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IGF-I Stimulation of Extracellular Acidification Is Not Linked to Cell Proliferation for Autocrine Cells

Rose Marie Robinson,[1] R. Michael Akers,[2] and Kimberly E. Forsten[1,*]

[1]*Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA; and* [2]*Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg, VA*

Insulin-like growth factor-I (IGF-I) increases extracellular acidification rate (ECAR), a measure correlated with proliferation for nonautocrine cells. To evaluate the effect of autocrine IGF-I secretion on cell responsiveness, a cell line that secretes IGF-I was tested. SV40-IGF-I cells also registered concentration-dependent increases in ECAR; however, unlike the parental cell line, signal attenuation upon repeat challenges was not evident. Furthermore, SV40-IGF-I cells did not proliferate in response to IGF-I. We investigated if lack of proliferation was due to differences in the protocols of the assays ([^{3}H]thymidine incorporation and microphysiometry). We identified three key differences in the protocols: surface substrate, cell density, and fluid residence time. We found no increase in [^{3}H]thymidine incorporation for cells on either tissue-culture plastic or polycarbonate transwells. Control levels of [^{3}H]thymidine incorporation were cell-density-dependent, but IGF-I did not increase proliferation at any density studied. Finally, we investigated IGF-I stimulation for cells under microphysiometer flow conditions and found no proliferative response to IGF-I. We found that the cells do respond to IGF-I with increased amino acid uptake. These data suggest that IGF-I signaling is operational in the SV40-IGF-I cells, but the transduction pathway for IGF-I-induced proliferation is compromised, despite the fact that these cells respond to fetal bovine serum with increased growth. Ongoing studies are focused on identifying which elements in the signaling cascade are altered by autocrine secretion of IGF-I.

Key Words: Insulin-like growth factor-I (IGF-I); microphysiometer; mammary epithelial cells; autocrine; MAC-T; SV40-IGF-I.

Introduction

Insulin-like growth factor-1 (IGF-I) is important for the normal growth and development of the mammary gland *(1–3)*. Recent reviews *(4–6)* have reported that IGF-I promotes mammary growth and development, whether IGF-I is supplied by the circulation or by local tissue. IGF-I has been shown to be synthesized in the mammary tissue of a variety of species *(5,7–9)*, including bovine *(10)* and human *(11)*. Moreover, much of the mitogenic activity of mammary extracts is attributed to IGF-I *(12)*. While the majority of IGF-I is thought to be produced within the stromal cells of the tissue *(13,14)*, secretion of IGF-I or IGF-II by mammary epithelial cells has also been reported *(15–17)*.

Indeed, autocrine secretion of IGF has been proposed to be one means through which malignant transformation of cells can occur *(18)*. Interaction of the IGFs with the IGF-I receptor (IGF-IR) can activate intracellular pathways leading to cell proliferation *(19)* or cell transformation *(20–23)*. Several studies indicate that IGF-II rather than IGF-I is likely secreted by breast cancer cells *(24–26)*; however, both IGF-I and IGF-II can activate intracellular signaling by binding to IGF-IR.

Both primary cells and cell lines have been used extensively for in vitro studies of IGF-I activity. We recently reported *(27)* that the bovine mammary epithelial cell line, MAC-T, responded to IGF-I with increased extracellular acidification (ECAR), a measurement of cell activity made using microphysiometry *(28)*. This change in ECAR was specific for IGF-I and occurred within minutes. The response correlated well with IGF-I-induced proliferation. In this study, we focused on the SV40-IGF-I cell line, a MAC-T–based cell line transfected to secrete IGF-I under the SV40 constitutive promoter *(29)*. These cells express high levels of IGF-I, exhibit low levels of IGF-IR, and demonstrate marked secretion of IGF binding protein-3 (IGFBP-3) not seen with the parental cells. A lack of proliferative response by the SV40-IGF-I cell line to insulin suggested that the endogenous secretion of IGF-I had made the cells recalcitrant to exogenous IGF-I. In the present study, we sought to determine if the SV40-IGF-I cells were sensitive to exogenous IGF-I stimulation and to determine how exogenous IGF-I might impact cell activity.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Kimberly E. Forsten, Department of Chemical Engineering, Virginia Polytechnic Institute and State University, 133 Randolph Hall, Blacksburg, VA. E-mail: kforsten@vti.edu

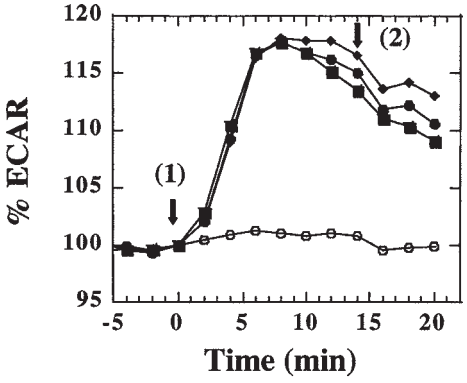

Fig. 1. Representative plot of ECAR response by SV40-IGF-I cells to IGF-I (5ng/mL). Transwells were seeded at 2×10^5 cells/cm^2 and equilibrated for 1 h in running buffer prior to addition of IGF-I. Tracings for four separate channels are shown. Channels received IGF-I at 5 ng/mL (◆, ●, ■) or 0 ng/mL (□) for 14 min followed by a switch to running buffer. The arrows indicate the valve switch to IGF-I *(1)* and the return switch to control running buffer *(2)*. Data normalized to baseline at the point designated 0 min.

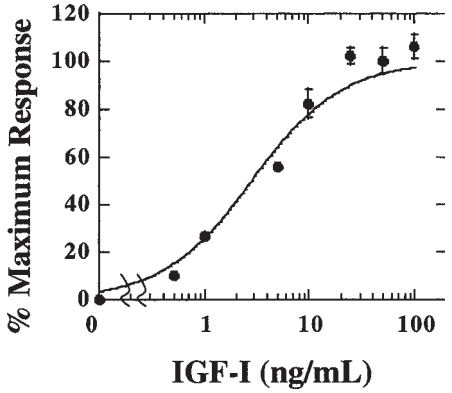

Fig. 2. Concentration-dependent effect of IGF-I on ECAR in SV40-IGF-I cells. Transwells were seeded at 2×10^5 cells/cm^2 and exposed to IGF-I concentrations from 0 to 100 ng/mL for 14 min. Maximal ECAR responses were seen with approx 50 ng/mL. The percentage of maximum response for each concentration was determined as the percentage of the 50 ng/mL response. Each point represents the mean ± SEM for at least three data points from at least two separate experiments, except for the 100 ng/mL point which is from two data points from one experiment. Individual experiments yielded similar results to composite figure.

Results

IGF-I Stimulation of the SV40-IGF-I Cell Line on the Microphysiometer

The Cytosensor® microphysiometer measures changes in ECAR in response to exogenous stimulant for a monolayer of cells *(28)*. The parental cell line, MAC-T, has previously been shown to respond to IGF-I with increased ECAR in a dose-dependent manner *(27)*. SV40-IGF-I cells also respond to IGF-I exposure with increased ECAR (Fig. 1). Preliminary optimization studies based on the parameters used for the parental cell line indicated that SV40-IGF-I cells could be seeded at a lower cell density (2×10^5 cells/transwell) to obtain comparable DNA and microphysiometer stimulation levels (data not shown). The shape of the ECAR curve is similar to the response curve for MAC-T cells, indicating a similar response time for both cell lines. This result was somewhat unexpected as the SV40-IGF-I cells have been shown to secrete at least 5.8×10^{-4} ng IGF-I per cell over a 72 h period *(29)* and one might anticipate interference in exogenous IGF-I binding due to endogenous IGF-I. However, SV40-IGF-I cells respond in a concentration-dependent manner to IGF-I with a maximal ECAR response at approx 50 ng/mL (Fig. 2). Peak ECAR values were higher for the SV40-IGF-I cells than for MAC-T cells, and the sensitivity was greater. The half-maximal effective concentration (EC_{50}) was 2.9 ng/mL IGF-I for SV40-IGF-I cells compared with 4.3 ng/mL IGF-I for MAC-T cells *(27)*. Furthermore, ECAR peak responses diminished for repeat challenges with IGF-I for the MAC-T cells. For example, a second challenge of MAC-T cells with IGF-I (10 ng/mL) diminished the response by approx 50% *(27)*.

Table 1
Response by SV40-IGF-I Cells with Repeated IGF-I Stimulation[a]

	IGF-I		
IGF stimulation	10 ng/mL	25 ng/mL	50 ng/mL
Challenge 1	100.0 ± 8.1	100.0 ± 0.2	100.0 ± 13.2
Challenge 2	94.8 ± 4.5	92.1 ± 2.1	90.9 ± 7.4
Challenge 3	92.6 ± 2.8		

[a] Results are reported as percentage of maximum response above normalized baseline for each concentration. Each result is the average of 4 different cell chambers from two different experiments. SEM is shown.

For the SV40-IGF-I cells, however, reduction in ECAR was minimal with repeated stimulation of the cells (Table 1).

We also investigated the effect of rIGFBP-3 on IGF-I stimulation of SV40-IGF-I cells on the microphysiometer (data not shown). Although IGFBP-3 is secreted by the SV40-IGF-I cells *(29)*, addition of exogenous rIGFBP-3 can reduce [^{125}I]-IGF-I binding at 4°C (data not shown). Partial inhibition (24–43%) of the ECAR response with the addition of rIGFBP-3 (0.5–200 ng/mL) to IGF-I (3 ng/mL) was seen on the microphysiometer. Exposure to rIGFBP-3 (0.5–50 ng/mL) alone did not result in an increased ECAR (data not shown), verifying the specificity of the IGF-I response. It is likely that the rIGFBP-3 binds to the IGF-I either rendering it unable to bind to the receptor or rendering it inactive in producing an ECAR response. Regardless, this finding suggests that the SV40-IGF-I response is mediated by IGF-I.

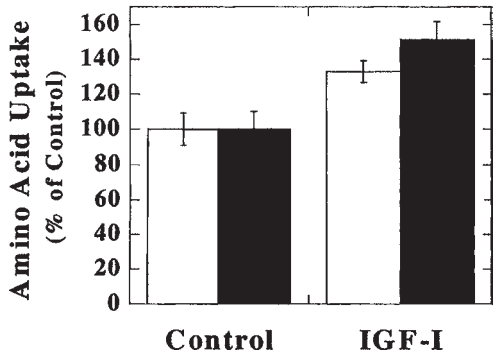

Fig. 3. Amino acid uptake determined by [^{14}C]AIB incorporation. SV40-IGF-I (■) and MAC-T (□)cells were plated at 2.6 × 10^4 cells/cm^2 and switched to serum free DMEM 24 h later. IGF-I (0 or 5 ng/mL) was added for 6 h. [^{14}C]AIB (4 μCi/mL) was added for 12 min. [^{14}C]AIB incorporation was quantified and results normalized to 100% control for individual cell lines. Mean ± SD is shown from at least duplicate samples. Results are representative of three separate experiments.

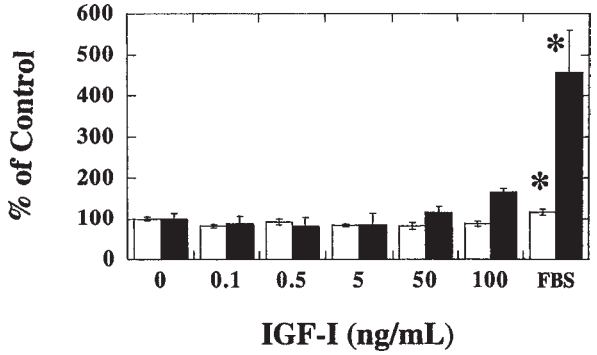

Fig. 4. [^{3}H]thymidine incorporation in response to IGF-I. SV40-IGF-I cells were cultured at 2.6 × 10^4 cells/cm^2 in the presence of 0, 0.1, 0.5, 5, 50, and 100 ng/mL IGF-I for 14 min (□) followed by fresh serum-free media for the remaining 15 h and 46 min or in the presence of IGF-I for 16 h (■). Response shown scaled to percentage of the control response (0 ng/mL IGF-I). Bars represent means ± SEM. Statistical analysis showed no significant difference between control and IGF-I. Addition of fetal bovine serum (FBS) did significantly stimulate proliferation (*) at both treatment times ($p < 0.05$). A repeat experiment yielded similar results.

Exogenous Addition of IGF-I Leads to Increased Amino Acid Uptake

Increased amino acid uptake in response to IGF-I has been demonstrated for bovine fibroblasts *(30)*. We investigated whether increased amino acid uptake in response to IGF-I occurred in both the parental and autocrine cells. Addition of exogenous IGF-I (5 ng/mL) resulted in increased amino acid uptake in both SV40-IGF-I and the parental MAC-T cell line as measured by α-[1-^{14}C]aminoisobutyric acid ([^{14}C]AIB) incorporation following 6 h of incubation at 37ºC (Fig. 3).

Exogenous Addition of IGF-I Does Not Lead to Increased Cell Proliferation

We examined the effect of IGF-I addition on SV40-IGF-I cell proliferation assayed by [^{3}H]thymidine incorporation using both a short (14 min) exposure time that correlated with the microphysiometer studies and a long (16 h) exposure time. Basal, or control, level of [^{3}H]thymidine incorporation was normalized to 100% (Fig. 4). There was essentially no response in proliferation with exogenous IGF-I as measured by [^{3}H]thymidine incorporation for either exposure time. However, addition of fetal bovine serum (FBS) increased proliferation (Fig. 4), verifying that the cells are responsive. The lack of IGF-I stimulation contrasts with our data for the parental cells *(27)*, but was not completely unexpected given that insulin (1 μg/mL) was previously shown to not affect proliferation *(29)*.

Cell Density Affects Basal Level but Not IGF-I Induction of Proliferation

Cell seeding for the microphysiometer and the proliferation studies differed by a factor of approx 8 (2 × 10^5 cells/cm^2 vs 2.6 × 10^4 cells/cm^2), so we investigated whether cell density might be responsible for the lack of proliferative response to IGF-I. Studies using [^{3}H]thymidine incorporation demonstrated that basal thymidine incorporation is higher at lower cell seedings on a per cell basis. However, there was no IGF-I stimulated proliferation at any of the densities tested (Fig. 5), confirming earlier work *(29)* showing that receptors were present on SV40-IGF-I cells and that cell binding on a per cell basis did not show a density dependence (data not shown).

Similar Lack of IGF-I-Induced Proliferation on Tissue-Culture Plastic and Polycarbonate Transwells

To determine if the growth substrate was responsible for the lack of a proliferative response to IGF-I, we compared the response on tissue-culture plastic (Fig. 4) with the response on polycarbonate membranes of transwells (used for microphysiometer studies) (Fig. 6) at the same lower cell density (2.6 × 10^4 cells/cm^2). SV40-IGF-I cells did not exhibit increased [^{3}H]thymidine incorporation in response to exogenous IGF-I on either surface; however, stimulation with FBS resulted in increased [^{3}H]thymidine incorporation. MAC-T cells responded to exogenous IGF-I with increased [^{3}H]thymidine incorporation on both growth surfaces (data not shown).

Cell Proliferative Response to IGF-I is Unchanged Under Flow

To obtain the sensitive pH measurement required for ECAR determination, the microphysiometer is designed to flush the cell environment between periods of pH measurement *(28)*. We postulated that this fluid exchange might remove endogenous IGF-I from the local environment, thereby creating an opportunity for exogenous IGF-I to stimulate the cells. Low cell seeding was used to maximize the

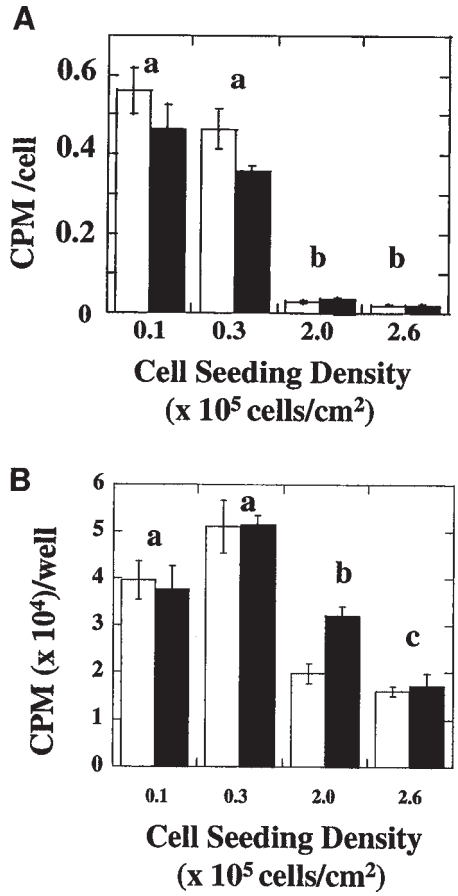

Fig. 5. (A) Cell seeding density effect on [^{3}H]thymidine incorporation. Direct cpm/cell shown for [^{3}H]thymidine incorporation of SV40-IGF-I cells at four different seeding densities. Bars represent means of three data points ± SEM. No significant differences found between control (□) and IGF-I (■) addition at any tested density. Significance between densities is designated by lower case letters. (**B**) Same as in panel **A** except plotted on a per well instead of a per cell basis. Two repeat experiments yielded similar results.

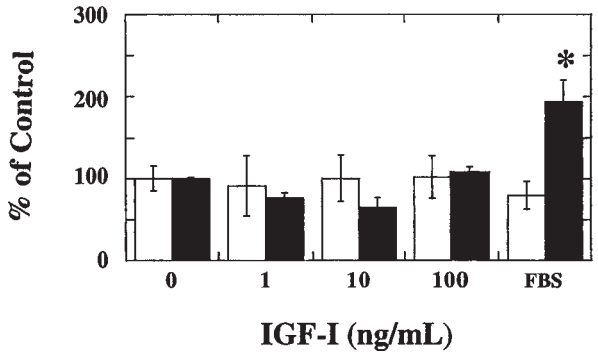

Fig. 6. [^{3}H]Thymidine incorporation by SV40-IGF-I cells (2.6 $\times 10^4$ cells/cm^2) grown on transwell polycarbonate membranes. Direct cpm [^{3}H]thymidine incorporation reported for cells exposed to 0, 1, 10, 100 ng/mL of IGF-I or 5% FBS scaled as percentage of the 0 ng/mL response on transwell polycarbonate membranes. Open columns (□) designate 14 min IGF-I or FBS exposure and solid columns (■) designate 16 h IGF-I or FBS exposure. Mean ± SEM is shown. Statistical analysis showed [^{3}H]thymidine incorporation was not significantly different at the $p < 0.05$ level for all treatments on transwells after 14 min. Significant difference was found only for the response to FBS on transwells after 16 h exposure (*). A repeat experiment yielded similar results.

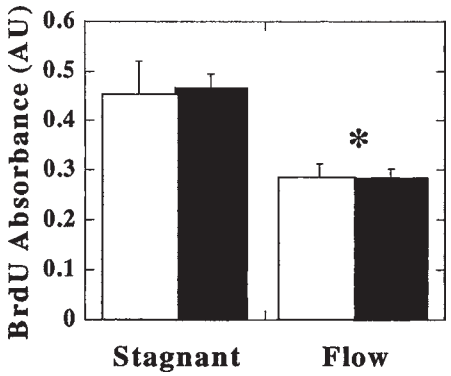

Fig. 7. BrdU incorporation by SV40-IGF-I cells exposed to IGF-I (50 ng/mL; (■)) under either stagnant or flow conditions. SV40-IGF-I cells seeded at 2.6×10^4 cells/cm^2 were maintained either in a 37ºC incubator for stagnant exposure or on the microphysiometer for flowing exposure (100 µL/min) during the 16 h IGF-I exposure time. Control (□) transwells received buffer without IGF-I. Differences between control and IGF-I exposure was nonsignificant for either condition. However, the rate of labeling was decreased ($p < 0.05$) with flow conditions (*). A repeat experiment yielded similar results.

possibility for cell proliferation (Fig. 5), and cells were examined under both flow and stagnant conditions. Incorporation of bromodeoxyuridine (BrdU) by control cells was decreased with flow, but there was no change in response with the addition of exogenous IGF-I for either condition (Fig. 7).

Discussion

IGF-I has a potent mitogenic effect on mammary epithelial cells *(24,31)*. It is manufactured in the liver as well as other cells and is available to mammary cells via endocrine, paracrine, and autocrine pathways. Normal mammary tissue growth and development depend on this growth factor *(2,3)*, and recent studies have shown that IGF-I stimulation of the IGF-IR can lead to mitosis and malignant cell transformation *(19,22,31)*. Using microphysiometry, we investigated whether exogenous IGF-I could alter ECAR of autocrine cells. We determined that SV40-IGF-I cells respond to exogenous IGF-I with an increase in extracellular acidification in a dose-dependent manner (Fig. 2) similar to the parental cell line *(27)*. That SV40-IGF-I cells would respond on the microphysiometer to exogenous IGF-I was surprising, as these cells had been shown to have reduced levels of receptors, to secrete high levels of IGF-I, and to be unresponsive to high levels of insulin *(29)*. Moreover, increased ECAR in response to exogenous ligand is not typical of autocrine systems. Epidermal growth factor (EGF) stimulation of ECAR was reduced in cells transfected to

secrete transforming growth factor-α, a ligand that signals via the EGF receptor *(32)*. ECAR, however, may not be directly tied to receptor levels. Increases in ECAR due to c-met stimulation by hepatocyte growth factor was higher in mesangial cells than in renal epithelial carcinoma A498 cells despite the fact that the A498 cells have higher c-met protein concentration *(33)*. While this may be specific to the A498 cells (as they also exhibited reduced responsiveness to EGF), it suggests that ECAR is linked to intracellular pathway activation, not just simple binding reactions. Thus, an increase in ECAR indicates some form of cellular activation. For example, heregulin activation of ECAR in mammary carcinoma cells was shown to require expression of both HER2 and HER3. However activation through HER2 could be mimicked by addition of antibodies that bound and clustered the receptors *(34)*. It is likely that the IGF-I induced ECAR we measured is specific, as addition of IGFBP-3 alone had no impact on extracellular acidification, and co-incubation resulted in a reduction in signal compared to IGF-I alone.

Although a dose-dependent ECAR response to exogenous IGF-I was demonstrated for both the parental *(27)* and the SV40-IGF-I cells, the overall response profile on the microphysiometer differed. The parental cell line response diminished with subsequent IGF-I challenges, while the response of SV40-IGF-I cells did not (Table 1). How this finding relates to cell signaling is unclear. Diminished responsiveness is not characteristic of growth factors such as epidermal growth factor *(35)*, although ECAR response does seem to vary greatly depending on what stimulating agent is responsible for the cellular activity *(28)*.

Given that the SV40-IGF-I cells respond to exogenous IGF-I with increased ECAR, we investigated whether longer-term cell responses could be stimulated by exogenous IGF-I. Amino acid uptake was increased in response to IGF-I (Fig. 3). However, [^{3}H]thymidine incorporation following either short (14 min) or long (16 h) IGF-I exposure did not differ from the control response (Fig. 4). SV40-IGF-I cells do bind IGF-I *(29)* and do show enhanced proliferation in response to serum. Decoupling of the proliferation response from growth factor binding has been shown previously with transfected mouse L cells that secrete IGF-I *(36)*. These cells demonstrate similar binding of IGF-I as parental cells, unlike SV40-IGF-I, but lack a proliferative response to exogenous IGF-I. This result suggests that binding and proliferation in response to IGF-I can be decoupled and that desensitization downstream can occur. Our data indicate that the intracellular desensitization does not prevent cell activation that results in extracellular acidification or induction of amino acid uptake.

It was, however, possible that the difference in SV40-IGF-I cell responsiveness in the microphysiometer assay compared to SV40-IGF-I cell responsiveness in the proliferation assay might be due to physical differences in the actual assay conditions. We examined factors in the experimental protocols that might alter cell responsiveness. These differences were identified as cell seeding density, growth substrate, and fluid residence time. Cell seeding densities for the studies differed by a factor of approx 8 with the proliferation studies being performed at the lower density. Cell density can impact IGF-IR expression levels *(37)*, which may impact IGF-I activity. For example, keratinocyte growth factor (KGF) receptor levels have been shown to be increased by high-density culture and these receptors are responsive to KGF *(38)*. SV40-IGF-I cells did not show density-dependent changes in cell surface IGF-I binding per cell (data not shown). Control levels of [^{3}H]thymidine incorporation were inversely correlated with cell density; however, addition of IGF-I did not induce proliferation at any density (Fig. 5).

Cell culture substrate can impact cell growth rate as well as the expression of various proteins. For example, the parental cell line, MAC-T, was shown to alter the production of urokinase-plasminogen activator depending on which matrix molecules the cells were plated *(39)*. The MCF-7 breast cancer cell line exhibits altered responsiveness to estrogen when cultured on laminin *(40)*. Furthermore, cell growth responses have even been shown to be dependent on the type of tissue culture material used *(41)*. The microphysiometer studies used cells cultured on a porous polycarbonate membrane rather than tissue culture plastic, and this could potentially have altered cell response. However, [^{3}H]thymidine incorporation assays performed on SV40-IGF-I cells grown on the polycarbonate membrane (Fig. 6) showed a similar lack of responsiveness to IGF-I as assays performed on SV40-IGF-I cells grown on tissue culture plastic (Fig. 4).

SV40-IGF-I cells secrete measurable quantities of IGF-I, which has been shown to be capable of stimulating proliferation of parental MAC-T cells *(29)*. It is conceivable that in the tissue culture environment, a buildup of IGF-I in the cell surface vicinity could lead to high enough concentrations whereby signaling is saturated by endogenous IGF-I, and, hence, addition of IGF-I has no effect. Certainly, secretion levels for SV40-IGF-I are high *(29)*, and these levels should be viewed as low estimates of the actual IGF-I released due to loss of IGF-I by binding *(32,42)*. The flow system of the microphysiometer reduces build-up time for the endogenous IGF-I, and this change in residence time for the endogenous ligand might have allowed exogenous IGF-I to stimulate IGF-IR present on the cell surface. However, just as under stagnant tissue culture plastic conditions, proliferation studies performed under flow conditions did not demonstrate IGF-I dependence.

Induced changes in IGFBP secretion and cell surface IGF-IR number in the SV40-IGF-I cells resulting from autocrine IGF-I have been shown *(29)*. Our work reveals that the presence of autocrine IGF-I has also induced changes in IGF-IR signaling, including the decoupling of the stimulation of proton secretion resulting from IGF-I

binding and the downstream stimulation of cell proliferation. Recent work by Santhanagopal and Dixon demonstrates that IGF-I stimulated changes in ECAR for osteoblastic cells are dependent on the phosphatidylinositol 3-kinase pathway *(43)*. We have further demonstrated that amino acid uptake by SV40-IGF-I cells is increased in response to IGF-I (Fig. 3). IGF-I–induced amino acid uptake in bovine dermal fibroblasts has been shown to be inhibited by LY294002, a specific inhibitor of PI-3 kinase activity *(30)*. This finding suggests that the PI-3 kinase pathway may be operational in SV40-IGF-I cells, and that failure to proliferate in response to exogenous IGF-I may lie in another signaling pathway. Ongoing studies in our laboratory are targeted at deciphering where and in which intracellular pathways desensitization in this mammary epithelial cell system has occurred.

Materials and Methods

Cell Culture

The SV40-IGF-I cell line was maintained in complete media composed of Dulbecco's modified Eagles media (DMEM), 10 mL/L antibiotic–antimycotic (100×), 1 mg/L gentamicin, 0.04 *M* $NaHCO_3$, and 10% fetal bovine serum (FBS). Normal cell maintenance involved plating cells at 5×10^5 cells per 100 mm dish and passaging via trypsin addition when cells were confluent. For microphysiometer studies, cells were plated at 1×10^6 cells per 100 mm dish and grown to confluency, typically 48 h. Cells were then seeded at the designated concentrations in transwells. For [^{3}H]thymidine incorporation studies and receptor-binding studies, cells were plated and grown to confluency, typically 72 h, and seeded at the designated concentrations in transwells, 24-well plates, or 12-well plates. Cells were used between passages 12 and 20.

DMEM, antibiotic–antimycotic (100×), gentamicin sulfate solution (10 mg/mL), calcium-free Dulbecco's phosphate buffered saline (DPBS), trypsin (2.5% lyophilized), and FBS were purchased from Gibco (Grand Island, NY). Enzyme free cell dissociation solution was purchased from Specialty Media (Phillipsville, NJ).

Microphysiometer Experiments

Human recombinant IGF-I was purchased from Peprotech (Rocky Hill, NJ). Human recombinant IGFBP-3 was purchased from Upstate Biotechnology (Lake Placid, NY). Materials obtained from Molecular Devices for use on the Cytosensor® microphysiometer included low buffer Roswell Park Memorial Institute (RPMI) media, capsule cup inserts, and spacer rings. Cells were seeded on tissue-culture-treated transwells (polycarbonate membrane, 12 mm diameter, 0.4 μm pore size) (Corning Costar Corporation, Cam-bridge, MA). Transwells were seeded with SV40-IGF-I cells at 2×10^5 cells/cm^2 in complete media. Cells were incubated at 37ºC for 24 h and the media replaced every 24 h with media without FBS for the next 72 h. RPMI (pH 7.4) supplemented with bovine serum albumin (BSA) (Fraction V, Sigma, St. Louis, MO) was used as the running buffer. Seeded transwell capsule cups were placed in the microphysiometer and equilibrated for approximately 1 h in running buffer pumped at 100 μL/min. Cells were subsequently challenged with IGF-I. Cell samples were challenged only once to determine dose-dependent responses.

Amino Acid Uptake Experiment

Amino acid uptake in response to IGF-I was measured using α-[1-^{14}C]aminoisobutyric acid ([^{14}C]AIB) (NEN, PerkinElmer Life Sciences, Boston, MA), based on a protocol from Conover et al. *(30)*. Briefly, cells were seeded at 2.6×10^4 cells/cm^2 in tissue culture wells in complete media. Cells were switched to FBS-free DMEM 24 h later. The amino acid uptake was conducted 48 h later. Cells were placed in buffer (0.5% BSA, 120 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* $MgSO_4 \cdot 7H_2O$, 15 m*M* $NaC_2H_3O_2$, 25 m*M* HEPES, 10 m*M* dextrose, pH 7.4) with IGF-I (0 or 10 ng/mL) and incubated at 37ºC for 6 h. [^{14}C]AIB was added and incubation proceeded for 12 min. Four washes with cold PBS and then overnight incubation with 0.3 *N* NaOH followed. Quantification was performed using a Liquid Scintillation Analyzer (Tri-Carb 2100 TR, Packard Instrument Co., Downers Grove, IL).

[^{3}H]Thymidine Incorporation Experiments

[^{3}H]Thymidine (ICN Pharmaceuticals, Irving, CA) uptake was measured as previously described *(29)*. Briefly, SV40-IGF-I cells were plated at designated concentrations. After 24 h, the cells were serum-starved for 72 h by replacing the media with DMEM without FBS. Cells were incubated with IGF-I for 16 h or 14 min. For the wells exposed to IGF-I for 14 min, the spent media was replaced with fresh media without FBS for the remaining incubation time. After 16 h, 100 μL of [^{3}H]thymidine (20 μCi/mL) was added to each well and incubation continued for 2 h. Wells were washed with DPBS followed by ice cold 10% TCA and 100% ethanol washes. Plates were then incubated overnight with 0.3 *N* NaOH. 3 *N* HCl was added to neutralize the NaOH, and the radioactivity of the samples from each well was quantified using a Liquid Scintillation Analyzer (Tri-Carb 2100 TR, Packard Instrument Co., Downers Grove, IL).

Bromodeoxyuridine (BrdU) Experiments

Transwells placed in 12-well plates were seeded with 2.6×10^4 cells/cm^2 to correspond with the seeding density on 24-well plates. After overnight adherence in DMEM with 10% FBS, the media was replaced with DMEM without FBS for 72 h. Cells were then exposed to IGF-I (0, 50 ng/mL) in fresh DMEM for 16 h. Detection of BrdU was per the manufacturer's instructions (Roche Diagnostics GmbH) with volumes of reagents increased to accommodate the larger volume of the transwell. The BrdU technique was verified as an effective nonradioactive alternative for use on the microphysiometer by comparing parallel [^{3}H]

thymidine incorporation studies under the same seeding and IGF-I exposure conditions. For cell proliferation studies under flow conditions, seeded transwells were placed on the microphysiometer and exposed to IGF-I (0, 50 ng/mL) in DMEM with 50 m*M* HEPES for 16 h. The BrdU assay protocol was adapted for use with the transwells on the microphysiometer by using the same reagent concentrations, but allowing the BrdU labeling reagent to be pumped across the transwells for 2 h under the same flow conditions used to obtain the IGF-I stimulation response. Seeded transwells in a 12-well plate maintained in a 37ºC incubator with DMEM with 50 m*M* HEPES were also assayed concurrently as a nonflow control.

Statistical Analysis

For microphysiometer ECAR responses, standard errors were calculated based on the Delta method. Calculation of the EC_{50} based on the response profiles for microphysiometry were based on a saturation model equation and fit using KaleidaGraph (version 3.5, Synergy Software). For [^{3}H]thymidine incorporation results, an ANOVA was completed using the GLM procedure of SAS (SAS Institute, 1989). A Bonferroni (Dunn) *T* test was performed to determine significance of differences between treatments.

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