CHAPTER III

CYTOSENSOR® MICROPHYSIOMETER SYSTEM OPTIMIZATION

3.1 Optimization of the Cytosensor® Microphysiometer System

The Cytosensor® Microphysiometer System is designed to measure the extracellular acidification rate (ECAR) of cultured cells in real time (Section 1.2-6). Because of the variability in the different growth parameters of living cells, the system parameters must be optimized for each cell line to get the best response (i. e. maximal change in acidification rate) from a baseline ECAR. Once the response using an agent in question (such as IGF-I) has been optimized, variations in the response as the result of additional agents can be compared with the change in ECAR from the initial agent. Our optimization of this system included seeding densities of SV40-IGF-I cells (autocrine for IGF-I) and MAC-T cells (negligible endogenous IGF-I secretion), IGF-I challenge concentrations, IGF-I challenge durations, and the number of challenge repetitions the cells would tolerate.

3.1.1 Optimization of The Seeding Densities of SV40-IGF-I and MAC-T Cells for the Microphysiometer Studies

The User's Manual for the Cytosensor® Microphysiometer System recommends that as a starting point for optimal seeding density, $3x10^5$ cells should be seeded to each capsule cup membrane. Seeding densities reported in the literature are: CHO-K1 cells with $3x10^5$ cells/capsule cup (Chan, *et al*., 1995), PC12 cells with $3.5x10^5$ cells/capsule cup (Pitchford, *et al*.,1995), and CHO-K1 cells with 10^6 cells/capsule cup (Taylor, *et al*., 1996). Furthermore, it is recommended that the cell monolayer on the capsule cup membrane be 75 to 85% confluent (Molecular Devices, 1995). 100% confluency would provide more cells for stimulation, however some cell lines require less than 100% confluency so that the stimulating agent may reach the intercellular space in sufficient quantity (Lucy Gray, personal communication). Alternatively, 100% confluency may

result in downregulation due to contact inhibition. The SV40-IGF-I and MAC-T cell lines had not previously been optimized for the microphysiometer and the following studies were undertaken.

3.1.1-1 Experimental Results for Optimal Initial Seeding Density for the SV40-IGF-I Cell Line

Figure 3.1 shows the % ECAR for 2 channels of SV40-IGF-I cells (passage 15) seeded at $3x10^4$ cells/ml. This lower than recommended seeding density was chosen because it resulted in an approximate 80% confluency of cells after 24 hr of incubation. The receptors were then serum starved for 24 hr to increase the responsiveness of the receptors to exogenous IGF-I. In this initial experiment a control channel receiving no IGF-I was run simultaneously with a channel receiving 50 ng/ml IGF-I. The challenge with IGF-I began at 8 min on the graph. It should be noted that recorded time zero on this and subsequent graphs are not actual experimental start times since for each experiment an equilibration period of at least one hour has passed before challenges are initiated. Time zero on each graph has been designated shortly before the challenge for readability.

The normalized signal shows a maximum signal of the stimulated cells 12% above the baseline. It was desirable to get the highest peak possible from IGF-I stimulation to maximize comparison with addition of other agents in conjunction with the IGF-I. In comparison the control rises to a maximum approximately 4% above the normalized baseline. During an experimental run, drift of cell signal is normal, and during the one hour equilibration period, the cell signal is watched for stabilization of this drift. The signal may achieve a nearly flat line, but more typically drifts gradually upward for several hours (10% upward drift during 1 hr is typical).

To increase the % ECAR of the SV40-IGF-I cells, the capsule cups were seeded with a higher density of cells, and the serum starvation period was increased to 72 hr. As can be seen in Figure 3.2, SV40-IGF-I cells (passage 19) seeded at densities of 1 and $2x10^5$

cells/ml with increased serum starvation time yielded ECAR at 20% and 29% respectively above the normalized baseline when challenged with only 5 ng/ml IGF-I. IGF-I challenges began at 6 min on the graph.

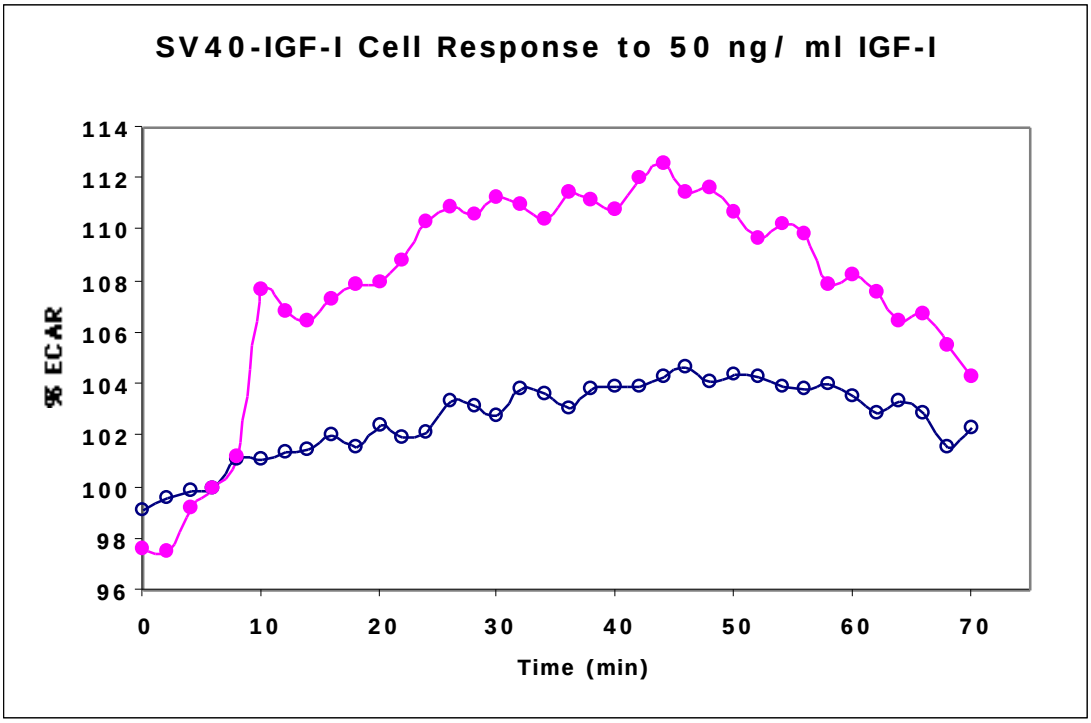

Figure 3.1 *Comparison of SV40-IGF-I cells (passage 15) seeded at 3x10^4 cells/ml and serum starved for 24 hr with (●) and without (❍) a 36 min 50 ng/ml IGF-I challenge beginning at 8 min on the graph. % ECAR was normalized.*

To verify that 2x10^5 cells/ml would be used as our optimal seeding density for the SV40-IGF-I cell line, cells seeded at 0.5, 1, 2, and 5x10^5 cells/ml and serum starved for 72 hr were investigated (Figure 3.3). As can be seen, the maximum % ECAR occurred at the seeding density of 2x10^5 cells/ml. The lowest seeding density of 5x10^4 cells/ml showed the lowest peak, and peaks increased in height with the highest peaks at a seeding density of 2x10^5 cells/ml. In addition, the higher density also had a longer sustained response. At the highest density of 5x10^5 cells/ml, there was essentially no response to IGF-I and a

a progressive downward trend in the % ECAR. However, the cells were very crowded on the membrane and likely were overcrowded and dying. Peak responses are tabulated in Table 3.1.

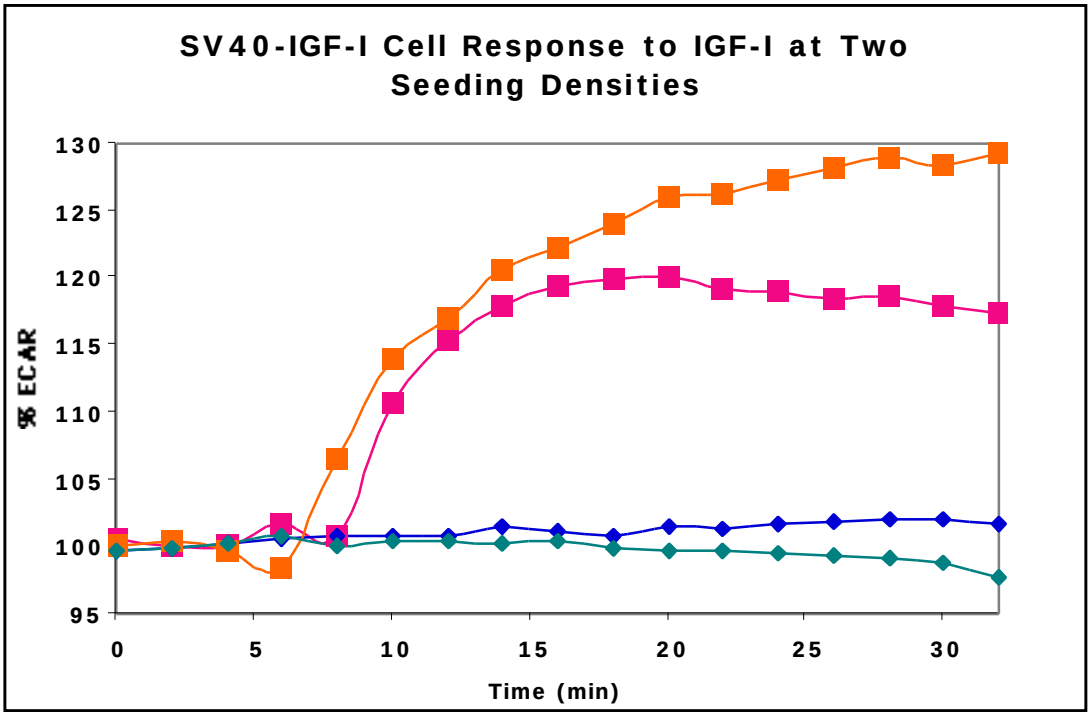

Figure 3.2 *Two seeding densities: 1*x10^5(●) *and 2x10*^{5} (■) *cells/ml of SV40-IGF-I cells (passage 19) serum starved for 72 hr were exposed to 20 min of 5ng/ml IGF-I starting at 6 min on the graph. Each density had a control channel (❍, ❍ respectively) that received no IGF-I for comparison. % ECAR was normalized for all channels.*

The data was analyzed using Microsoft® Excel and fit to a polynomial regression curve. The slope of the regression curve was found significant as compared to a slope of zero with a significance of 0.001 ($p < 0.05$ is significant). As a result of these experiments, SV40-IGF-I cells were considered to have an optimal seeding density of 2x10^5 cells/ml.

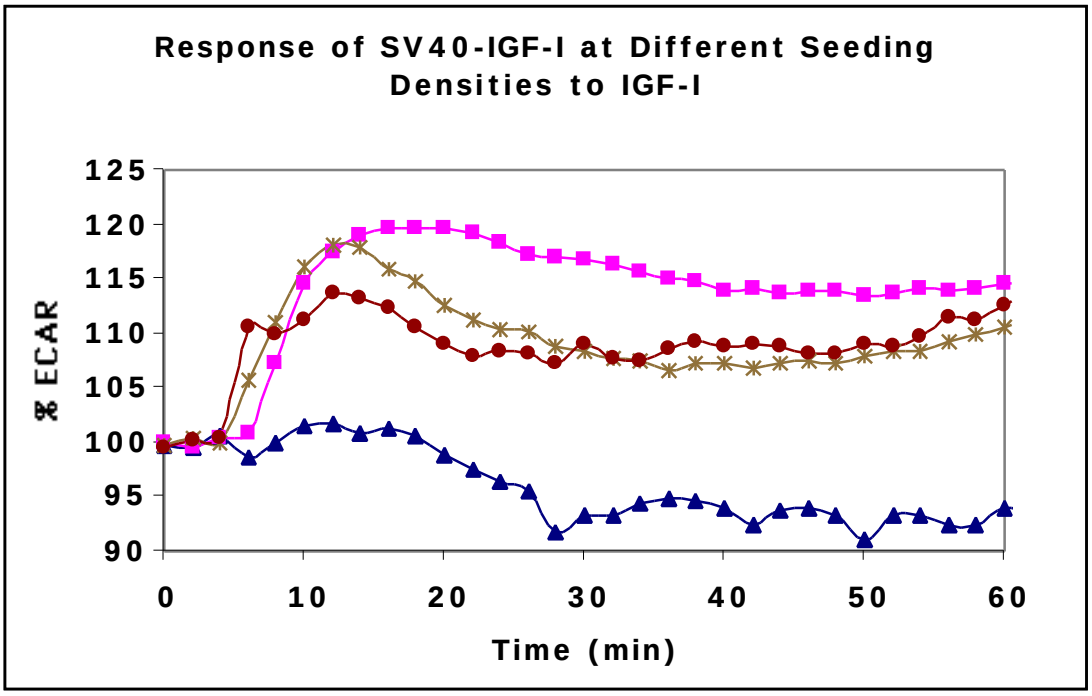

Figure 3.3 *SV40-IGF-I cells (passage 22) exposed to 14 min of 5ng/ml IGF-I starting at 14 min on the graph at 4 different initial seeding densities (5 x 10^5 (▲), 2 x 10^5 (■), 1x 10^5 (✳), and 5 x 10^4 (●)). Values are the averages of two channels except for 5 x10^5 cells/ml which represents one channel. The highest % ECAR was found for the cells seeded at 2x10^5 cells/ml. % ECAR was normalized for all channels.*

TABLE 3.1

% ECAR for 5 ng/ml IGF-I Challenges of SV40-IGF-I Cells at Different Seeding Densities As Seen in Figure 3.3

Seeding Density	% ECAR *	Standard Deviation
5x10^5 cells/ml	101.7	
2x10^5 cells/ml	120.3	± 1.5
1x10^5 cells/ml	118.4	± 0.4
5x10^4 cells/ml	114.1	± 1.6

** % ECAR is the average of two peaks except for the seeding density 5x10^5 cells/ml for which there was only one channel run.*

3.1.1-2 Experimental Results for Optimal Initial Seeding Density for the MAC-T Cell Line

Similar studies were initiated to determine optimal cell seeding density for the MAC-T cells (Figure 3.4). A comparison was made for the initial seeding densities of MAC-T cells (passage 4) seeded at 2, 5, and $7x10^5$ cells/ml. The cells were serum starved for 72 hours to have the identical serum starvation period as the SV40-IGF-I cells. The IGF-I challenge began at 6 min on the graph with a concentration of 5 ng/ml and lasted for 14 min. Data was normalized over the first three points displayed so that % ECAR response first appears at 6 min.

The maximum % ECAR was 12.8 % above the normalized baseline at the initial seeding density of $5x10^5$ cells/ml. Looking at the graph, the initial "rabbit ear" peak observed at $2x10^5$ cells/ml is not uncharacteristic of the response of the cells to IGF-I. For unexplained reasons, and as can also be seen in Figure 3.5, an initial "rabbit ear" response in the % ECAR was often seen in the first data points when the cells were exposed to the IGF-I challenges. The wobbly pattern of the response after the peak shows some instability in the signal which was also noted on another channel. The highest seeding density of $7x10^5$ cells/ml did not peak as high as $5x10^5$ cells/ml, possibly as a result of cell crowding and contact inhibition. The seeding density of $5x10^5$ cells/ml gave the highest peak at 12.8% ECAR.

Two channels of MAC-T cells (passage 4) seeded with $5x10^5$ cells/ml were exposed to 5 ng/ml IGF-I for 22 min as shown in Figure 3.5. The maximum % ECAR was approximately 11 % above baseline for both channels. This was comparable to the 12.8 % peak in Figure 3.4. Again, the initial "rabbit ear" appears in the signal. In an experiment not shown, a double "rabbit ear" appeared when there was a significant pH difference in the running buffer when switched to the challenge agent. This indicated that a single peak is likely not due to any change from running buffer pH. The % ECAR

as a result of IGF-I stimulation was consistently found to be lower for the MAC-T cells than for the SV40-IGF-I cells even after increasing the seeding density of the MAC-T cells.

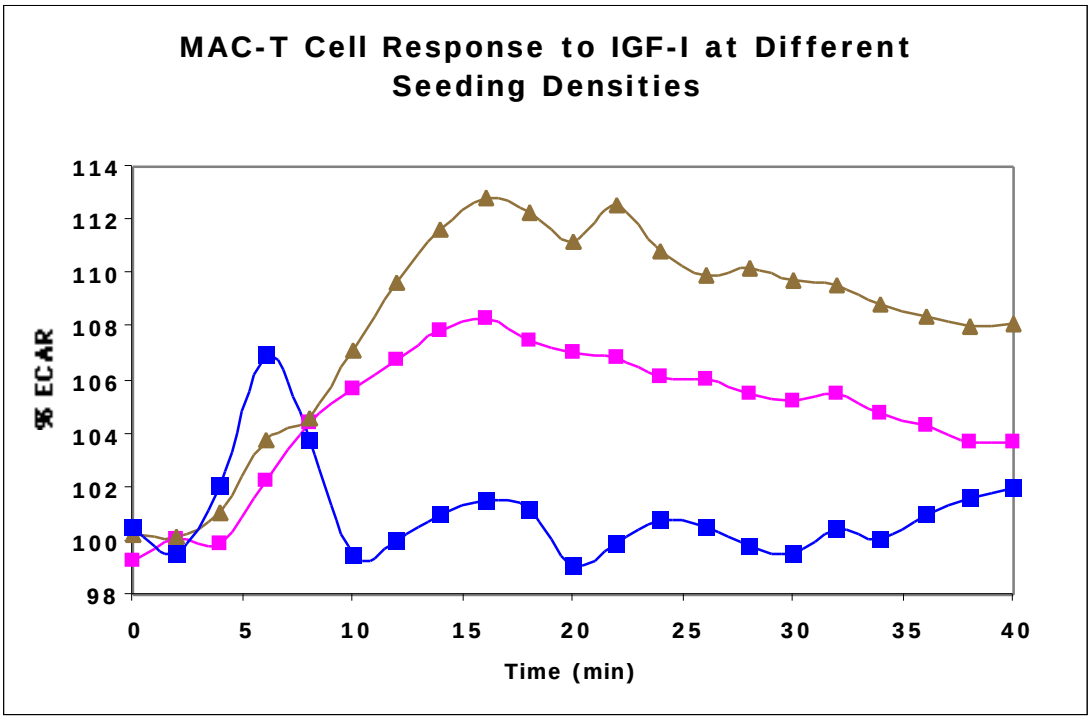

Figure 3.4 *% ECAR at different seeding densities (2 x10,* (■)*, 5 x10* (▲)*, and 7x10^5* (■) *cells/ml) of MAC-T cells (passage 4). IGF-I challenges at a concentration of 5 ng/ml began at 6 min on the graph and lasted for 14 min. Maximal % ECAR was found at the seeding density of 5x10^5 cells/ml. % ECAR was normalized for all channels.*

As a result of these experiments, MAC-T cells were considered to have an optimal seeding density of 5x10^5 cells/ml.

The lower % ECAR was not expected for the MAC-T cell line since the MAC-T cells have more receptors on the cell surfaces than the SV40-IGF-I cells, and the MAC-T cells do not produce endogenous IGF-I. The higher seeding density required for the MAC-T

cells suggested that these cells did not proliferate as quickly as the SV40-IGF-I cells on the capsule cup membranes, but additional studies were needed to investigate the cell number during the experimental runs (Section 3.1.1-3).

In a later experiment (Section 4.2.1) for which new cultures of SV40-IGF-I and MAC-T cells were maintained at the test location, similar % ECAR responses were obtained from both lines under the optimized conditions described in this chapter. This suggests the MAC-T cells may have been more vulnerable to environmental stressors (i.e. CO_2 deprivation, physical jostling during transportation) than the SV40-IGF-I cells.

3.1.1-3 Quantifying the Number of Cells on the Capsule Cup Membrane

In optimizing the initial seeding density for each cell line, it was noted that a higher initial seeding density ($5x10^5$ cells/ml) was required for MAC-T cells than was required for the SV40-IGF-I cells ($2x10^5$ cells/ml). In addition, even with a higher initial seeding density, the MAC-T cells did not have as high an increase in ECAR as compared to the SV40-IGF-I cells. In order to have a meaningful comparison of the responses of the autocrine (SV40-IGF-I) system with the paracrine (MAC-T) system, it seemed desirable to have the same number of cells on the capsule cup membranes at the time of testing. At the very least, it was desirable to quantify the cells on the membranes since 96 hr of time passed between initial seeding and testing. It had been noted that SV40-IGF-I cells generally proliferated more quickly than MAC-T cells on tissue culture plates. Future tests will be done to confirm this.

Quantification of cells growing on the capsule cup membrane was done by first harvesting the cellular DNA from the membrane and finding the DNA content by DNA assay (Section 2.3). The number of cells was then determined from a correlation of cell number as a function of DNA content for the two cell lines.

To establish the relation of DNA content to cell number, a reliable means for counting cells had to be determined. Resuspended cells had the tendency to form cell clumps,

particularly at higher concentrations and more for the SV40-IGF-I cells than for the MAC-T cells. Figure 3.6 shows the results of cell counts for SV40-IGF-I (passage 21) cells diluted serially on the basis of hemacytometer cell counts. (Dilutions were prepared and measurements taken by Leonard Jenkins, undergraduate research assistant in chemical engineering.) The Coulter® Counter was much faster than the hemacytometer

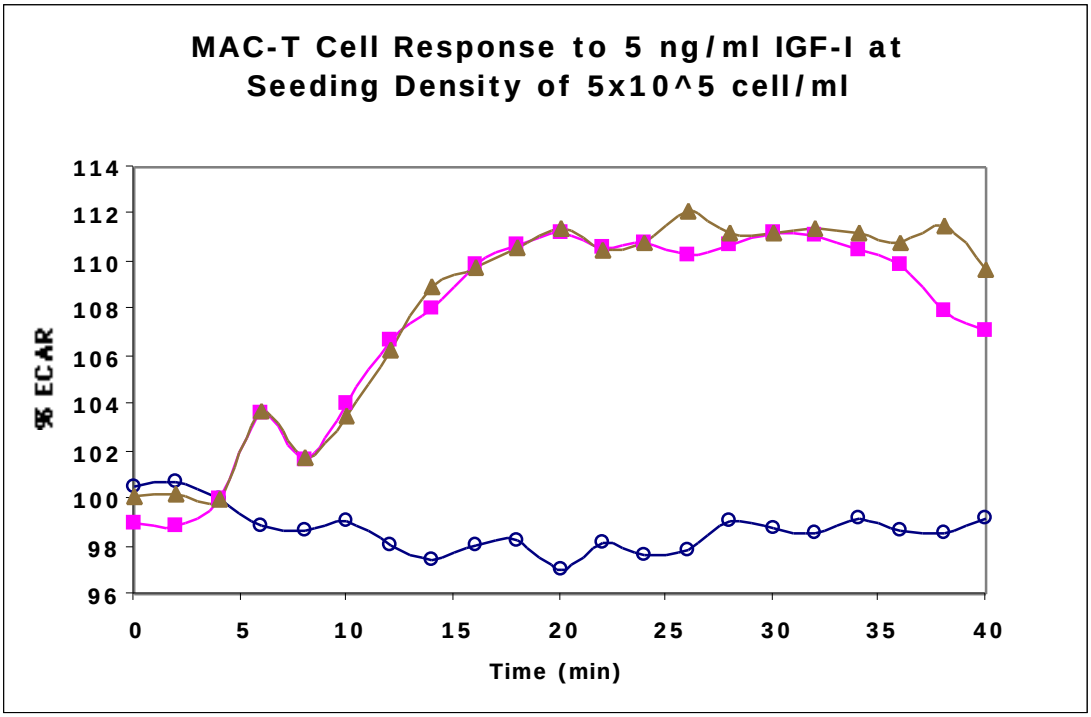

Figure 3.5 *% ECAR response for MAC-T cells (passage 4) seeded at 5x10^5 cells/ml (■, ▲) exposed to 5 ng/ml IGF-I for 22 min starting at 6 min on the graph. The control channel (○) received no IGF-I. Data normalized to baseline.*

method and the resulting trendline was very close to the trendline of the anticipated concentrations. For this reason, future cell counts were done using the Coulter® Counter. Values obtained by the hemacytometer were lower than expected and may be due to the significant cell clumping which occurred during the experiment.

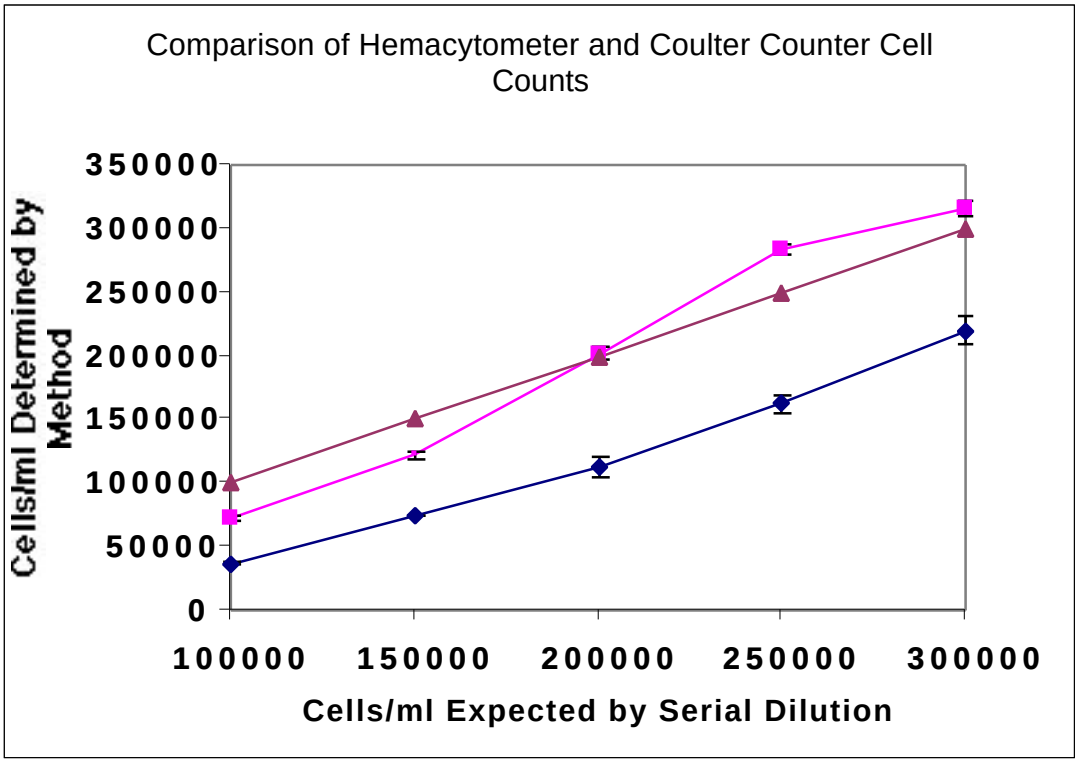

Figure 3.6 *Comparison of cell counts of SV40-IGF-I (passage 21) dilutions using the hemacytometer* (◆) *and the Coulter® Counter* (■). *The serial dilution trendbar represents the expected cell count based on the original hemacytometer count of cells prior to serial dilution* (▲). *Standard error bars are shown for triplicate data for each hemacytometer and Coulter® Counter point. Serial dilution values are based on the initial hemacytometer counts of suspended cells prior to formation of the cell pellet for resuspension.*

To establish the DNA content per cell count correlation, dilutions of SV40-IGF-I and MAC-T cells were prepared from very confluent plates. Three separate experiments were run with triplicate measurements at each dilution. The cells were taken at different passages, and since the cells on the capsule cup membranes are crowded, cells were harvested under crowded conditions to mimic similar DNA content.

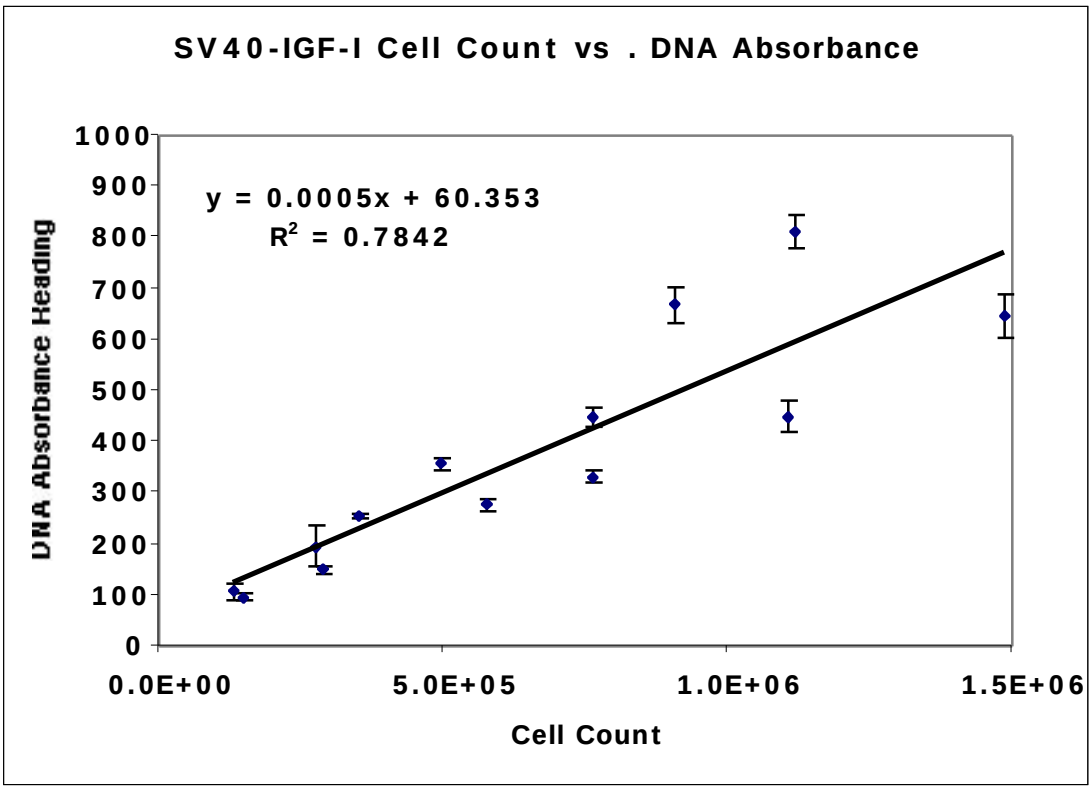

Figure 3.7 *Each point represents the average of 3 readings. Points are combined from three experiments using SV40-IGF-I cells plated at 1 million cells per plate but at different passages and different incubation times: passage 13 cells 3 days old, passage 4 cells 7 days old and passage 10 cells 5 days old. All plates were confluent. The trendline is computed by linear regression in Microsoft® Excel.*

To reduce the amount of clumping in the cell suspensions, different techniques for resuspending the cells after trypsinization were used. Because Ca^{+2} contributes to cell clumping (Steve Ellis, Virginia Tech, personal communication), DPBS was used instead of media. Ultimately it was found that by resuspending the cell pellet initially in 1 ml of cold DPBS (4°C) after centrifuging and bringing up the volume with additional cold

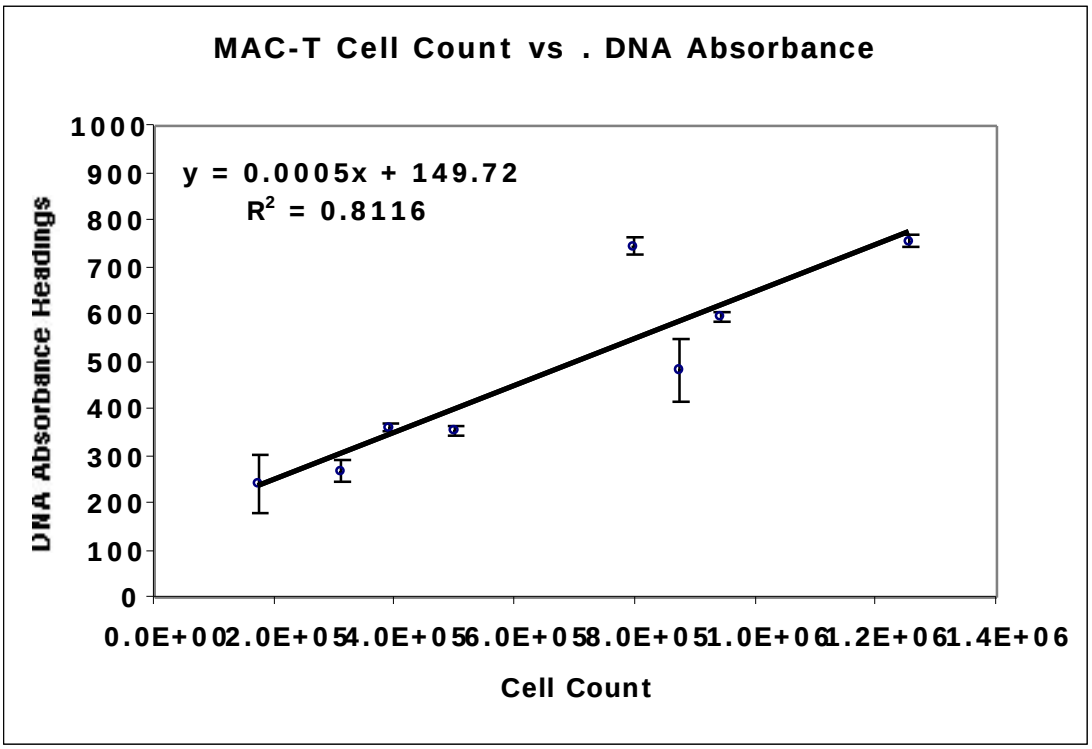

Figure 3.8 *Each point represents the average of 3 readings. Points are combined from three experiments using MAC-T cells plated at 1 million cells per plate but at different passages and different incubation times: passage 13 cells 4 or 5 days old, passage 4 cells 7 days old and passage 10 cells 5 days old. All plates were confluent. The trendline is computed by linear regression in Microsoftâ Excel.*

DPBS, clumping did not occur. The cell dilutions were prepared by rapidly diluting with additional cold DPBS in a serial manner. Clumping was not discernible to the eye, but in the highest concentrations (500,000 to 1,000,000 cells/ml) it was noted that the aperture of the Coulter® Counter would block and had to be flushed during sampling.

Figures 3.7 and 3.8 show the results of cell counts from confluent plates of SV40-IGF-I and MAC-T cells counted in triplicate using the Coulter® Counter. Samples to correspond with each data point were measured in duplicate for DNA absorbance. Each data point is from 3 combined experiments.

3.1.2 Optimization of IGF-I challenge concentration

Challenges with various concentrations of IGF-I were performed to determine which concentration would give a good response (% ECAR) and would allow the response from the addition of other agents to be discerned. Since repeat challenges with IGF-I leads to an attenuated response, it was desirable not to have so high a concentration that the attenuated response would not return to a baseline within a reasonable amount of time. (A reasonable amount of time was considered to be about an hour). Our idea was that we would be able to do repeat challenges on the same set of capsule cups to maximize replicates. Cells were seeded onto the capsule cup membranes at the optimal concentrations as determined in Section 3.1.1.

The following two figures demonstrate the basis for selecting 5 ng/ml as an optimized IGF-I challenge concentration. IGF-I challenge concentrations were chosen in the increments 0.5, 5, and 50 ng/ml.

As can be seen in Figure 3.9, the challenge by 50 ng/ml of IGF-I stimulated the cells to a higher % ECAR compared to the other concentrations. The cell signal at the higher stimulation did not recover to a baseline in line with the other two concentrations between the multiple 14 min challenges. The peaks appeared to deteriorate at this high concentration with the fourth and fifth challenges. The channels receiving 0.5 and 5 ng/ml did not have such a high peak, but appeared to retain better % ECAR peaks after multiple challenges.

Figure 3.10 shows a comparison of single 14 min challenges of 0.5 and 5 ng/ml of IGF-I. Each challenge concentration was run in duplicate to demonstrate the repeatability of the data on two different channels. All four channels returned to similar baselines after about

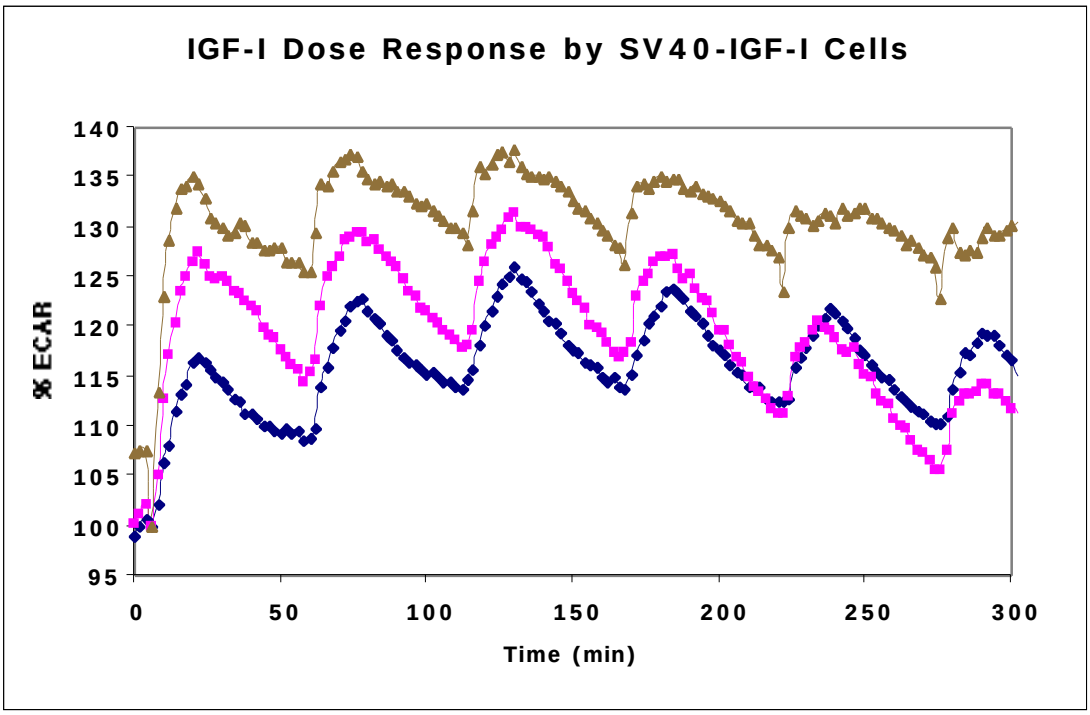

Figure 3.9 *Each channel of SV40-IGF-I cells (passage 25) plated at 2 x 10^5 cells/ml and serum starved for 72 hrs was challenged for 14 min by 0.5* (◆), *5* (■), *or 50* (▲) *ng/ml of IGF-I followed by 40 min of washout before the next 14 min challenge. Data was normalized to a baseline prior to the first challenge.*

60-80 min of washout. The average peak values for the first challenges from SV40-IGF-I cells in Figures 3.9 and 3.10 were 117.3 ± 0.3 S.E. for 0.5 ng/ml IGF-I and 125.1 ± 1.2 S.E for 5 ng/ml IGF-I.

The best concentration of IGF-I given our constraints was determined to be 5 ng/ml. Later studies included 2 and 3 ng/ml concentrations for IGF-I challenge with other agents. These concentrations are of the same order of magnitude as 5 ng/ml and do not

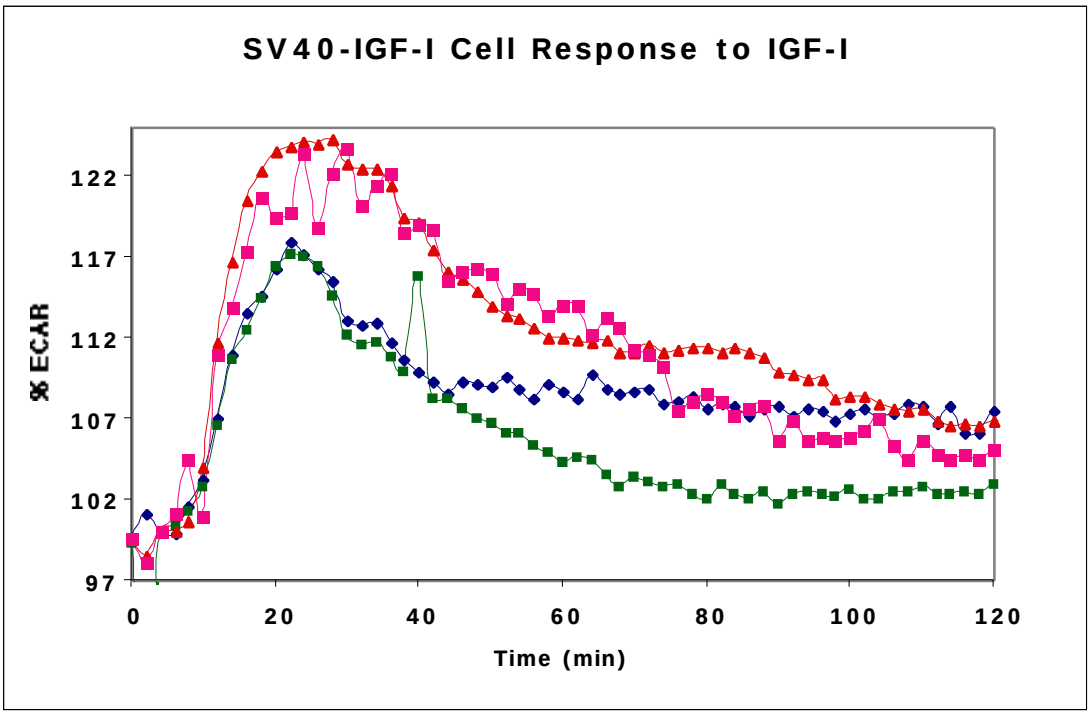

Figure 3.10 *% ECAR response by SV40-IGF-I cells (passage3) plated at 2 x 10^5 cells/ml and serum starved for 72 hrs at 0.5* (◆, ■) *and 5* (▲, ■) *ng/ml IGF-I shows a dose response with return to baseline after 60 to 80 min of washout. Data normalized prior to IGF-I exposure.*

maximize the response, allowing the addition of other agents to show stimulatory or inhibitory effects on the IGF-I binding. Since the goal of continuing studies is the comparison of the response of the MAC-T (paracrine) cell line with the SV40-IGF-I (autocrine) cell line, this optimized concentration was considered to be the working concentration for both cell lines.

3.1.3 Optimization of IGF-I challenge times

Multiple challenges of cells by growth factors such as IGF-I result in an attenuated response on the % ECAR graph. Long challenge times result in a long recovery of the signal to a baseline (Molecular Devices, 1996). A very short challenge time does not

allow the response to fully develop. The goal for determining an optimal challenge time was to determine the time required to achieve a significant peak, yet allow the response to return to a baseline in about an hour for a subsequent challenge. As can be seen from the first challenge peaks in Figure 3.11, a lower peak occurs for 6 min exposure time (119.4% ECAR) compared to challenge times of 12 min (124.2 % ECAR) or 18 min (129.6 % ECAR).

Peaks for 12 and 18 min were higher with possible deterioration with repeated 18 min exposures. Overall, this appeared to be a good working range. As was seen in Figure 3.10, a return to baseline occurred in 60 to 80 min with a 14 min challenge. The challenge time of 14 min was therefore chosen as an optimized challenge time.

3.1.4 Number of repetitions tolerated by cells

For some cell-receptor systems, particularly those with transient responses such as CHO cells transfected with the muscarinic M1 receptor, the cells may be challenged repeatedly to gather response data (Baxter, et al., 1994, Molecular Devices, 1996). However, repeated stimulation of the SV40-IGF-I cells as well as the MAC-T cells showed a decreased peak response with each exposure to IGF-I. In Figure 3.11 and quantitated in Table 3.2, it can be seen for the SV40-IGF-I cells that each stimulation resulted in a lower peak at 3 different stimulation durations. For this reason, in the analysis of cell response to IGF-I stimulation alone and in the presence of other agents, only the first peak from a capsule cup of cells was considered as a measured response.

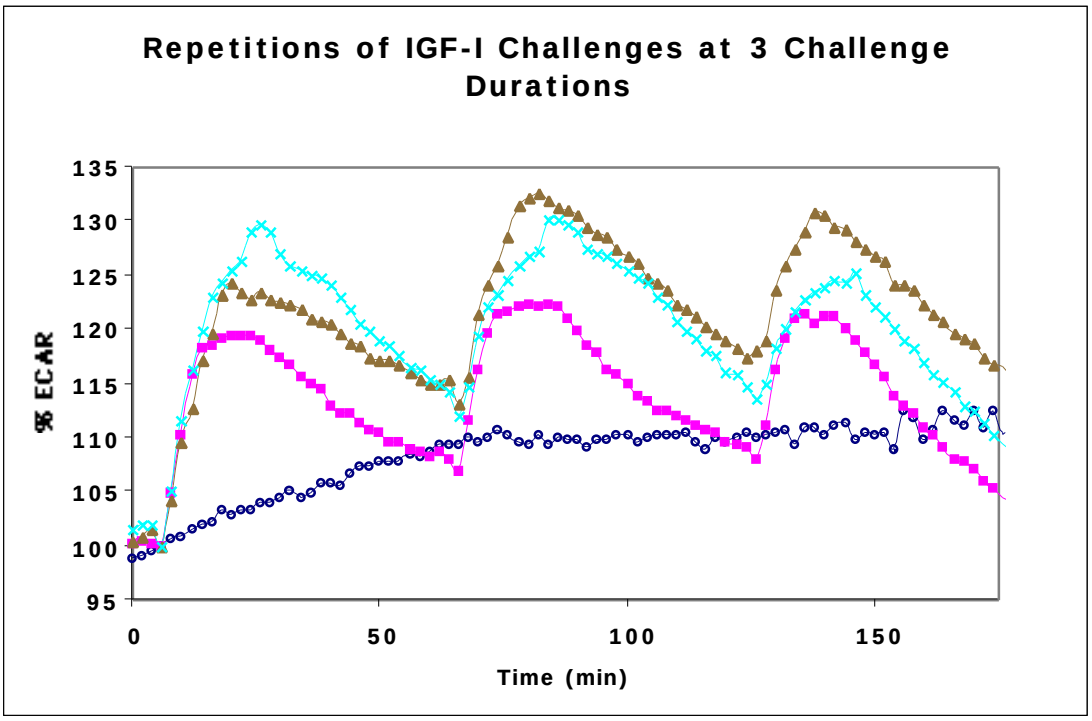

Figure 3.11 *Three channels of SV40-IGF-I cells (p26) plated at 2 x 10^5 cells/ml and serum starved for 72 hrs were challenged with 5 ng/ ml IGF-I for 6 (■), 12 (▲), or 18 (×) min per channel. The control channel (○) received no IGF-I. All channels were normalized to baseline prior to the first IGF-I exposure. Each challenge peak is lower than the one before at each exposure duration. % ECAR for first challenge peaks are 119.4, 124.2, and 129.6 for 6, 12, and 18 min exposures respectively.*

Table 3.2 Diminishing peak with each subsequent challenge as seen in figure 3.11

Challenge Exposure Time

Peak	6 min	12 min	18 min
1	119.4	124.2	129.6
2	114.3	117.2	115.9
3	112.2	113.4	110.6

Peak values are for single points from figure 3.11. Each peak height was measured from the point prior to stimulation by IGF-I on the response curve for each individual exposure time.

3.1.5 Staining of Capsule Cup Membranes to Verify Cell Confluency

The capsule cups from the microphysiometer runs were retained for crystal violet staining as described in Section 2.4-6. The capsule cups were carefully removed from the microphysiometer after a run and placed in a 12 well plate without draining the buffer from the capsule cups. These were refrigerated and then stained the same day. Staining consistently revealed uniform coverage of the membranes without evidence of tears or irregularities.

3.1.6 Summary

In summary optimal seeding densities were determined to be 2 x 10^5 cells/ml for SV40-IGF-I cells, and 5 x 10^5 cells/ml for MAC-T cells. Cells were seeded with DMEM plus FBS and allowed to adhere over the next 24 hrs followed by a 72 hr serum starvation period to sensitize the IGF-IR to IGF-I. Optimal challenge time was determined to be 14 min with an optimal IGF-I challenge of the order of magnitude of 5 ng/ml. % ECAR data was taken only for the first IGF-I stimulation of the cells on an experimental run due to diminished peaks with subsequent runs.