Chapter IV. Cell Binding Studies

Transcytosis of IGF-I may be the primary function for vascular endothelial cells, with regard to IGF-I tissue distribution. For this reason, investigation of how locally produced proteoglycans affect IGF-I binding to endothelial cells were initiated as binding is likely to be a key step in transcytosis. The bovine aortic endothelial (BAE) cell system was chosen for investigation.

The cell binding studies are different than the cell-free assays discussed in Chapter III due to the presence of the BAE cells themselves. The cell-free assays are designed to explore only the specific interactions between the molecules included. The binding studies introduce many other complicating factors than are not present in the cell-free assays. Cells may secrete IGF-I, IGFBP-3 and p9 HS and may do so in response these molecules. There may be up- or down-regulation of cell surface receptors which may affect final binding of IGF-I to the cell surface. Cell binding studies have been simplified via temperature regulation. Investigation done at 4°C should prevent both protein/proteoglycan synthesis/ secretion and receptor trafficking, but not significantly affect binding[67].

A. NaOH Effect on Cell Release and Viability

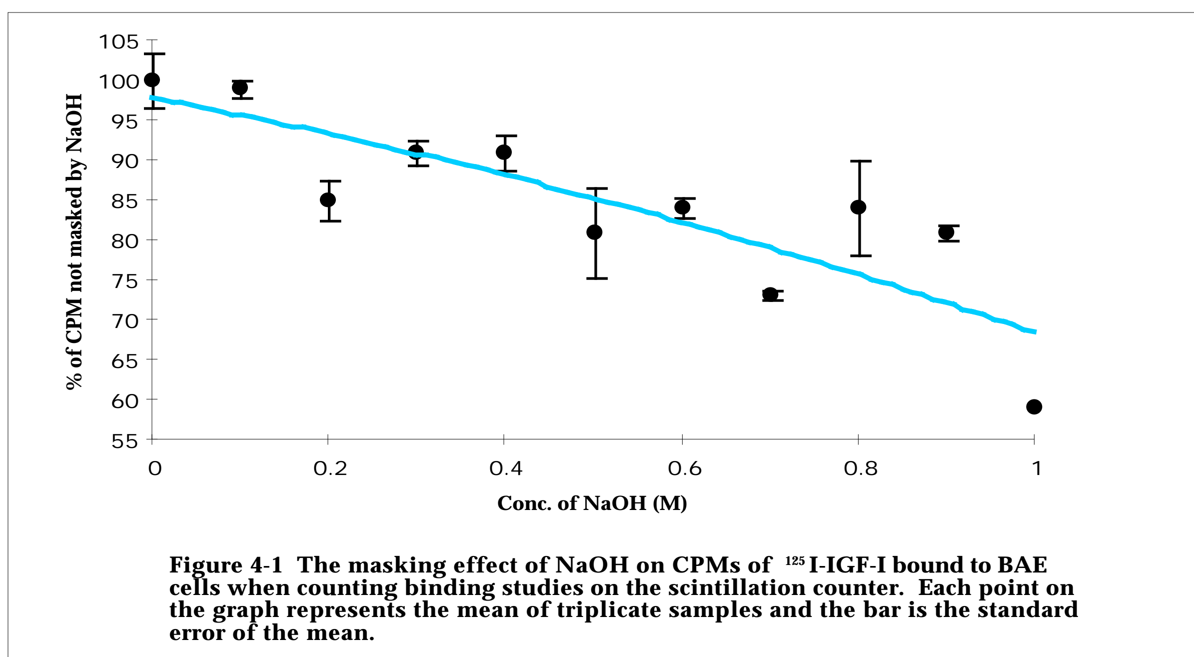

Figure 4-1 The masking effect of NaOH on CPMs of ^{125}I-IGF-I bound to BAE cells when counting binding studies on the scintillation counter. Each point on the graph represents the mean of triplicate samples and the bar is the standard error of the mean.

As discussed in Chapter III, radiolabels can be masked with chemical or color quenching when counted with a scintillation counter. Initial binding studies showed that masking may occur with the presence of sodium hydroxide (NaOH). NaOH was needed in binding studies for BAE cell release from the 24-well dishes. A series of experiments were performed in order to determine the optimal time and NaOH concentration needed for BAE cell release and subsequent quantification with a liquid scintillation counter. Concentrations of NaOH (0.1 - 1 M, 0.5 ml) were added to BAE cells with ^{125}I-IGF-I for 20 minutes. Large concentrations of NaOH mask the radiolabel while 0.1 - 0.4 M NaOH showed approximately 98% of the actual counts, (Figure 4-1). The actual cell count when compared with trypsinized cells was low for all concentrations of NaOH, (Figure 4-2). However, this is likely due to the sudden increase in sodium, which at excess concentrations can make cells burst. It should be noted that NaOH was only used after the conclusion of the actual binding study. Any effect of pH due to NaOH should not affect cell surface binding.

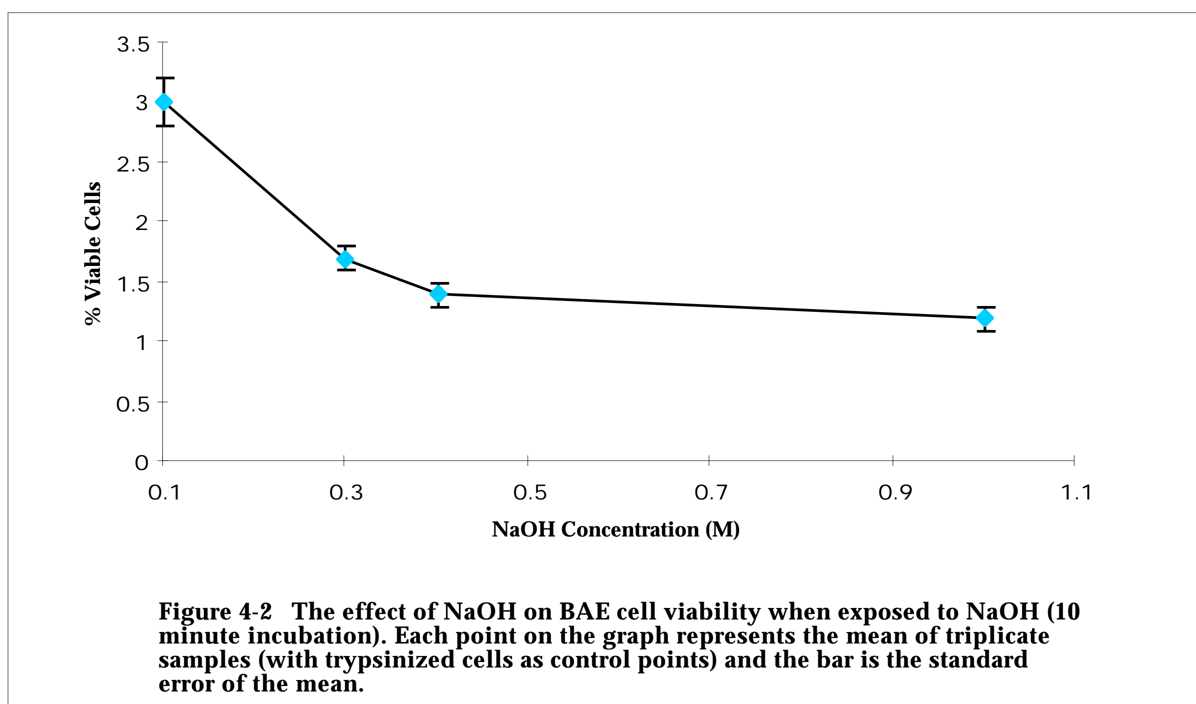

Figure 4-2 The effect of NaOH on BAE cell viability when exposed to NaOH (10 minute incubation). Each point on the graph represents the mean of triplicate samples (with trypsinized cells as control points) and the bar is the standard error of the mean.

The effect of incubation time on 0.3 M NaOH subjected cells did not change drastically from 5 minutes to 20 minutes, (Figure 4-3).

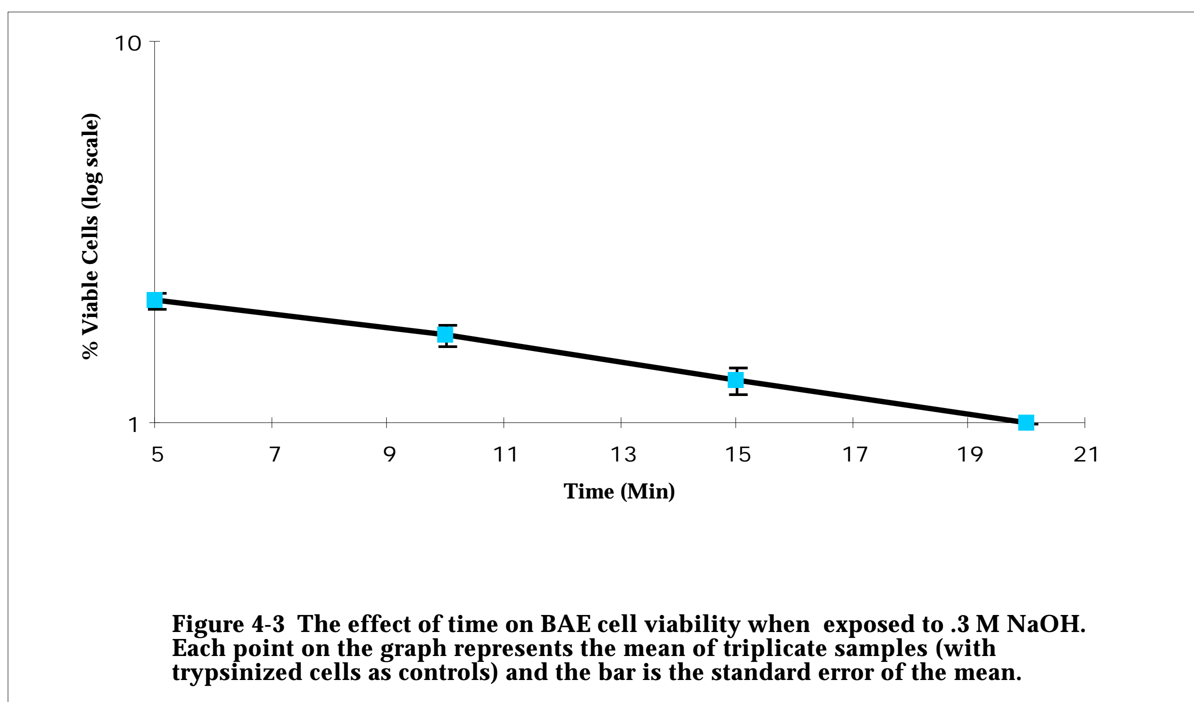

Figure 4-3 The effect of time on BAE cell viability when exposed to .3 M NaOH. Each point on the graph represents the mean of triplicate samples (with trypsinized cells as controls) and the bar is the standard error of the mean.

Consequently, 0.3 M NaOH was selected as the BAE cell releasing agent for binding studies. It is important to note that this study is based on use of a scintillation counter. Cell binding studies counted with a gamma counter are unaffected by NaOH concentration.

B. Experimental Results

The focus of the binding studies was on determining how p9 HS addition, either in the presence or absence of IGFBP-3, impacts IGF-I binding to the cell surface of BAE cells. Non-specific binding was determined via addition of excess long R^3 IGF-I which binds normally to IGF-IR, but binds to IGFBP-3 with much lower affinity[68]. Subtraction of non-specific binding from the amount of ^{125}I-IGF-I bound to the cells in all other sample wells was done. It should be noted that non-specific binding consistently constituted only 20% of bound counts and that any endogenous IGFBP-3 surface binding would be included. All results were based on triplicate well studies.

Figures 4-5 through 4-11 represent various cell binding experiments. Each experiment included its own control value of ^{125}I-IGF-I bound to BAE cells. These values were taken as 100% and all additions to ^{125}I-IGF-I (for example, ^{125}I-IGF-I + 4.7 x 10^{-12} M p9 HS, ^{125}I-IGF-I + 1.2 x 10^{-11} M p9 HS, etc.) were analyzed as a percentage of the control 100% value. This allowed for data merging of several experiments into one figure and a clearer presentation of the results. Cell studies involved between 15,000 and 20,000 cells per well with each well containing 0.5 ml of binding fluid.

IGF-I BAE Cell Binding in Presence of IGFBP-3

IGFBP-3 decreased IGF-I binding to BAE cell surface. Briefly, BAE cells were incubated with IGFBP-3 (0 - 2.2 x 10^{-8} M) and ^{125}I-IGF-I (5.7 x 10^{-10} M) for 2.5 hours at 4°C in binding buffer, (See Section 2.J). NaOH facilitated cell removal from cell plates. Cells were counted for bound ^{125}I-IGF-I on a gamma counter. The addition of IGFBP-3 did not affect IGF-I cell surface binding at low concentrations (0 - 5.6 x 10^{-10} M IGFBP-3). A larger increase of exogenous IGFBP-3 (2.8 x 10^{-9} M - 2.2 x 10^{-8} M) resulted in a significant decrease (P   0.05) of IGF-I cell surface binding, (Figure 4-4).

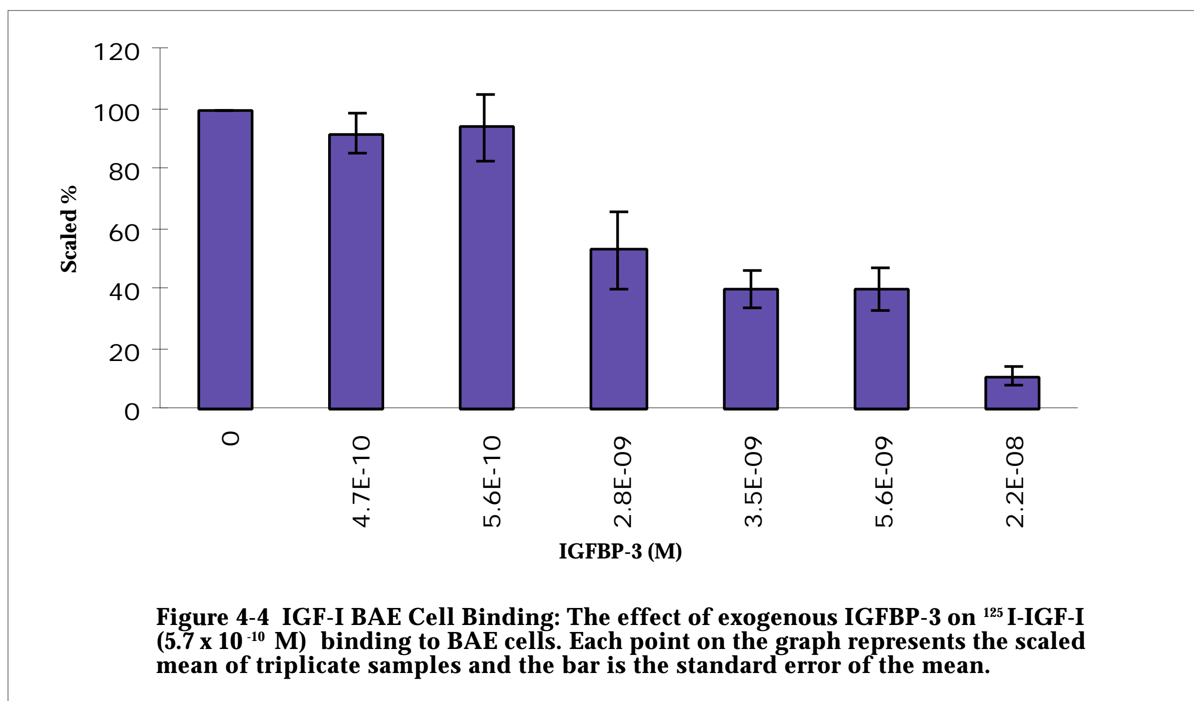

Figure 4-4 IGF-I BAE Cell Binding: The effect of exogenous IGFBP-3 on ^{125}I-IGF-I (5.7 x 10^{-10} M) binding to BAE cells. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

The decrease of IGF-I surface binding may have been due to the higher affinity of IGF-I for solution IGFBP-3 than for surface bound IGFBP-3 which has been seen for GM10 and porcine smooth muscle cells[46,47]. The affinity of IGF-I for IGFBP-3 (6×10^{-9} M) is greater than the affinity of IGF-I for IGF-IR (0.2 - 1×10^{-9} M)[37].

IGF-I BAE Cell Binding in Presence of p9 HS

p9 HS increased IGF-I cell surface binding when higher molar concentrations of p9 HS were present. To determine the effect of p9 HS on IGF-I cell surface binding, BAE cells were incubated with p9 HS (0 - 8×10^{-10} M) and ^{125}I-IGF-I (5.7×10^{-10} M) for 2.5 hours at 4°C in binding buffer, (See Section 2.J). Addition of NaOH facilitated cell removal from plates. A gamma counter was used to quantify bound ^{125}I-IGF-I. The addition of p9 HS did not affect IGF-I cell surface binding at low concentrations (0-1.8×10^{-10} M p9 HS). However, higher concentrations of p9 HS (2×10^{-10} M - 8×10^{-10} M) resulted in a significant increase (P   0.05) in IGF-I cell surface binding, (Figure 4-5).

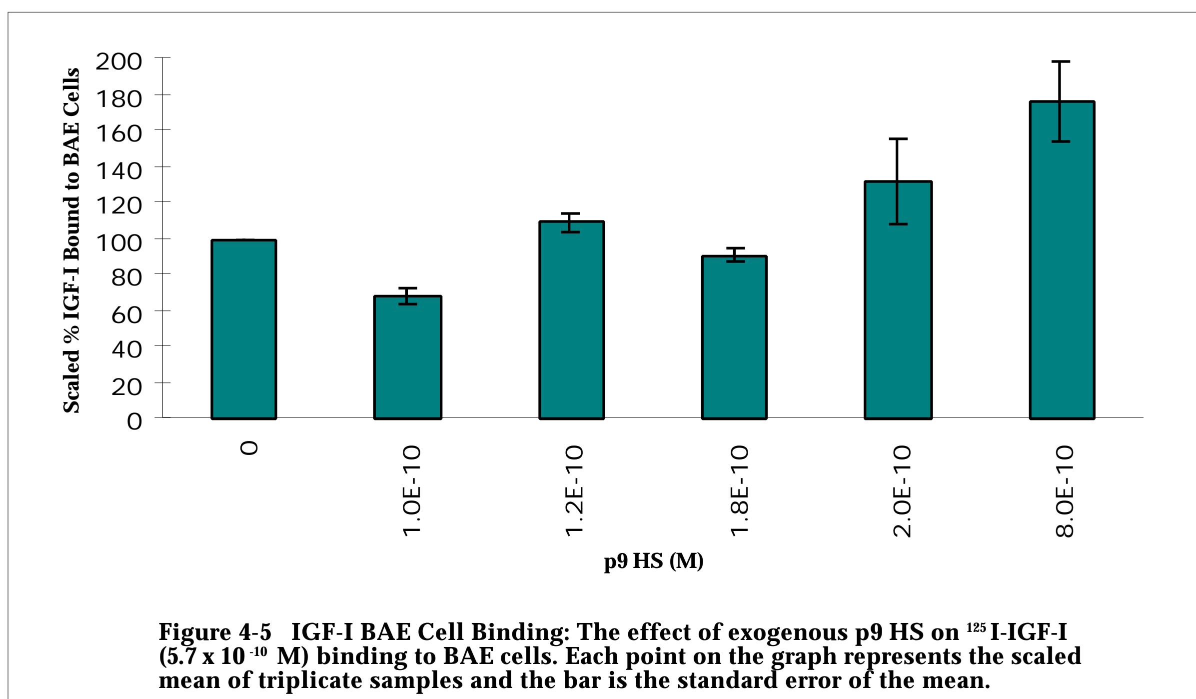

Figure 4-5 IGF-I BAE Cell Binding: The effect of exogenous p9 HS on ^{125}I-IGF-I (5.7×10^{-10} M) binding to BAE cells. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

IGF-I BAE Cell Binding in Presence of Both IGFBP-3 and p9 HS

p9 HS countered the decreasing effect IGFBP-3 had on IGF-I binding to BAE cells. To determine the effect of p9 HS on IGF-I cell surface binding in the presence of IGFBP-3, BAE cells were incubated with p9 HS(0 - 2×10^{-10} M), IGFBP-3 (0 - 2.2×10^{-8} M) and ^{125}I-IGF-I (5.7×10^{-10} M) for 2.5 hours at 4°C in a binding buffer, (See Section 2.J). ^{125}I-IGF-I BAE cell surface binding, in the absence of IGFBP-3 and p9 HS, was taken as the control (100%) and all additions to ^{125}I-IGF-I were analyzed as a percentage of the control value. The addition of p9 HS increased IGF-I cell surface binding in the presence of all concentrations of IGFBP-3, (Figure 4-6).

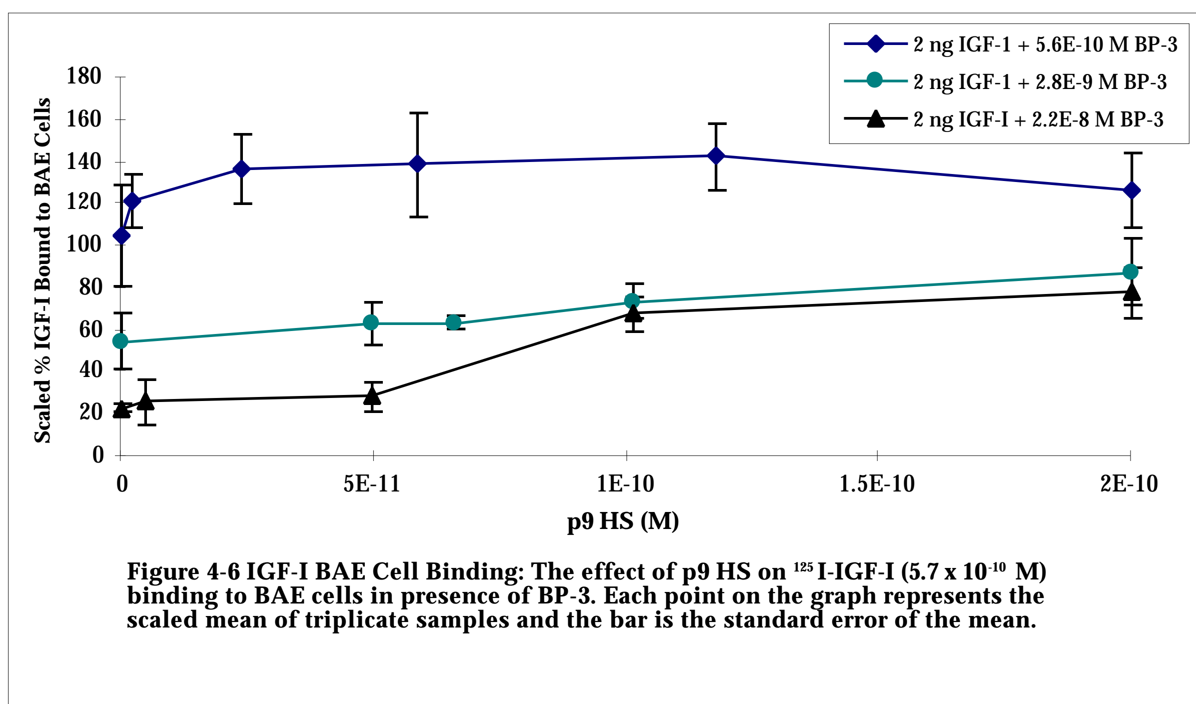

Figure 4-6 IGF-I BAE Cell Binding: The effect of p9 HS on ^{125}I-IGF-I (5.7×10^{-10} M) binding to BAE cells in presence of BP-3. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

The lowest concentration of IGFBP-3 (5.6×10^{-10} M) did not significantly inhibit (P 0.05) ^{125}I-IGF-I surface binding and the addition of p9 HS to ^{125}I-IGF-I and IGFBP-3 increased ^{125}I-IGF-I surface binding only slightly (40%). At 2.8×10^{-9} M IGFBP-3, ^{125}I-IGF-I surface binding was 40% inhibited and the addition of p9 HS helped to counterbalance the inhibition with a maximum 20% recovery increase at 2×10^{-10} M p9 HS. The highest concentration of IGFBP-3 (2.2×10^{-8} M) investigated inhibited ^{125}I-IGF-I cell surface binding by 80%. The addition of p9 HS decreased the inhibitory affect by 45% at 2×10^{-10} M p9 HS.

Figure 4-7 helps to make evident the positive effect p9 HS had on ^{125}I-IGF-I binding in the presence of IGFBP-3. IGFBP-3 decreased ^{125}I-IGF-I cell surface binding with no p9 HS present. In all concentrations investigated, the addition of p9 HS raised ^{125}I-IGF-I cell surface binding in the presence of IGFBP-3. However, there was never total recovery to the initial value of ^{125}I-IGF-I cell surface binding in the absence of IGFBP-3 even at the highest molar concentration of p9 HS added to the system. p9 HS was present in a lower molar concentration than IGFBP-3 throughout the range of p9 HS and IGFBP-3 explored. It is possible that p9 HS might have been able to fully recover ^{125}I-IGF-I cell surface binding to the original binding value and might have actually increased ^{125}I-IGF-I cell surface binding if present at an equimolar concentration to IGFBP-3 present. This was not investigated.

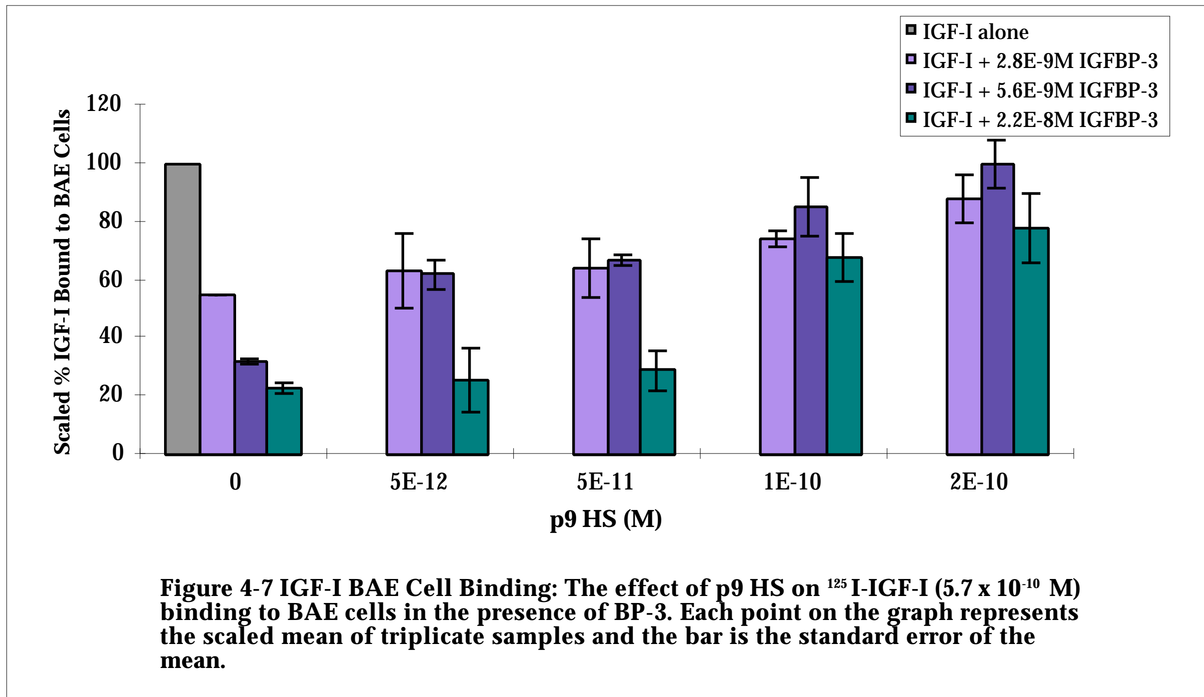

Figure 4-7 IGF-I BAE Cell Binding: The effect of p9 HS on ^{125}I-IGF-I (5.7 x 10^{-10} M) binding to BAE cells in the presence of BP-3. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

IGFBP-3 decreased IGF-I cell surface binding even in the presence of p9 HS. p9 HS did, however, help to decrease the inhibitory effect that IGFBP-3 had on IGF-I cell surface binding. To determine if lower concentrations of IGFBP-3 supported or impeded IGF-I cell surface binding in the presence of p9 HS, BAE cells were incubated with IGFBP-3 (0 - 2.2 x 10^{-9} M), p9 HS (0 - 8 x 10^{-10} M) and ^{125}I-IGF-I (5 .7 x 10^{-10} M) for 2.5 hours at 4°C in binding buffer. With no IGFBP-3 present, p9 HS (2 x 10^{-10} M - 8 x 10^{-10} M) increased ^{125}I-IGF-I cell surface binding by 40% and 90%, respectively. The addition of

IGFBP-3 (5 x 10^{-11} M - 2 x 10^{-9} M) resulted in a steady decrease of ^{125}I-IGF-I surface binding. The two highest concentrations (1.1 x 10^{-9} M and 2.2 x 10^{-9} M IGFBP-3) examined resulted in almost a total inhibition of ^{125}I-IGF-I surface binding even with p9 HS present, (Figure 4-8). The total inhibition of IGF-I surface binding at the higher concentrations of IGFBP-3 may again be due to the unbalanced molar concentrations of p9 HS and IGFBP-3 present. There may have not been enough p9 HS in the system to offset the inhibitory affect IGFBP-3 had on ^{125}I-IGF-I surface binding.

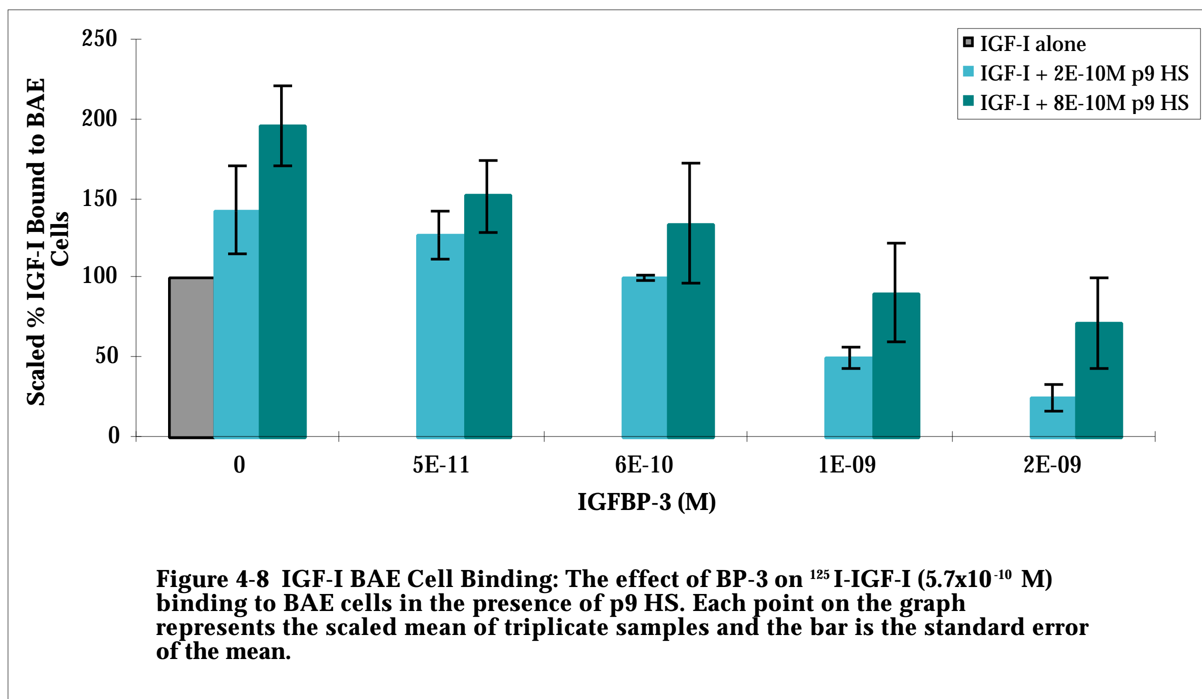

Figure 4-8 IGF-I BAE Cell Binding: The effect of BP-3 on ^{125}I-IGF-I (5.7x10^{-10} M) binding to BAE cells in the presence of p9 HS. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

IGFBP-3 Cell Surface Binding

IGFBP-3 binds to the surface of BAE cells. To ascertain if IGFBP-3 bound to cells, BAE cells were pre-incubated with IGFBP-3 (0-2.2 x 10^{-8} M) for 2.5 hours at 4°C in binding buffer. Cells were then washed with binding buffer (1 ml/well twice) to remove any unbound IGFBP-3. Binding buffer (.5 ml) was added to each well and cells were again incubated for 2.5 hours at 4°C with ^{125}I-IGF-I (5.7 x 10^{-10} M). Cells were washed with binding buffer (1 ml/well twice) to remove any unbound ^{125}I-IGF-I. Cells were counted for bound ^{125}I-IGF-I on a gamma counter. It is likely that IGFBP-3 does bind to BAE cells as pre-incubation led to an increase of ^{125}I-IGF-I bound at all concentrations of

IGFBP-3 investigated,(Figure 4-9). ^{125}I-IGF-I in the absence of IGFBP-3 was used as the control value (100% = 3.7 x 10^{-12} M bound). An interesting point to note is that at very high concentrations of IGFBP-3, ^{125}I-IGF-I cell binding actually started to decrease. This could be due to IGFBP-3 saturating the available binding sites on BAE cells or from disassociation of IGFBP-3 from the surface of the cell at the high concentrations. ^{125}I-IGFBP-3 binding to BAE cells was not investigated.

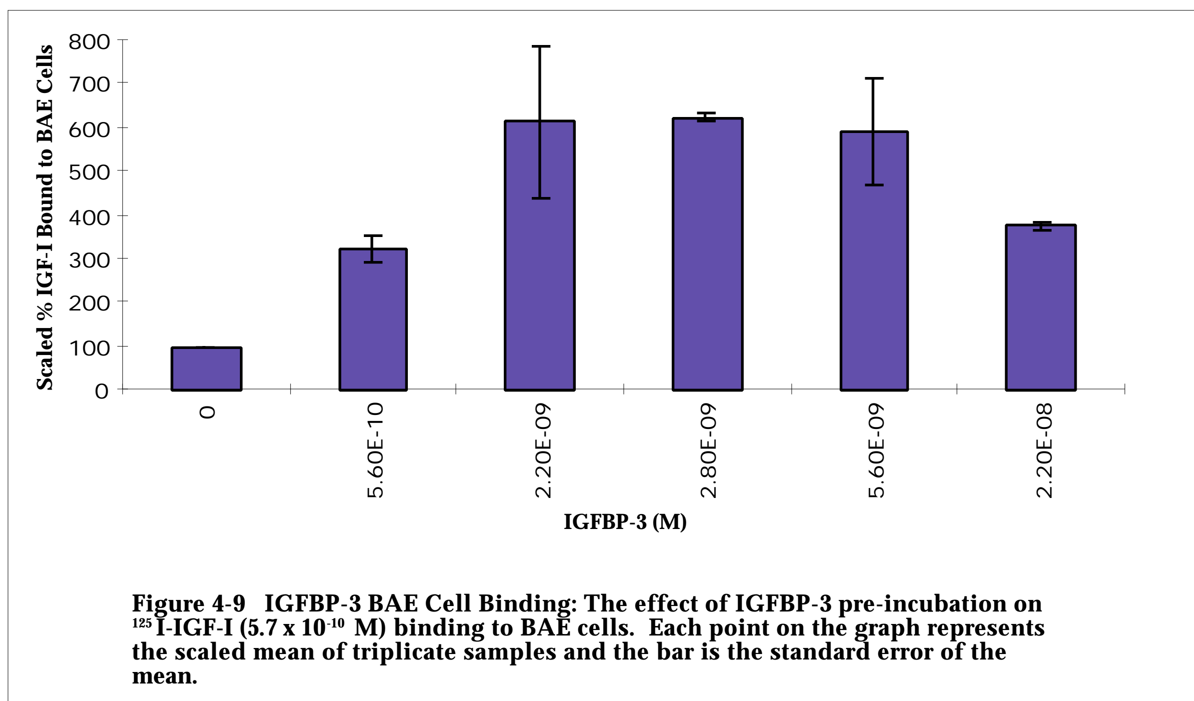

Figure 4-9 IGFBP-3 BAE Cell Binding: The effect of IGFBP-3 pre-incubation on ^{125}I-IGF-I (5.7 x 10^{-10} M) binding to BAE cells. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

p9 HS Binding to BAE Cells

To determine if p9 HS bound to cells, BAE cells were pre-incubated with p9 HS (0-8 x 10^{-10} M) for 2.5 hours at 4°C in binding buffer. Cells were then washed with binding buffer (1 ml/well twice) to remove any unbound p9 HS. Binding buffer (.5 ml) was added to each well and cells were again incubated for 2.5 hours at 4°C with ^{125}I-IGF-I (5.7 x 10^{-10} M). Cells were washed with binding buffer (1 ml/well twice) to remove any unbound ^{125}I-IGF-I. Cells were counted for bound ^{125}I-IGF-I on a gamma counter. It is likely that p9 HS does bind to BAE cells since there is an increase of ^{125}I-IGF-I bound at most concentrations of p9 HS, (Figure 4-10). ^{125}I-IGF-I in the absence of IGFBP-3 was used as the control value (100% = 4.3 x 10^{-12} M bound). It is interesting to

note that at very high concentrations of p9 HS (8×10^{-10} M), ^{125}I-IGF-I cell binding was inhibited. This could be due to p9 HS saturating the open binding sites on BAE cells or from disassociation of p9 HS from the surface of the cell at the high concentrations. A higher concentration of p9 HS was used in this experiment than previous ones. p9 HS may not bind ^{125}I-IGF-I as acutely as IGFBP-3 binds ^{125}I-IGF-I since there was a larger amount of surface complexes when cell were incubated with IGFBP-3 as compared with p9 HS . This may be due to a higher binding affinity of IGF-I for IGFBP-3 than for p9 HS or because p9 HS was present in lower molar concentrations than IGFBP-3 for the similar experiments. ^{35}S-p9 HS binding to BAE cells was investigated due to the low concentration of the material.

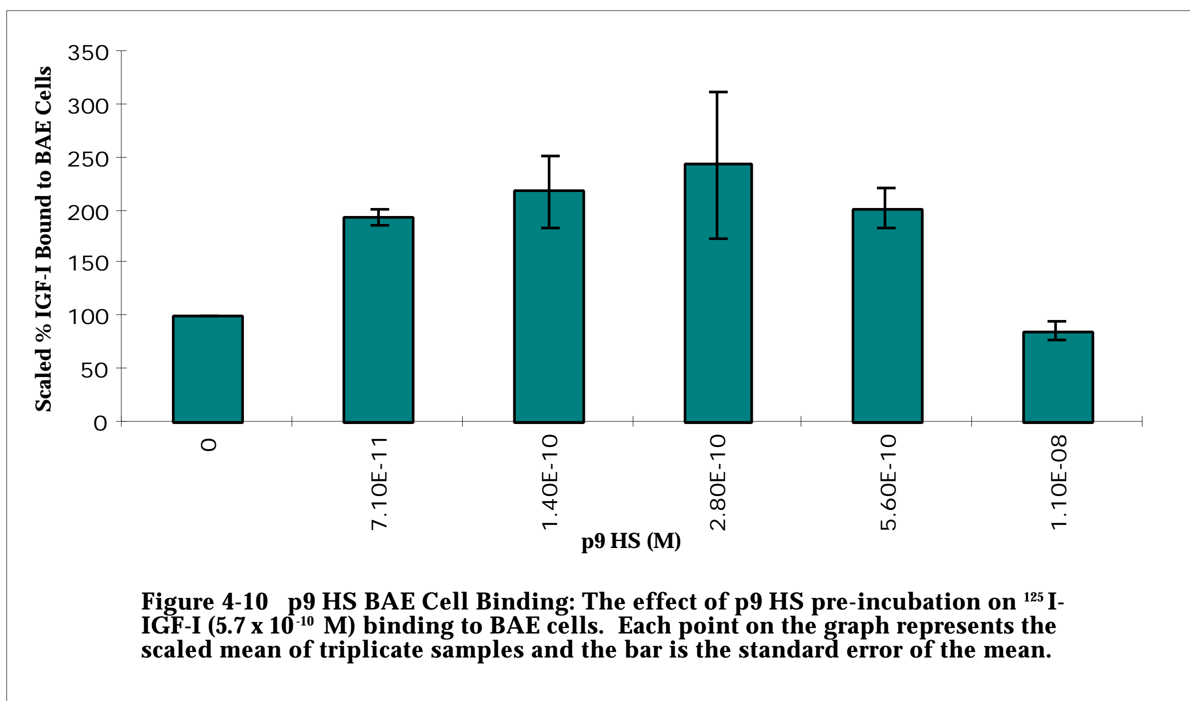

Figure 4-10 p9 HS BAE Cell Binding: The effect of p9 HS pre-incubation on ^{125}I-IGF-I (5.7×10^{-10} M) binding to BAE cells. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

Effect of p9 HS and IGFBP-3 Pre-Incubation on IGF-I Binding to BAE Cells

Figure 4-11 shows the investigation of various pre- and post-incubation steps with IGFBP-3 and p9 HS. A pre-incubation step involves a 2.5 hour incubation with IGFBP-3 (2.8×10^{-9} M) and/or p9 HS (1.4×10^{-10} M) before the radiolabeled ligand (5.7×10^{-10} M - 1.1 x 10-9 M ^{125}I-IGF-I) was added. Pre-incubation with IGFBP-3 increased ^{125}I-IGF-I BAE cell binding by 175%. Pre-incubation with p9 HS increased ^{125}I-IGF-I BAE cell

binding by 125%. The post addition of p9 HS to the pre-incubation and post-incubation stage of IGFBP-3 resulted in a significant increase (P   0.05) of 200% and 50% ^{125}I-IGF-I BAE cell binding. The pre-incubation of both IGFBP-3 and p9 HS resulted in an increase of 300% ^{125}I-IGF-I BAE cell binding, while the addition of IGFBP-3 post-incubation to p9 HS pre-treated cells inhibited binding by 12%. The simultaneous addition of IGFBP-3 and p9 HS with ^{125}I-IGF-I (post-incubation) still showed an increase of 50% ^{125}I-IGF-I cell surface binding, which was a severe decrease compared to when BAE cells were pre-treated with IGFBP-3 and p9 HS. p9 HS helps to increase ^{125}I-IGF-I cell surface binding in the presence of IGFBP-3 (pre- and post-incubation). IGFBP-3 helps to increase ^{125}I-IGF-I cell surface binding in the presence of IGFBP-3 (pre-incubation). However, when IGFBP-3 is added post-incubation to pre-treated p9 HS BAE cells, inhibition of IGF-I surface binding is observed. This may be due to a high affinity of IGF-I for solution IGFBP-3[46,47] which would not allow IGF-I to reach the surface bound p9 HS.

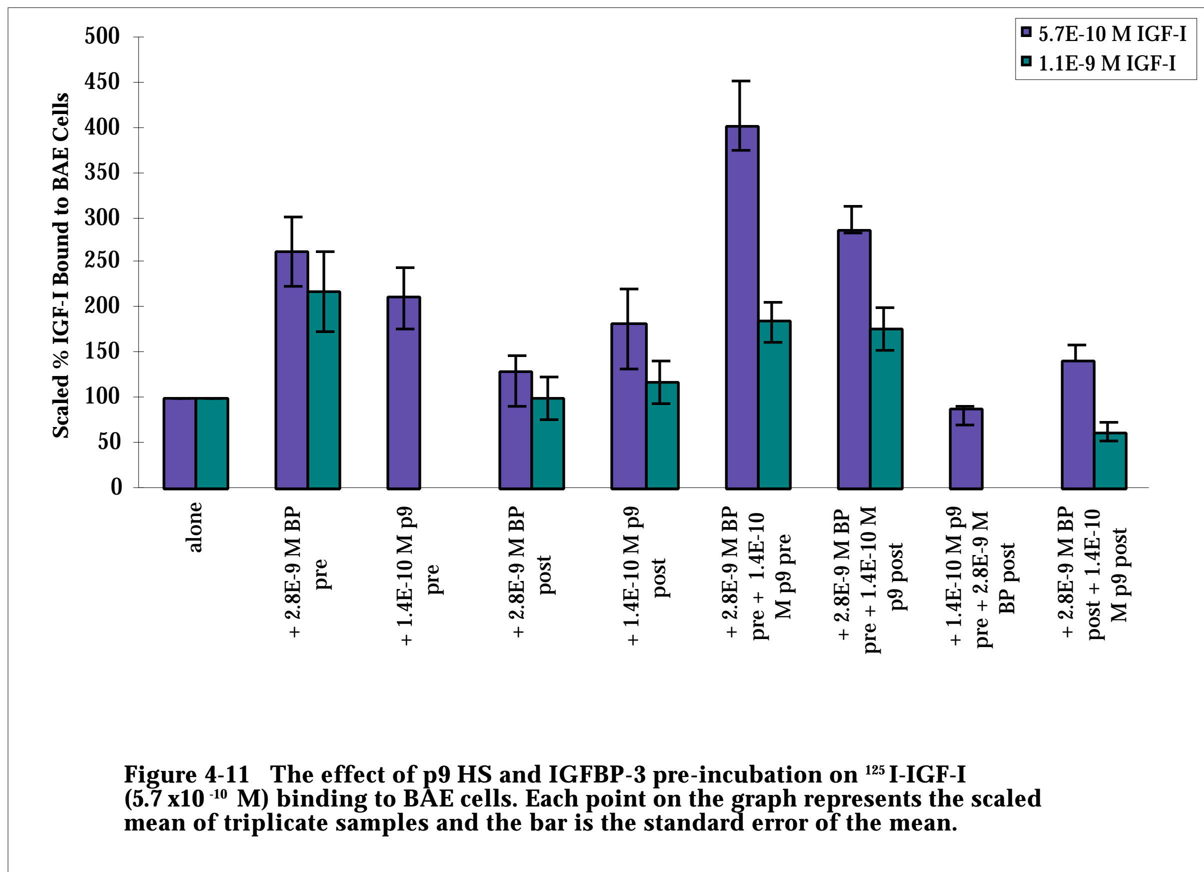

Figure 4-11 The effect of p9 HS and IGFBP-3 pre-incubation on ^{125}I-IGF-I (5.7×10^{-10} M) binding to BAE cells. Each point on the graph represents the scaled mean of triplicate samples and the bar is the standard error of the mean.

The cell binding studies suggest that IGFBP-3 and p9 HS do bind to BAE cells in the absence of IGF-I and that this binding increases IGF-I cell surface binding. Pre-incubation increased IGF-I binding and the affect appears to be additive. When IGFBP-3 and p9 HS were added before IGF-I, IGF-I surface binding was increased. When IGFBP-3 and p9 HS were added at the same time as IGF-I, IGFBP-3 inhibited IGF-I cell surface binding. This may be due to the higher affinity of IGF-I for solution IGFBP-3 than for surface bound IGFBP-3 if pre-incubation resulted in a significant increase in surface binding sites. When p9 HS and IGF-I were added at the same time, p9 HS increased the amount of IGF-I that bound to the cell surface. p9 HS helped to compensate for the inhibitory effect IGFBP-3 had on IGF-I cell surface binding when added simultaneously. Likewise, IGFBP-3 decreased IGF-I cell surface binding gradually as the concentration increased in the presence of p9 HS. If IGFBP-3 was present in a higher concentration than p9 HS, there was total inhibition of IGF-I cell surface binding. If p9 HS was greater or equal in concentration to IGFBP-3, p9 HS could help to decrease the inhibitory affect of IGFBP-3, but a slight decrease of IGF-I surface binding was still observed.

C. Future Direction

There are many biologically interesting experiments that could be done in the future with p9 HS and IGFBP-3 in regard to regulation of IGF-I cell surface binding. The regulatory activity of p9 HS may change with the enzymatic digestion of p9 HS with Heparinase I (Hep I) and Heparintinase III (Hep III). -elimination reaction conditions free GAG chains from core proteins which may also affect p9 HS activity. For example, it has been shown with bFGF binding to vascular smooth muscle cells that the whole proteoglycan is more effective than the free GAG chains[33]. The protein preparation of p9 HS resulted in a low concentration of p9 HS with a low CPM/ng. The low amount of radioactivity made the exploration of ^{35}S-p9 HS binding to BAE cells difficult. Labeling p9 HS with ^{125}I would make this binding study possible. ^{125}I-IGFBP-3 binding to BAE cells should be investigated. The influence of pre-incubation with p9 HS or IGFBP-3 on receptor activity may affect IGF-I binding to BAE cells. In all cases, further experimentation will help to elucidate the effect p9 HS and IGFBP-3 have on IGF-I cellular activity.