Chapter II. Materials and Methods

A. Cell Cultures:

Bovine aortic endothelial (BAE) cells were used between passage 5 and 12. BAE, isolated as described[58]. All cells were maintained in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM, low glucose (1 gm / L), Cellgro, Herndon, VA), supplemented with penicillin (100 U / ml, streptomycin (100 µg / ml), glutamine (2mM) and 10% calf serum (Cellgro, Herndon, VA). Confluence was judged to occur when 95 -100% of the culture plate was covered with the typical "cobblestone" appearance of endothelial monolayer cell growth, (Figure 2-1). The cell number was determined by counting trypsin-suspended cells (Cellgro, Herndon, VA) with a Coulter Counter (Model Z1, Coulter Electronics, Inc., Miami, FL).

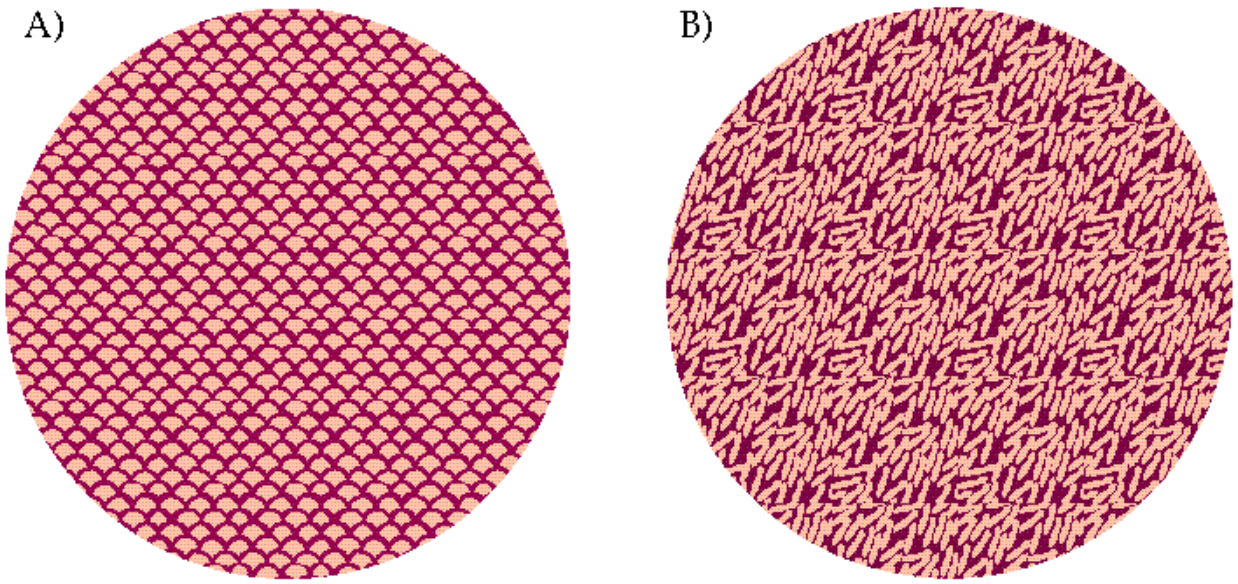

Figure 2-1 Schematic illustration depicting the A) "cobblestone" confluence of BAE cells and B) the spindle-like confluence of SMC.

B. Materials Used:

^{125}I-IGF-I, prepared by Chloramine-T procedure[59], was a generous gift of Dr. R. M. Akers (Dairy Science, Virginia Polytechnic Institute and State University). Specific activity was between 40 and 48 µCi/µg. Long R^3 IGF-Iwas obtained from Gropep (Adelaide, SA, Australia). IGFBP-3 and IGF-I were obtained from Upstate (Lake Placid, NY). Initial ^{125}I-IGFBP-3, iodinated by Chloramine-T method[59], was obtained from Diagnostic Systems Laboratories (Webster, TX) with a specific activity of 8.75 µCi/ml. In addition, IGFBP-3 was obtained from Upstate (Lake Placid, NY) and generously labeled, following the Bolton-Hunter procedure, by Dr. M.A. Nugent (Boston University School of Medicine) with a specific activity of 46 µCi/µg. Gelatin, HEPES, Sepharose gels CL-2B and CL-6B and Q-Sepharose were obtained from Sigma (St. Louis, MO). Dimethylene blue (DMB) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Bovine Kidney Heparan Sulfate standards were obtained from Sigma (St. Louis, MO). Bovine Serum Albumin (BSA) was obtained from Fisher Scienitific (FairLawn, NJ). Bovine aortic endothelial cells were a generous gift from Dr. M. A. Nugent (Boston University School of Medicine).

C. Conditioned Medium:

Confluent BAE cells at passage 9 were established in DMEM supplemented with 10% calf serum and maintained in culture 2-3 days before conditioning. Conditioned medium was prepared by incubating the cells with serum-free DMEM for 1 hour at 37°C as a wash step. Cells were then incubated for 24 hours at 37°C in serum-free DMEM, or in serum-free DMEM with ^{35}SO4 (2 mCi/mL) obtained from NEN Life Science Products (Boston, MA). Media was collected and centrifuged for 30 minutes at 3000 rpm to remove any cell debris.

D. Purification of p9 HS:

Anionic exchange chromatography was used to purify the proteoglycan fraction (p9 HS) from the conditioned medium. 1 M urea was added to conditioned media and the media was loaded on a Q-Sepharose (Sigma) column (1.5 x 3.5 cm) (equilibrated in .15 M tris-HCl buffered saline (TBS) (50 mM Tris, pH 8.0, 150 mM NaCl, 1M urea)). The

conditioned media was loaded at 1 ml/min, after which anionic proteins and glycoproteins were removed by washing the column with 0.3 M TBS (50 mM Tris, 300 mM NaCl, 1 M urea) at a flowrate of 1 ml/min for approximately 30 minutes. Following, the GAG containing fraction was eluted with 1.5 M TBS (50 mM tris, 1500 mM NaCl, 1M urea) at a flowrate of 1 ml/min. The GAG content was determined using the DMB assay, a spectrophotometer based assay for sulfated GAG, using a bovine kidney heparan sulfate standard[60]. Free sulfate and excess salt were removed by dialysis against TBS (Spectra-Por cellulose ester membrane with a 1,000 molecular weight cut off (MWCO)). The resulting material will be referred to as p9 HS with a concentration of 6 ng/µL; and the ^{35}S p9 HS concentration was 1.4 ng/µL.

E. Gel Chromatography:

According to previous data with HS samples run on a Sepharose CL-2B column (separation between 70,000 and 40 million MW), HS should be included within the gel and separate into two broad fractions[33]. ^{35}S p9 HS was run over a CL-2B column (24,200 CPM) at .5 ml/min and seen to be included and have two separate peaks. Fractions were counted on a Packard Tri-Carb 2100TR Scintillation Counter (Meriden, CT), (Figure 2-2).

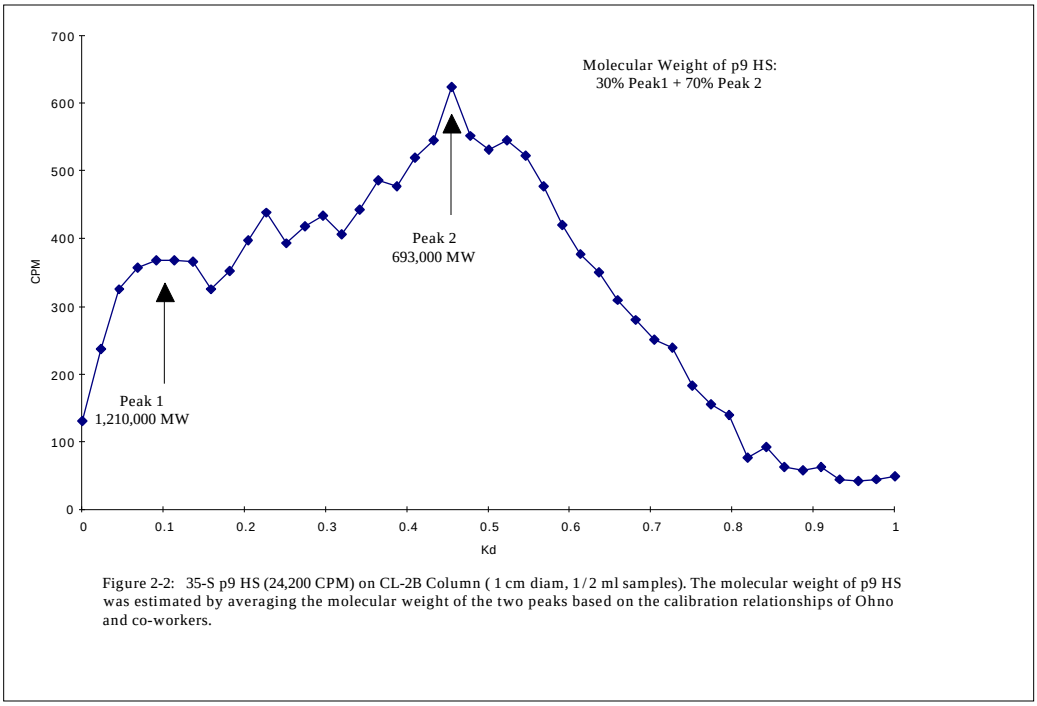

Figure 2-2: 35-S p9 HS (24,200 CPM) on CL-2B Column (1 cm diam, 1/2 ml samples). The molecular weight of p9 HS was estimated by averaging the molecular weight of the two peaks based on the calibration relationships of Ohno and co-workers.

The labeled 0.3M wash from purification steps was run on the same CL-2B column as a means of finding the end of the column, V_t, (Figure 2-3). It should be noted that the proteoglycan purification process does not separate the specific type of proteoglycans. Previous enzymatic digestion with Heparinase I (Hep I), Heparitinase (Hep III) and Chondroitinase ABC on similarly obtained material suggest that peak 1 consisted of essentially only heparan sulfate and peak 2 contained a mixture of heparan sulfate and chondroitin sulfate[28].

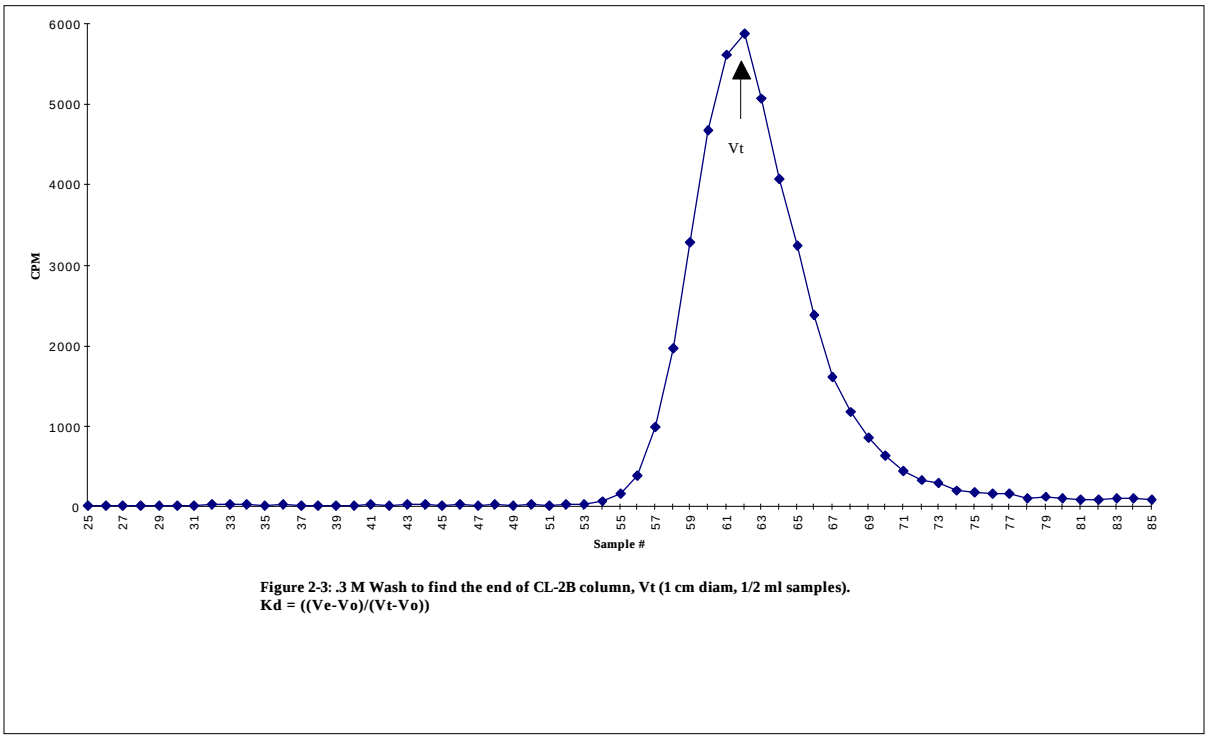

Figure 2-3: .3 M Wash to find the end of CL-2B column, Vt (1 cm diam, 1/2 ml samples). Kd = ((Ve-Vo)/(Vt-Vo))

The molecular weight of p9 HS was determined using calibration relationships derived for cartilage proteoglycan subunits from chick limb-bud chrondocyte cell cultures by Ohno and co-workers[61]:

$$\log MW = -K_{av}(1.65 \pm 0.27) + (6.58 \pm 0.08)$$

A molecular weight for p9 HS was determined after running p9 HS on a CL-2B column and determining an average K_{av}. It was determined that 30% of the material was in the first peak and 70% in the second peak yielding an average K_{av} of 0.27 and an overall molecular weight of 8.5×10^5 g / mol.

F. *Biodot Assay:*

The Zeta-Probe membrane was sized to fit the Bio-Dot apparatus (Bio-Rad, Hercules, CA) and pre-soaked in 0.15M TBS with 1 mg / ml BSA for 30 minutes. The Zeta-Probe membrane was clamped between a plastic sealing gasket and the 96-well sample template and sealed tightly with screws in a cross-diagonal fashion. The plastic sealing gasket lies on top of the gasket support plate which sits in the vacuum manifold. The vacuum manifold has a three way flow valve, which allows for on and off control during the assay. (Figure 2-4)

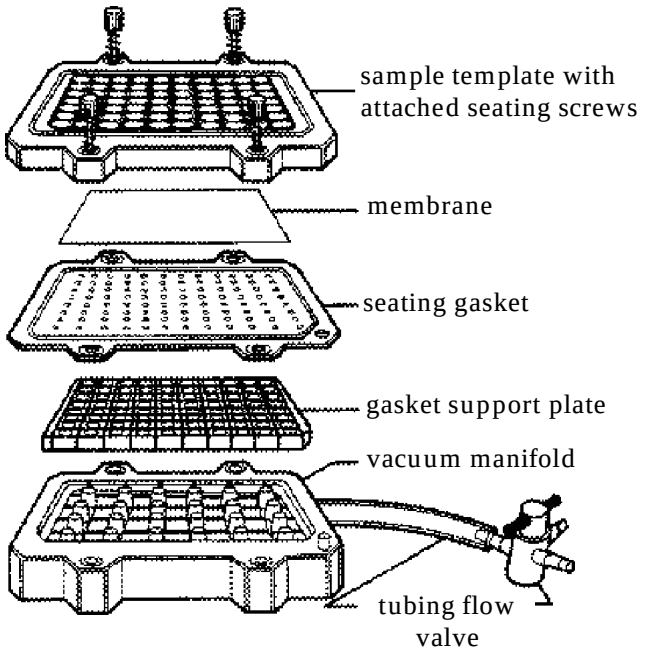

Figure 2-4 Diagram of the assembly of the Bio-Dot apparatus. (*Bio-Rad Instruction Manual*)

After the membrane was secured in the apparatus, 200 µL of 0.15M TBS, 1 mg / ml BSA was added to all 96 wells and pulled via vacuum across the membrane to insure complete wetness. The vacuum valve was then closed and samples added to individual wells containing 200 µL 0.15M TBS, 1 mg / ml BSA. After incubation, for some set time, samples were pulled through the membrane via vacuum and washed with buffer to insure all free sample has passed through the membrane. The amount of ^{125}I-IGF-I, ^{125}I-

IGFBP-3 and ^{35}S p9 HS retained on the membrane was quantified using a liquid scintillation counter.

G. *Charcoal Assay:*

The binding affinity of IGF-I for IGFBP-3 and p9 HS was examined using a method described by Booth and co-workers[62] in which free ^{125}I-IGF-I was bound by the charcoal and pelleted while IGF-I / IGFBP-3 or IGF-I / p9 HS complexes are left in the supernatant. It has been shown that charcoal is as effective a way to isolate free from bound IGF-I as a precipitate with polyethylene glycol and concanavalin A or antibody to IGFBP[63].

IGFBP-3 (0 - 20 ng) and p9 HS (0 - 50 ng) were incubated for 1 hour at room temperature with ^{125}I-IGF-I in 0.2 ml TBS, 1 mg / ml BSA in a microcentrifuge tube. Samples were then placed in an ice water bath and 0.2 ml of ice-cold 0.15M TBS, 2% BSA, 5% charcoal was added to each vial to separate bound from free IGF-I. The mixtures were incubated in the ice water bath for 15 minutes and then centrifuged at 9900 rpm for 5 minutes to pellet the charcoal. The amount of free and bound IGF-I was quantified by counting the supernatant and the pellet using a Beckman 5500 Gamma Counter (Irvine, CA).

H. *IGFBP-3-p9 HS Binding; Gel Chromatography:*

Binding of ^{125}IGFBP-3 and p9 HS was observed by gel chromatography. ^{125}IGFBP-3 was loaded on a Sepharose CL-6B column (10,000 and 4 million MW globular proteins) at a flowrate of 1 ml / min. Binding was initiated by incubating p9 HS (50 - 150 ng) with ^{125}IGFBP-3 (18,000 CPM / 1.2 ng) at room temperature for 1 hour and then running the sample over the CL-6B column. 1 minute samples were collected and counted in a scintillation counter.

I. Capacity Binding:

The capacity of ^{35}S p9 HS to bind IGFBP-3 and ^{125}I-IGFBP-3 to bind p9 HS was investigated using a nitrocellulose binding assay[64]. Nitrocellulose discs (0.5 cm) were

placed in individual wells in a 24 well plate. IGFBP-3 (0 - 48 ng) or p9 HS (0-30 ng) was added to nitrocellulose discs (each concentration was scaled to insure addition of 5µL of mixture directly onto the disc and not onto the surface beneath the disc) and incubated at 37°C for 27 hours until discs were completely dry. A quick wash with .15 M TBS with 4% BSA was done to wet the membrane discs. Labeled p9 HS or IGFBP-3 (200 µL volume binding buffer) was incubated with the discs for 16 hours at 4°C. Binding buffer was aspirated and the discs were counted in scintillation vials.

J. *Cell Binding Studies*:

BAE cells, passage 8 to passage 11, were plated at a density of 5 x 10^4 cells / well in 24-well dishes. The cells were grown in DMEM supplemented with 10% calf serum, penicillin (100 U / ml), streptomycin (100 ug / ml) and glutamine (2 mM) (Cellgro, Hendon, VA) in a 5% CO_2, 37°C environment until confluent. At confluency, cells were starved of serum components by a switch to serum-free DMEM for 72 hours. This step deprived the cells of any IGF-I found in serum-enriched DMEM and allowed for receptor levels to return to "normal". The cells per well were determined on the day of the experiment by counting trypsin suspended cells using a Coulter Counter. This was done to insure that all cell wells had approximately the same number of cells and tht experiments were done on roughly the same number of cells each time. The serum-free DMEM was removed from the test wells and 1 ml of cold binding buffer (DMEM, 25 mM HEPES, 0.05% Gelatin) was added as a wash. Binding buffer (0.5 ml) was then added to each well as the incubation solution and refrigerated for 10 minutes at 4°C. Samples were added in triplicates and incubated at 4°C for 2.5 hours. Unbound ^{125}I-IGF-I was removed by washing the monolayers 2 times with cold binding buffer (1 ml / well / wash). Bound ^{125}I-IGF-I was then extracted via 2 washes (one 20 minute, one rapid) with 0.3M NaOH (0.25 ml / well / wash). ^{125}I-IGF-I was determined in all samples by counting in a Beckman 5500 Gamma Counter (Irvine, CA).

J. Statistical Analysis

All experiments consisted of at least three samples for every data point. Error bars on all graphs represent standard error. Means of the samples are evaluated ($P \leq 0.05$) for significant changes.