endothelium by transcytosis in order to reach many of their target tissues (Bar *et al.*, 1986). However, it is unclear whether IGF-I remains bound to IGFBP-3 during this process. Also, the role of HSPGs in regulation of accessibility of this complex to the surface is unknown. Since IGFBP-3 is known to have a putative heparin binding domain (Arai *et al.*, 1994), it is possible that P9 binds to IGFBP-3, and induces a conformational change which renders the binding protein incapable to maintain its complex form with IGF-I. A binding study could be conducted in which P9 HS is co-incubated with BAE cells and a pre-formed complex of labeled IGF-I and IGFBP-3. With the aid of Recombinant DNA technology, deletion or substitution mutants of P9 HS could be constructed which have a lower affinity to IGFBP-3. A co-incubation of the above complex with these could then be used as a comparison. Since three reagents are involved, the results will be difficult to interpret, however, valuable insight may be gained on the nature of IGF-I binding to BAE cells.

5.3 Cell free assays :

Two methods were used to study the binding of molecules in cell free environment. The cetlypiridnium chloride precipitation technique yielded significant precipitation of P9 HS (85%, see Chapter IV for details). However, this assay proved to be unsuitable for conducting binding studies, since the concentration of P9 HS used was lower than the minimum required for meaningful results (see Chapter IV). Also, studies were done to determine the feasibility of conducting binding studies with IGFBP-3 and IGF-I using modified CAFAS assay. It was seen that IGFBP-3 retention of 23% on the membrane was too high to be used. IGF-I, on the other hand, showed a lower retention value of 10%, making it a suitable reagent for use, though with a certain degree of uncertainty.

Hence, there is a clear need for a new cell free assay. Koedam *et al.* (1997) have developed a solid phase binding assay which could be used to study the interaction between IGF-I and IGFBP-3, as well as the effects of antibodies and heparin. Recombinant IGFBP-3 was immobilized on plastic microtitre wells, after which radiolabelled IGF-I or -II was allowed to bind. The assay was specific, since IGF-I did not bind on prior addition of albumin, which blocked the available binding sites. A major problem with this method is that the immobilization step may sterically restrict the access of IGFBP-3 binding sites to IGF-I or heparin. However, Hickson *et al.* (1997) and Fowlkes *et al.* (1996) have used this procedure successfully, and it may be worthwhile to conduct a first hand evaluation of its efficiency.

Heding *et al.* (1996) have used a BIAcore™ instrument (Pharmacia Biosensor AB, Uppasala, Sweden) to measure the high affinity binding of unlabeled IGFs and IGF analogues to recombinant unglycosylated IGFBP-3. The BIAcore technology made it possible to visualize the macromolecular interactions directly in real time (Fagerstam *et al*, 1992). The BIAcore™ is a biosensor based instrument that uses surface plasmon resonance as the detection principle. One molecule of the interaction to be studied is immobilized covalently to a sensor chip, and the other (ligand) is passed over the chip in solution. A computer screen displays a signal proportional to the mass of ligand bound to the surface. Such a study may require a significant financial commitment, however, it has been successfully employed to analyze cell free interactions within the IGF-I system, as shown by Heding and co-workers.

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Appendix A : Dissociation Constant for IGFBP-3

A.1 Specific binding curve analysis

The method of determination of the dissociation constant (K_D) for IGFBP-3 is discussed here. The equation used for this analysis was :

(2) $L + R = C$;

where L = the ligand (IGFBP-3) concentration in the media,

R = the number of receptors on the surface of a cell, and

C = the number of receptor-ligand complexes formed.

Conservation mass of the ligand and receptor yields :

(3) $Lo = L + C$;

(4) $Rt = R + C$;

where Lo = the total amount of ligand added, and

Rt = the total number of receptors on the surface of the cell.

At equilibrium law of mass action yields :

(5) $C K_D = L R$

Two distinct cases arise :

(a) Assuming no ligand depletion from the extracellular media, and using the conservation equation (4) in (5) above yields the following equation :

(6) $Lo = C K_D/(Rt - C)$;

(b) Assuming ligand depletion from the extracellular media and using the conservation equations (3) and (4) in (5), we arrive at a quadratic solution, the positive root was discarded since the subsequent parameters were negative in value :

(7) $C = \{(Lo + K_D + Rt) - \sqrt{[(Lo + K_D + Rt)^2 - 4\ Lo\ Rt]}\}/2$

In order to conduct a least squares analysis for both cases (a) and (b), and find the corresponding values of K_D, the following experimental data was obtained :

^{125} I-IGFBP-3 (Lo, ng)	Binding Complex (C, ng)
1.0	0.0106
2.5	0.01485
5.0	0.02718
10.0	0.03061

Mathematica® was used for the mathematical analysis. The least squares fit yielded the following results :

Case (a) : K_D = 7.31 ng or 3.1×10^{-10} M

Case (b) : K_D = 7.25 ng or 3.1×10^{-10} M

A sample calculation is as follows :

7.31 ng/(0.5 ml) x mol/47000ng x 1000ml/L = 3.1×10^{-10} M

Mathematica® Routine :

<<Statistics ` NonLinearFit` /* The built in package is installed */

data = {{1, 0.0106}, {2.5, 0.015}, {5, .027}, {10, 0.031}}; /* data is input */

noligdep = NonLinearFit[data, (Lo Rt/(Lo + K_D)), {Lo}, {K_D, Rt}]; /* regresion for case (a) */

The output was :

0.062 Lo / (7.31 + Lo)

ligdep = NonLinearFit[data, {(Lo + K_D + Rt) - √[(Lo + K_D + Rt)^2 - 4 Lo Rt]}/2, {Lo}, {K_D, Rt}]

/* regression for case (b) */

The output was :

$\{ 7.31 + Lo - \sqrt{[-0.25\ Lo + (7.31 + Lo)^2]}\}/2$

A.2 : Total binding curve analysis

The above values for K_D were obtained by modeling the specific binding values for IGFBP-3 obtained by subtracting the nonspecific binding from total binding values. However, the use of calf serum as opposed to excess unlabelled ligand (IGFBP-3) may have lead to inaccurate readings for the nonspecific binding (and hence the specific binding) values. This is because calf serum contains a large excess of several other growth factors and proteins which may interfere with the IGFBP-3 interaction with the cell surface. Thus, a separate analysis was conducted to model the total binding of IGFBP-3, and evaluate equilibrium constants for both specific and non specific binding events.

Assuming nonspecific binding to be non saturable, and a linear function of the ligand concentration (Lauffenburger and Lindermann, 1993), we get

(8) $L = B,$

where B is the concentration of the nonspecifically bound ligand. This yields the corresponding equilibrium equation

(9) $B = Kn\ L,$

where Kn is the equilibrium association constant for nonspecific IGFBP-3 binding.

As in appendix A.1, two distinct cases arise :

(a) Assuming no ligand depletion occurs and combining equations (2), (4), (5), (8) and (9), an equation relating the total binding to the ligand concentration is obtained

(10) $$B + C = \frac{Lo\ Rt}{(Lo + K_D)} + Kn\ Lo$$

(b) Assuming ligand depletion occurs and combining equations (2), (3), (4), (5), (8) and (9), an equation relating the total binding to the ligand concentration is obtained :

(11) $B+C = Lo\ (\ Kn\ K_D + Rt)/(K_D + Kn\ K_D + Rt)$

Here it was assumed that the number of receptor-ligand complexes forming was much less than the total number of receptors present.

In order to conduct a least squares analysis and find the value of K_D and Kn, the following experimental data was obtained :

^{125} I-IGFBP-3 (Lo, ng)	Total Binding (B + C, ng)
1.0	0.022
2.5	0.031
5.0	0.054
10.0	0.076
15.0	0.101
25.0	0.145

Mathematica® was used to conduct a nonlinear least squares analysis of the above data, The values obtained were as follows (for a sample calculation, see appendix A.1). :

Case (a)

Kn = 0.0044 ng or 1.9×10^{-13} M

K_D = 1.54 ng or 0.066×10^{-9} M

Case (b)

Kn = 0.0044 ng or 1.9×10^{-13} M

K_D = 1.52 ng or 0.064×10^{-9} M

Mathematica® routine :

<<Statistics ` NonLinearFit` /* The built in package is installed */

data = {{ 1, 0.022}, {2.5, .031}, {5, 0.054}, {10, 0.076}, /* Data is input */
{15, 0.101}, {25, 0.145}};

NonLinearFit[data, (x Rt) / (x + K_D) + Kn x, {x}, {Kn, K_D, Rt}] /* Regression for case (a)*/

Output was :

(10) $$0.0043533\,x + \frac{0.0389333\,x}{1.53853 + x}$$

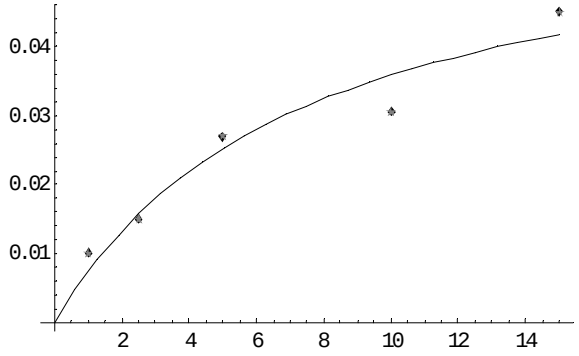
NonLinearFit[data, Kn Lo + {K_D + Lo + Rt - √[(K_D + Lo + Rt)^2 - 4 Lo Rt]}/2} (1-Kn), {x}, {Kn, K_D, Rt}] /* Regression for case (b)*/

Output was :

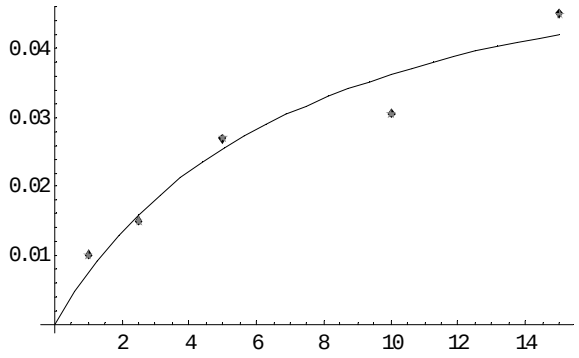
(11) $0.0044\ Lo + 0.5\ \{1.56 + Lo - \sqrt{[-0.16\ Lo + (1.56 + Lo)^2]}\}$

The following are graphical representations of the regression analysis conducted in this Appendix. Each figure is labeled according to the case it represents.

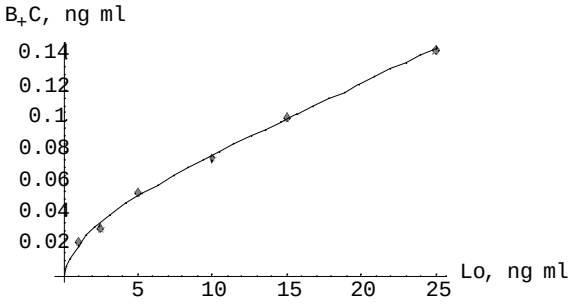
A.1 a



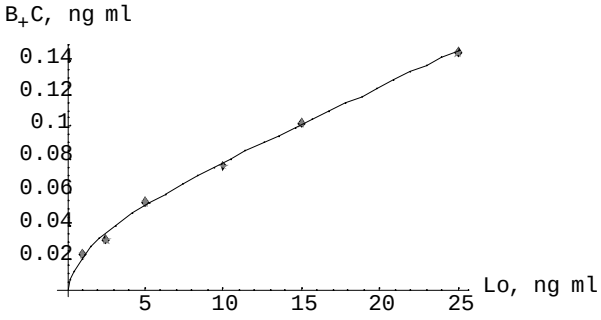
A.1 b


0.04
0.03
0.02
0.01
2
4
6
8
10
12
14

A.2 a


B+C, ng ml
0.14
0.12
0.1
0.08
0.06
0.04
0.02
5
10
15
20
25
Lo, ng ml

A.2 b


B+C, ng ml
0.14
0.12
0.1
0.08
0.06
0.04
0.02
5
10
15
20
25
Lo, ng ml

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