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HSG Cells Differentiated by Culture on Extracellular Matrix Involves Induction of S-Adenosylmethione Decarboxylase and Ornithine Decarboxylase

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The human salivary gland (HSG) epithelial cell line can differentiate when cultured on extracellular matrix preparations. We previously identified >30 genes upregulated by adhesion of HSG cells to extracellular matrix. In the current studies, we examined the role of one of these genes, the polyamine pathway biosynthetic enzyme S-adenosylmethionine decarboxylase (SAM-DC) and the related enzyme, ornithine decarboxylase (ODC), on HSG cell differentiation during culture on extracellular matrix. HSG cells cultured on fibronectin-, collagen I gel-, and Matrigel-coated substrates for 12–24 h upregulated SAM-DC and ODC mRNA expression and enzyme activity compared to cells cultured on non-precoated substrates. After 3–5 days, HSG cells grown on Matrigel- or collagen I gel-coated substrates acquired a differentiated phenotype: the cells showed changes in culture morphology and increased expression of salivary gland differentiation markers (vimentin, SN-cystatin, and α-amylase). Further, culturing the cells on substrates precoated with an anti-β1-integrin-antibody promoted differentiation-like changes. HSG cells cultured on collagen I- or Matrigel-coated substrates rapidly entered the cell cycle but showed decreased cell proliferation at longer times. In contrast, cell proliferation was enhanced on fibronectin-coated substrates compared to cells on non-precoated substrates. Treatment with the polyamine synthesis inhibitors, difluoromethylornithine (DFMO), and methylglyoxal bis-(guanylhydrazone) (MGBG), inhibited cell proliferation and delayed ^{3}H-thymidine incorporation in HSG cells cultured on all of the substrates. Further, inclusion of DFMO and MGBG inhibited or delayed acquisition of the differentiated phenotype in HSG cells cultured on Matrigel- or collagen I gel-coated substrates. This suggests that the adhesion-dependent expression of SAM-DC and ODC contributes to extracellular matrix-dependent HSG cell differentiation. J. Cell. Physiol. 9999: 1–9, 2004. © 2004 Wiley-Liss, Inc.

Cellular differentiation is a complex process requiring cooperation between cell growth and cell signaling pathways. The human salivary gland (HSG) epithelial cell line can be reliably induced to differentiate in vitro by culture on the complex extracellular matrix protein preparation, Matrigel (Royce et al., 1993; Hoffman et al., 1996; Zheng et al., 1998; Jung et al., 2000). HSG cells are derived from intercalated ductal cells (Shirasuna et al., 1981) which are believed to be the stem cells that differentiate to give rise to the acinar and myoepithelial cells of the salivary gland (Eversole, 1971). Under normal culture conditions, HSG cells present an undifferentiated epithelial-like morphology. However, when cultured for 3–5 days on complex extracellular matrices such as Matrigel (or Vitrogen 100) they show morphologic differentiation and induce expression of salivary gland differentiation markers such as vimentin, salivary cystatin, and α-amylase (Hoffman et al., 1996; Zheng et al., 1998). Adhesion of HSG cells to extracellular matrix proteins is mediated by integrin adhesion molecules (Lafrenie et al., 1998). Integrins have been implicated as signal transduction molecules capable of altering cellular metabolism in response to changes in adhesion to extracellular matrix (reviewed in Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Lafrenie and Yamada, 1998; Giancotti and Ruoslahti, 1999; Hynes, 2002). To identify the linkage between integrin-dependent cell adhesion and differentiation of HSG cell cultures, a population of genes upregulated 6 h following adhesion to the extracellular matrix proteins, fibronectin, and collagen I, were identified (Lafrenie et al., 1998). One of these adhesion-responsive genes was S-adenosylmethionine decarboxylase (SAM-DC, RL10) which has been shown to be a limiting factor in polyamine biosynthesis (Pegg, 1988) and increased expression of SAM-DC and ornithine decarboxylase (ODC) are among the earliest events associated with cellular proliferation (Pegg and McCann, 1982, 1992).

The polyamines, putriscine, spermidine, and spermine are important and highly regulated cellular constituents that are required for cell growth and differentiation (Tabor and Tabor, 1984; Pegg, 1988; Heby and Persson, 1990; Janne et al., 1991; Marton and Pegg, 1995). ODC and SAM-DC catalyze the first steps in polyamine biosythesis. ODC catalyzes the formation of putrescine from ornithine while the decarboxylation of S-adenosylmethionine by SAM-DC results in the donation of aminopropyl groups for spermine and spermidine synthesis (Tabor and Tabor, 1984). Inhibitors of polyamine biosynthesis such as the specific inhibitor of ODC, difluoromethylornithine (DFMO), or the specific inhibitor of SAM-DC, methylglyoxal bis-(guanylhydrazone) (MGBG), show that polyamine biosythesis is critical for modulating cellular proliferation (Pegg and McCann, 1992; Thomas et al., 1996). Inhibition of polyamine biosynthesis has also been found to inhibit carcinogenesis in several experimental systems (Sunkara and Rosenberger, 1987; Verma, 1990).

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The expression of ODC and SAM-DC are highly controlled and can be regulated by a variety of agents that stimulate cellular proliferation such as growth factors, hormones, and tumor promoters (Pegg, 1988; Gawel-Thompson and Greene, 1989; Hurta et al., 1993, 1996; Soininen et al., 1996; Desiderio et al., 1998; Bielecki and Hurta, 2000). Transcriptional regulation of the ODC and SAM-DC gene promoters involves the Ras (Hurta, 2001; Voskas et al., 2001a), protein kinase C (Desiderio et al., 1998; Pintus et al., 1998; Song et al., 1998) and MAP kinase pathways (Patel et al., 1997[Q2]; Hurta, 2000; Voskas et al., 2001b). ODC and SAM-DC expression also appears to be controlled at post-transcriptional and translational levels (Kahana and Nathans, 1985; Katz and Kahana, 1987; Sertich and Pegg, 1987; Hurta et al., 1993, 1996; Wallon et al., 1995; Soininen et al., 1996).

In this study, we examined the role of polyamine biosynthetic enzymes in adhesion-dependent differentiation of HSG cells. We examined the effect of HSG cell adhesion to extracellular matrix on ODC and SAM-DC expression, cellular growth, and cellular differentiation. Further, we examined the effects of the specific ODC and SAM-DC inhibitors, DFMO and MGBG, respectively, on HSG cell growth and differentiation.

MATERIALS AND METHODS

HSG cell culture and differentiation

HSG cells (Shirasuna et al., 1981) were maintained in Dulbecco's modified Eagle medium (DMEM, Princess Margaret Hospital, Toronto, Ont., Canada) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, CO), 100 U/ml penicillin, and 100 μg/ml steptomycin (Life Technologies, Burlington, Ont., Canada). Cells were harvested with 10 mM EDTA in PBS and suspended in culture media containing 10% FCS. Cells were plated at medium density (2.5×10^4 cells/cm^2) on non-precoated culture dishes or culture dishes coated with 10 μg/ml human plasma fibronectin (Invitrogen, Burlington, Ont., Canada) for 16 h at 4°C, 2.5 mg/ml bovine collagen type I gel (Vitrogen 100, Collagen Canada, Toronto, Ont., Canada), or Matrigel (a gift from H.K. Kleinman, NICDR, Bethesda, MD) and cultured for 1, 3, or 5 days. For some experiments, HSG cells were pretreated with 1 μg/ml anti-β1 integrin antibody (clone mAb13, a gift from K.M. Yamada, NICDR, Bethesda, MD) prior to addition to the various substrates. HSG cells were also cultured on substrates that were precoated with anti-β1 integrin antibodies. The substrates were coated overnight with 1 μg/ml anti-β1 antibody, clone mAb13 (an anti-functional antibody that inhibits cell adhesion) (Akiyama et al., 1989; Mould et al., 1996) or anti-β1 antibody, clone K-20 (an antibody that does not inhibit adhesion, Life Technologies) (Mould et al., 1998) and then the cells added and cultured for 3 days. The monolayers were visualized using a Zeiss Axiophot microscope fitted with a videocamera and images were digitally recorded using Northern Eclipse computer software.

Cell attachment to immobilized proteins

HSG cells were resuspended at 10^5 cells/ml in DMEM media supplemented with 1% BSA. Non-tissue culture 96-well plates were coated with 50 μl of 10 μg/ml fibronectin, gelatin, Matrigel, collagen type I (Vitrogen 100), or BSA (Roche, Laval, Que., Canada) for 16 h, and then non-specific adhesive sites were blocked with 50 μl of 1 mg/ml BSA for 2 h. The wells were washed with PBS and 50 μl of the cell suspension (5×10^3 cells) was added to each well and incubated at 37°C for 1 h. For some studies, anti-integrin antibodies (1 μg/ml) that block adhesion via the β1 (clone mAb 13), α5 (clone mAb 16; Akiyama et al., 1989), α6 (clone GoH3, AMAC Inc., Westbrook, ME), α2 (clone P1E6; Wayner et al., 1988), α3 (clone P1B5), α4 (clone P4G9), or αv (clone VNR147) integrin subunits (Life Technologies) were added to the cells prior to incubation with the substrates. The wells were washed three times with PBS and the number of adherent cells counted per high power microscope field. All experiments were conducted in quadruplicate and data was analyzed using a Students's t-test; P values <0.05 were considered significant.

Northern blot analysis

HSG cells were cultured on non-precoated, fibronectin-, collagen I gel-, and Matrigel-coated substrates in culture media for various times. The cells were harvested in 4 M guanidine isothiocyanate, 50 mM sodium citrate, 0.1% sodium sarcosyl, and 0.1% β-mercaptoethanol, and RNA was extracted three times with water-saturated phenol/chloroform, and then precipitated with ethanol (Chomczynski and Sacchi, 1987). In some experiments, the HSG cells were cultured in the presence of the ODC inhibitor, DFMO (5 mM, Calbiochem, San Diego, CA), or the SAM-DC inhibitor, methylglycoxal bis-(guanylhydrazone) (10 mM MGBG, Sigma-Aldrich, St. Louis, MO). Fresh inhibitor was added daily. Total RNA (30 μg) was subjected to electrophoresis on 1% agarose gels containing formaldehyde and then transferred to Nytran membranes (Schleicher and Schuell, Xymotech Biosystems, Toronto, Ont., Canada). The membranes were hybridized with ^{32}P-labeled (Prime-It II kit, Stratagene, La Jolla, CA) cDNA fragments corresponding to SAM-DC, ODC, vimentin, SN-cystatin, α-amylase, or GAPDH (American Type Culture Collection, Rockville, MD) at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA as described (Lafrenie et al., 1998). The blots were washed once with 4× SSPE, 0.1% SDS at room temperature for 30 min followed by 30 min washes in 0.5× SSPE, 0.1% SDS once at 42°C and once at 55°C. The blots were then exposed to Hyperfilm-MP (Amersham-Pharmacia, Oakville, Ont., Canada) at −80°C.

Immunoblot analysis

HSG cells cultured on non-precoated, fibronectin-, collagen I gel-, Matrigel-coated substrates or substrates coated with anti-β1 integrin antibodies (clone mAb13 or clone K-20, as described above), in the presence of 10% FCS were harvested and lysed in RIPA buffer (1% Triton X-100, 0.5% SDS, and 0.5% sodium deoxycholate in PBS, pH 7.5) and protease inhibitors (Roche). Cell lysates were subjected to electrophoresis on 10% polyacrylamide gels containing SDS and transferred to nitrocellulose filters (Schleicher and Schuell). The filters were blocked by incubation in 3% BSA in Tris-buffered saline, pH 7.5, and 0.1% Tween-20 (TBST) and then incubated with antibodies against ODC, vimentin, or actin (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.5% BSA in TBST. The filters were washed, incubated with the appropriate anti-IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology), the HRP detected by incubation in Supersignal Reagent (Pierce Chemical Co., Rockford, IL), and then exposed to Hyperfilm-ECL X-ray film (Amersham-Pharmacia).

SAM-DC and ODC activity assays

HSG cells were harvested, plated at 2.5×10^4 cells/cm^2 on non-precoated, fibronectin, or Matrigel-coated substrates, and cultured for 2–48 h in culture media. For some experiments, the cells were incubated with 5 mM DFMO or 10 mM MGBG prior to adhesion. The cells were harvested and lysed in 50 mM HEPES, pH 7.4, 2.5 mM dithiothreitol, and 1 mM EDTA and lysate corresponding to 10^5 cells analyzed for SAM-DC (Shantz and Pegg, 1998) and ODC activities (Coleman and Pegg, 1998). Briefly, SAM-DC activity was determined by incubation of the cell lysate in 50 mM sodium phosphate, pH 7.5, 1.25 mM DTT, 3 mM putrescine, 0.2 mM S-adenosylmethionine, and 2 nCi/ml S-adenosyl-L-[carboxy-^{14}C]-methionine at 37°C for 60 min. ODC activity was determined by incubation of the cell lysate in 50 mM Tris-HCl, pH 7.5, 4 μM pyridoxal-5-phosphate, 0.25 mM DTT, 0.4 mM L-ornithine, and 2 nCi/ml L[-1-^{14}C]-ornithine at 37°C for 60 min. The reaction was stopped by addition of 5 N sulfuric acid. The evolved $^{14}CO_2$ was collected in a central well containing 0.25 ml of 1 M hyamine hydroxide for >1 h. The hyamine was neutralized by the addition of 1 N acetic acid, mixed with scintillation fluid, and counted on a liquid scintillation counter. Experiments were performed in quadruplicate.

Analysis of HSG cell growth

HSG cells, cultured in DMEM containing 10% FCS on non-precoated, fibronectin-, collagen I gel-, or Matrigel-coated substrates for 1–5 days, were harvested with 10 mM EDTA in PBS, washed and resuspended in PBS. In some experiments, the HSG cells were cultured for 3 days in the continued presence of DFMO or MGBG. Fresh inhibitor was added daily. The cell number was determined by a hemocytometer count of viable cells following trypan blue-staining.

Analysis of ^{3}H-thymidine incorporation

Cell proliferation was also measured by determining the incorporation of ^{3}H-thymidine (Denton, 1998). HSG cells were cultured on non-precoated, fibronectin-, collagen I gel-, or Matrigel-coated substrates in media supplemented with 10% FCS for various times and then pulse-labeled with 50 μCi/ml methyl-[^{3}H]-thymidine (Mandel, NEN) for 4 h intervals over 32 h. In some experiments, the HSG cells were cultured in the continued presence of 5 mM DFMO or 10 mM MGBG. The media was removed, cellular macromolecules were precipitated by treatment with 5% trichoroacetic acid, and the precipitate was washed with methanol. The precipitates were then harvested in formic acid and counted in a scintillation counter. To normalize for differences in ^{3}H recovery from cells cultured on the different substrates, the sum of ^{3}H-thymidine incorporation over the 32 h experiment was set at 100% for the untreated condition. Data is presented as the percentage of total ^{3}H incorporation measured for each 4 h labeling pulse.

Cell-cycle analysis

HSG cells, cultured for various durations on non-precoated, fibronectin-, collagen I gel-, or Matrigel-coated substrates were harvested, washed, and then fixed by incubation in 70% ethanol. The cells (10^6 cells/ml) were then incubated in 10 μg/ml propidium iodide and subjected to flow cytometric analysis on an EpicsElite Flow Cytometer (Becton-Dickenson[Q3]). The fluorescent profiles were fitted to various cell-cycle DNA content parameters utilizing the MultiCycle computer software and the relative proportions of cells in the G_1, S, and G_2/M phases of the cell cycle were determined.

RESULTS

HSG cell adhesion and differentiation

HSG cells cultured on Matrigel undergo morphological changes consistent with differentiation to ductal and acinar phenotypes (Royce et al., 1993; Hoffman et al., 1996). In the current experiments, HSG cells cultured on Matrigel for 3–5 days were shown to undergo dramatic changes in culture morphology forming a reticular network of duct-like structures in association with multicellular aggregates (Fig. 1A). HSG cells cultured on Matrigel-coated substrates for 5 days showed large multicellular structures that resembled intact salivary glands with large asci-like aggregates connected by duct-like structures. HSG cells cultured for 3 days on collagen I gels formed monolayers of rounded cells with highly refractile cell–cell boundaries, while after 5 days the cultures formed three-dimensional "domes." HSG cells cultured on non-precoated, gelatin-, or fibronectin-coated substrates formed confluent monolayers with well-defined cell–cell boundaries. HSG cells cultured on Matrigel or on collagen I gels showed upregulated expression of vimentin and the salivary gland differentiation markers SN-cystatin, and α-amylase mRNA. Vimentin expression was upregulated by threefold and expression of SN-cystatin and α-amylase was upregulated by fivefold and eightfold, respectively (based on three independent experiments), after 5 days of culture on Matrigel-coated substrates compared to cells cultured on non-precoated, gelatin- (not shown), or fibronectin-coated substrates (Fig. 1B). We detected two mRNA bands of 1.5 and 1.8 kb that hybridized to the SN-cystatin probe and the expression of both of these species were enhanced to a similar extent. Changes in vimentin expression were observed after only a single day of culture on Matrigel or collagen I (not shown).

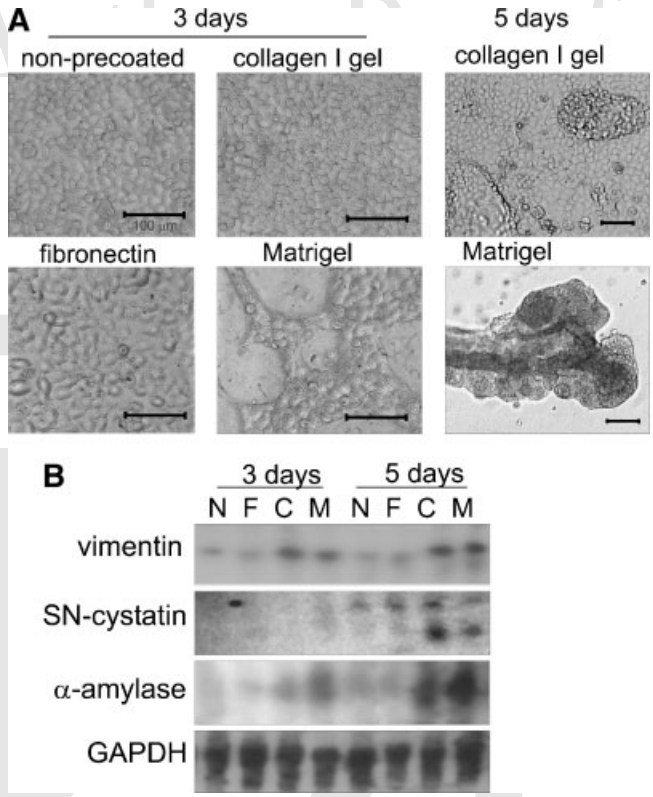


Fig. 1. The human salivary gland (HSG) cells cultured on extracellular matrix preparations undergo differentiation. Part A: HSG cells were cultured on the indicated substrates for 3 or 5 days. Microscopic images were recorded using phase-contrast microscopy. Scale bars are 100 μm. Part B: HSG cells were cultured on non-precoated (N), fibronectin- (F), collagen I gel- (C), and Matrigel-coated (M) substrates for 3 or 5 days. Total RNA was purified and subjected to Northern blot analysis for vimentin, SN-cystatin, α-amylase, and GAPDH mRNA expression.

Integrin-dependent cell adhesion to extracellular matrix components

The role of integrin adhesion molecules in mediating HSG cell adhesion to the various extracellular matrix-coated substrates was examined by measuring cell adhesion in the presence of various anti-integrin antibodies. The adhesion of HSG cells to Matrigel-, collagen I gel-, or gelatin-coated substrates was inhibited 84%–87% ($P < 0.05$) by inclusion of the anti-β1 integrin antibody (clone mAb13), and was inhibited by 41%–52% ($P < 0.05$) by inclusion of anti-α2 (Fig. 2A). Inclusion of the anti-α6 antibody inhibited HSG cell adhesion to Matrigel by 25% ($P < 0.05$) but did not affect adhesion to gelatin or collagen I gels. The adhesion of HSG cells to fibronectin was inhibited by 90% ($P < 0.05$) by the inclusion of the anti-β1 (clone mAb13) antibodies or 84% ($P < 0.05$) by the anti-α5 monoclonal antibodies but not by anti-α2, anti-α3, or anti-α6 antibodies. (Inclusion of the non-functional anti-β1 integrin antibody, clone K-20, did not inhibit adhesion to any substrate (not shown).)

Pretreatment of the HSG cells with anti-functional antibodies against the β1 integrin subunit (clone mAb13) prior to culture on Matrigel was sufficient to block the

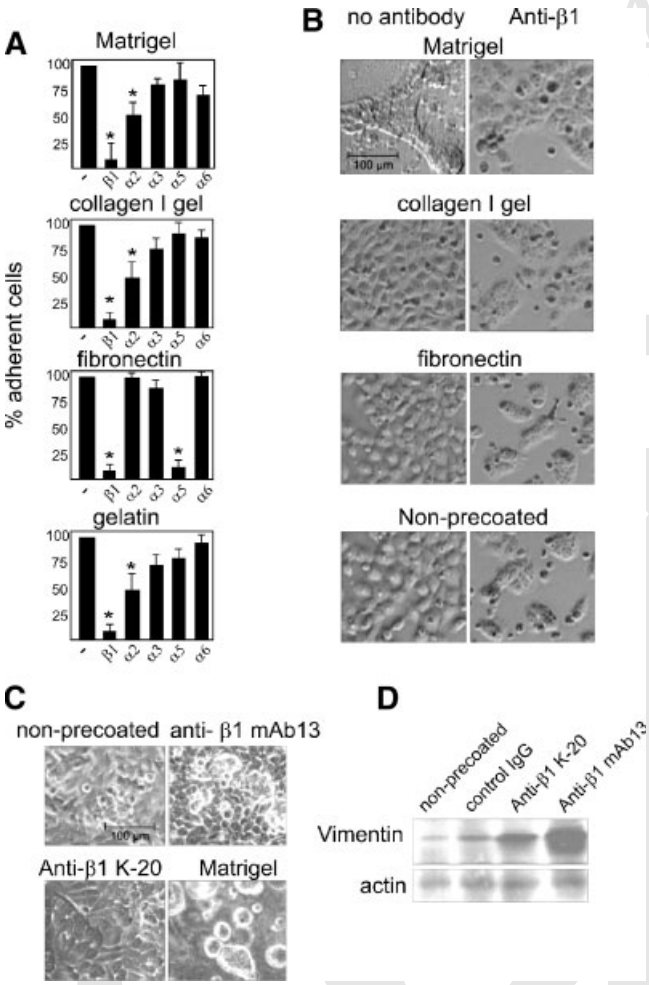


Fig. 2. HSG cell interaction with extracellular matrix preparations is mediated by integrin adhesion molecules. Part A: HSG cells were adherent to fibronectin-, gelatin-, collagen I-, or Matrigel-coated substrates in the absence (−) or presence of adhesion blocking antibodies against the various (β1, α2, α3, α5, or α6) integrin subunits. The number of adherent cells was determined by microscopy and expressed as the percent of cells adhered in the absence of antibody (control). Significant ($P < 0.05$) inhibition of adhesion is indicated (*). Part B: Adhesion-blocking anti-β1 integrin antibodies (clone mAb13) were added to the HSG cells prior to culturing for 3 days on non-precoated or Matrigel-coated substrates. The morphology of the HSG cell cultures were recorded by phase contrast microscopy. Part C: HSG cells were cultured for 3 days on non-precoated substrates, substrates precoated with adhesion-blocking (anti-functional, clone mAb13) β1 integrin antibodies, antibodies that did not alter adhesion (anti-β1 integrin, clone K-20), or Matrigel and then examined for changes in morphology. Part D: Cell lysates from HSG cell cultured on antibody-coated plates (as in part C) were subjected to immunoblot analysis with an anti-vimentin antibody.

changes in culture morphology and the formation of the duct-like structures that correlate with in vitro differentiation of HSG cells (Fig. 2B). Cells treated with antibody mAb13 spread poorly on all of the substrates. To further examine the role of integrin-dependent adhesion on HSG cell differentiation, the cells were cultured on substrates precoated with anti-integrin antibodies. HSG cells adhered to and were able to spread on substrates precoated with anti-β1 integrin antibodies (both anti-functional clone mAb13 and non-functional clone K-20). Further, cells cultured for 3 days on substrates precoated with the anti-functional anti-β1 integrin antibody, mAb13, showed characteristics consistent with the early stages of differentiation: cultures showed substantial changes in culture morphology, with the formation of asci-like structures, similar to cells cultured on Matrigel (Fig. 2C), and expressed increased levels of vimentin protein (Fig. 2D). However, cells cultured on the non-functional anti-β1 integrin antibody, clone K-20, control IgG, and non-precoated substrates remained as compact monolayers and did not express elevated levels of vimentin protein.

HSG cell adhesion enhanced expression of polyamine biosynthetic enzymes and cell growth

It was previously shown that SAM-DC (RL10) was one of the genes upregulated by culturing HSG cells on fibronectin- or collagen I-coated substrates for 3–6 h compared to non-precoated substrates (Lafrenie et al., 1998; Lam et al., 2001). Therefore, we examined the role of SAM-DC expression in HSG cell differentiation. The current results showed that HSG cells adherent to fibronectin-, collagen I gel-, and Matrigel-coated substrates for 12 h enhanced the expression of SAM-DC and ODC mRNA by at least threefold or fivefold, respectively (Fig. 3A) and enhanced ODC protein expression by 2.5-fold (Fig. 3B) compared to cells adherent to

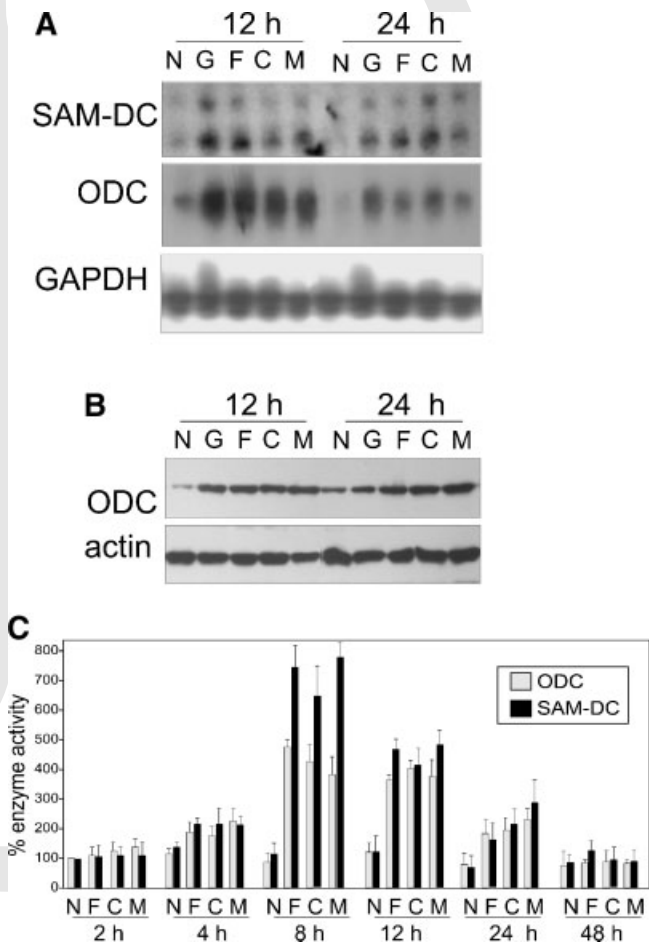


Fig. 3. Adhesion of HSG cells to extracellular matrix preparations promotes SAM-DC and ODC expression. HSG cells were cultured on non-precoated (N), gelatin- (G), fibronectin- (F), collagen I gel- (C), or Matrigel-coated (M) substrates for 12 or 24 h. Part A: Total RNA was purified and subjected to immunoblot analysis for SAM-DC, ODC, and GAPDH mRNA expression. Part B: Cell lysates were prepared and subjected to immunoblot analysis with antibodies against ODC or actin. Part C: The SAM-DC and ODC enzyme activities were measured in HSG cells cultured on the indicated substrates for various times and expressed as % of the activity in HSG cells adherent to non-precoated substrates for 2 h based on quadruplicate determinations. * Q5 denotes a significant ($P < 0.05$) increase in enzyme activity.

non-precoated substrates. Enhanced expression was maintained for at least 24 h. However, HSG cells cultured on fibronectin-, collagen I gel-, and Matrigel-coated substrates for 3 days and longer showed similar levels of SAM-DC and ODC mRNA expression (not shown).

SAM-DC and ODC enzyme activity were induced in HSG cells adherent to fibronectin-, collagen I gel-, or Matrigel-coated substrates for 4–24 h compared to cells adherent to non-precoated substrates (Fig. 3C). The extracellular matrix-dependent increase in SAM-DC and ODC activity was the greatest after 8 h and was enhanced ~7.5- or 4.5-fold, respectively, compared to cells cultured on non-precoated substrates. After 48 h, SAM-DC and ODC activity returned to basal levels. HSG cells cultured on non-precoated substrates did not show changes in SAM-DC or ODC activity over time. Inclusion of MGBG inhibited SAM-DC activity by at least 70% in HSG cells cultured on extracellular matrices for 8 h and inclusion of DFMO inhibited ODC activity by at least 80% in cells cultured on extracellular matrices for 8 h (not shown).

HSG cells cultured on non-precoated, fibronectin-, collagen I gel-, and Matrigel-coated substrates grew at different rates. HSG cells cultured on fibronectin-coated substrates had an increased growth rate, with a doubling time of less than 22 h, and grew to the highest number of cells (1.4×10^5 cells/cm^2) by day 5 (Fig. 4A). Cells grown on non-precoated substrates had a slower growth rate (doubling time of ~30 h) and grew to a lower number of cells (8.0×10^4 cells/cm^2) than cells cultured on fibronectin. HSG cells grown on collagen I gel- or Matrigel-coated substrates grew rapidly and almost doubled over the first day of culture but then the growth rate slowed and they grew to a lower number of cells by day 5 (4.5×10^4 or 7.0×10^4 cells/cm^2, respectively). In addition, treatment of HSG cells with the polyamine biosynthetic enzyme inhibitors, DFMO or MGBG, inhibited HSG cell growth. Treatment with DFMO or MGBG for 3 days significantly ($P < 0.05$) decreased cell growth on non-precoated, fibronectin-, collagen I gel-, and Matrigel-coated substrates by approximately 30% (Fig. 4B). Since, it appeared that HSG cells cultured on collagen I gels or Matrigel showed an initial burst in cell growth before slowing, we measured the incorporation of ^{3}H-thymidine into replicating DNA over the first 36 h of culture. HSG cells cultured on fibronectin-, collagen I gel-, and Matrigel-coated substrates displayed a peak of DNA replication (28%–38% of the thymidine label was incorporated during the 12–16 h labeling pulse) that was earlier than in cells cultured on non-precoated substrates (peak of thymidine incorporation at 24–28 h) (Fig. 4C). Treatment of HSG cell cultures with DFMO or MGBG also altered the incorporation of ^{3}H-thymidine into the cellular DNA. For example, the pulse of ^{3}H-thymidine incorporation seen in HSG cells cultured on fibronectin-, collagen I gel-, or Matrigel-coated substrates for 12–16 h was inhibited by 40%–50% ($P < 0.05$) in the presence of DFMO or MGBG. Measuring changes in cellular DNA content using flow cytometry also showed that HSG cells cultured on fibronectin-, collagen I gel-, and Matrigel-coated substrates entered the cell cycle (increase in S phase) earlier than cells on non-precoated substrates (Fig. 5). At the 8 h time point, cells cultured on collagen I gel or Matrigel had a greater proportion of cells in S phase (15% and 12%, respectively) and G_2/M phases (35% and 36%, respectively) than cells cultured on non-precoated substrates (6% S phase and 17% G_2/M phase).

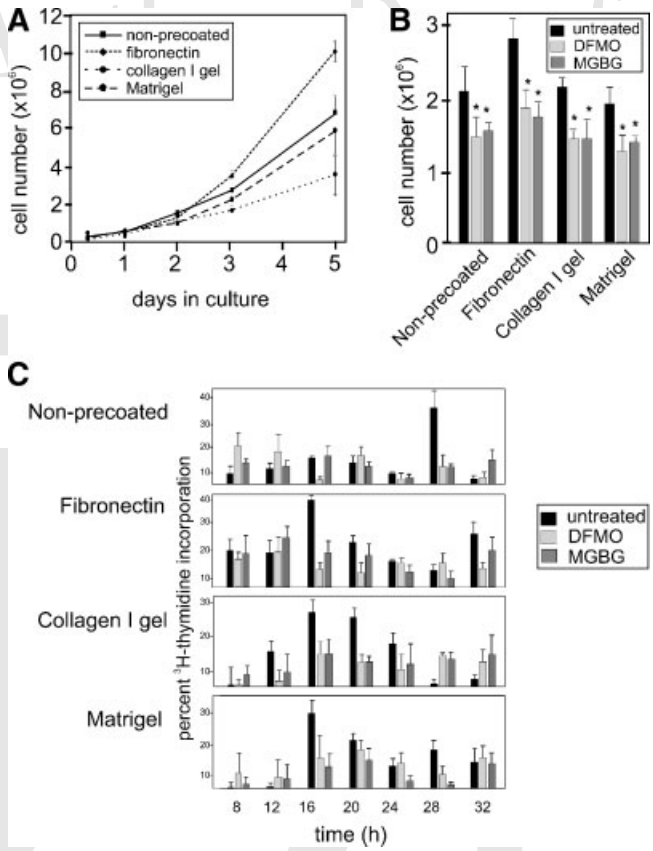
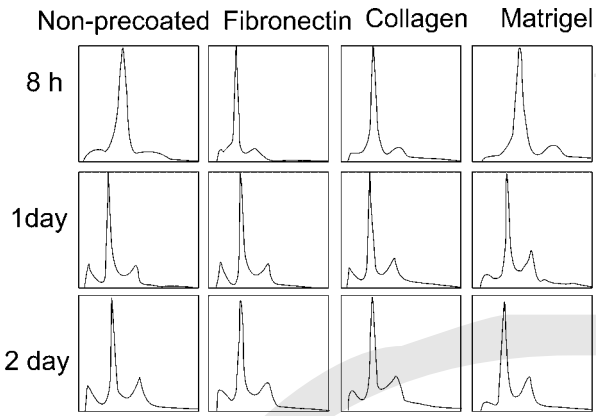


Fig. 4. The effects[Q6] of different extracellular matrices and polyamine biosynthesis inhibitors on HSG cell growth. Part A: HSG cells cultured on non-precoated, fibronectin, collagen I gel-, and Matrigel-coated substrates were harvested and counted using a hemocytometer to determine the changes in cell number during time in culture. Part B: HSG cells cultured on the different substrates for 2 days in the absence or presence of DFMO or MGBG were harvested and counted to determine the effects of the inhibitors on cell growth. Part C: HSG cells were cultured on the different substrates and then labeled with ^{3}H-thymidine for sequential 4 h pulses over the 36 h experiment. The incorporation of ^{3}H-thymidine by the cultures of HSG cells was measured in the absence or presence of DFMO or MGBG and the data expressed as the percent total incorporation of ^{3}H-thymidine for each labeling pulse.

Effect of polyamine biosynthesis inhibitors on HSG cell differentiation

The effects of the polyamine synthesis inhibitors, DFMO and MGBG, on HSG cell differentiation were measured in cells cultured on collagen I gel- and Matrigel-coated substrates. The inclusion of DFMO or MGBG altered the morphology of HSG cell cultured on Matrigel-coated substrates (Fig. 6A). In the presence of DFMO or MGBG, the cells did not organize into duct- or acinar-like structures to the same extent as in untreated cultures. While the cells did show some changes in culture organization creating a “honey-comb-like” pattern in parts of the cultured monolayer, the treated cells never showed the three-dimensional organization of ducts and asci-like aggregates seen in untreated HSG cells. HSG cells cultured on non-precoated, fibronectin, or collagen I-coated substrates in the presence of DFMO or MGBG showed similar culture morphologies but a general decrease in cellularity.

Inclusion of DFMO or MGBG also inhibited the expression of the salivary gland differentiation markers. In untreated HSG cells, the expression of vimentin, SN-cystatin, and α-amylase was upregulated in cells



	8 h			1 day			2 days		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Non-precoated	77	6	17	44	11	45	42	22	36
Fibronectin	62	18	20	42	7	51	50	21	29
Collagen I gel	50	15	35	56	23	22	52	26	30
Matrigel	52	12	36	57	22	22	60	18	22

Fig. 5. HSG cells cultured on the different substrates for 8 h, 1 day or 2 days were harvested, fixed, and stained with propidium iodide. The fluorescent profiles for the labeled cell populations were determined by flow cytometry and the proportion of cells in the different phases of the cell cycle determined (the table shows the average of three independent experiments).

cultured on collagen I gel- and Matrigel-coated substrates for 5 days (Fig. 6B). However, HSG cells cultured on collagen I gel- or Matrigel-coated substrates in the presence of DFMO showed a twofold downregulation of vimentin expression, an eightfold downregulation in α-amylase expression, and a fivefold downregulation in SN-cystatin expression. Similarly, HSG cells cultured on collagen I gel- or Matrigel-coated substrates in the presence of MGBG, showed downregulation of vimentin, α-amylase, and SN-cystatin by threefold, fivefold, and sixfold, respectively, compared to the untreated controls.

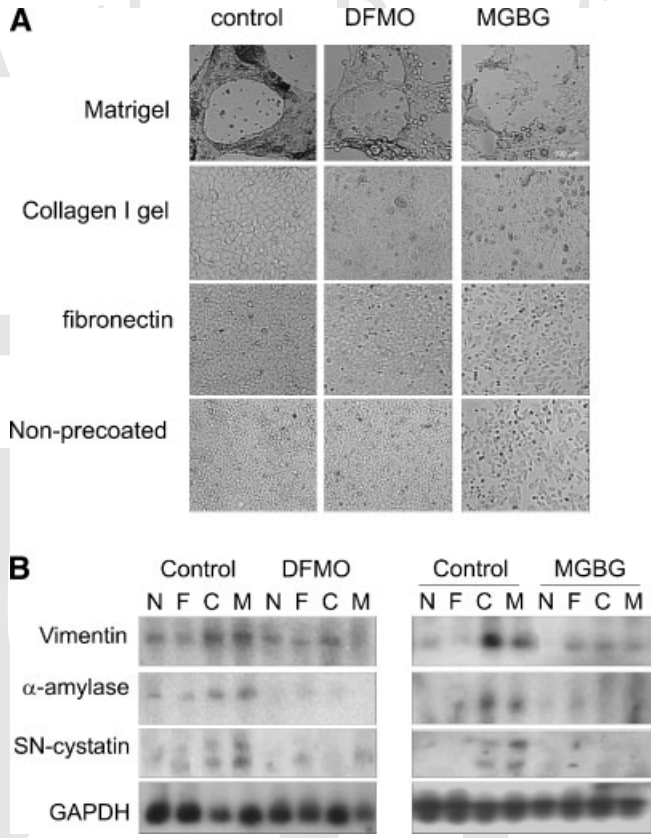


Fig. 6. Effect of polyamine synthesis inhibitors on extracellular matrix-dependent HSG cell differentiation. Part A: HSG cells were cultured on fibronectin- or Matrigel-coated substrates in the presence of DFMO or MGBG for 3 days. Cell morphology was examined and recorded using phase-contrast microscopy. Part B: HSG cells were cultured on non-precoated (N), fibronectin- (F), collagen I gel- (C), and Matrigel-coated (M) substrates for 3 days in the presence or absence of the polyamine inhibitors DFMO or MGBG. Total RNA was purified and subjected to Northern blot analysis for vimentin, SN-cystatin, α-amylase, and GAPDH mRNA expression.

DISCUSSION

The HSG cell line has been shown to undergo differentiation when cultured on Matrigel-coated substrates although the mechanisms underlying this differentiation remain largely unknown (Royce et al., 1993; Hoffman et al., 1996). In order to identify genes that might be intermediates in extracellular matrix-induced HSG cell differentiation, we isolated a population of genes that were upregulated by adhesion to extracellular matrix-coated substrates (Lafrenie et al., 1998). One of these genes was the polyamine biosynthetic gene, SAM-DC. Since polyamine biosynthesis is an important contributor to cell growth and differentiation (Choudhary et al., 1999), we examined the role of SAM-DC and the related polyamine biosynthetic gene ODC in matrix-induced HSG cell differentiation. In this study, we showed that HSG cells cultured on fibronectin-, collagen I gel-, or Matrigel-coated substrates for 12–24 h upregulated the polyamine biosynthetic enzymes SAM-DC and ODC by fivefold or eightfold, respectively, and upregulated SAM-DC and ODC enzyme activity by 4.5- or 7.5-fold in 8 h. In addition, HSG cells cultured on these substrates rapidly entered the cell cycle. However, when HSG cells were grown on collagen I gel- or Matrigel-coated substrates for 3–5 days, cellular proliferation slowed and the cultures acquired a differentiated phenotype.

HSG cells cultured on Matrigel for 5 days had many of the morphologic characteristics of intact salivary glands with large asci-like cellular aggregates connected via hollow duct-like structures. HSG cells on collagen I gel-coated substrates for 5 days showed more subtle changes in morphology. The cells appeared more rounded and the cultures formed some multicellular "dome" structures consistent with contact-induced differentiation (Pantschenko et al., 2000; Schreider et al., 2002). In contrast, HSG cells spread on the non-precoated, gelatin-, or fibronectin-coated substrates and did not form multicellular structures. In addition to changes in morphology, HSG cells grown on collagen I gel- or Matrigel-coated substrates for 3–5 days expressed increased levels of vimentin and the salivary gland differentiation markers SN-cystatin, and α-amylase, consistent with previous reports (Shirasuna et al., 1981; Royce et al., 1993; Hoffman et al., 1996; Zheng et al., 1998; Jung et al., 2000). HSG cells on non-precoated, fibronectin-, or gelatin-coated substrates did not show enhanced expression of salivary gland differentiation markers. Matrigel is the only substrate that promotes a completely differentiated morphology, however, both collagen I gel and Matrigel promoted expression of salivary gland differentiation markers. Thus, changes in

morphology were not mechanistically required for expression of the differentiation markers. Adhesion of the HSG cells to the substrate, mediated by integrin adhesion molecules, are required for in vitro differentiation since inclusion of an anti-β1 antibody that blocked cell/matrix adhesion (clone mAb13) also blocked the formation of duct-like structures by cells cultured on Matrigel-coated substrates. Alternately, culturing cells on substrates precoated with anti-functional β1 integrin antibodies (clone mAb13, that blocks ligand binding for all β1-containing integrins (Mould et al., 1996)) promoted the early stages of differentiation and enhanced expression of vimentin protein. Vimentin is expressed by myoepithelial cells in the immature acinus of the developing salivary gland (Ogawa, 2003) and is expressed during epithelial-mesenchymal transition that occurs early in differentiation. Thus, in vitro differentiation of HSG cells appears to be dependent on integrin-mediated adhesion.

Previous experiments have shown that culturing various cells on Matrigel-coated substrates can induce differentiation-like phenotypic changes. For example, endothelial cells and some epithelial cell lines have been shown to form duct-like structures on Matrigel-coated substrates (Grant et al., 1989; Schmeichel and Bissell, 2003). Murine mammary epithelial cells grown on Matrigel-coated substrates also enhance the expression of the tissue-specific differentiation markers casein and whey protein (Lin et al., 1995; Streuli et al., 1995) and hepatocytes grown on Matrigel or collagen I gels enhance expression of albumin (DiPersio et al., 1991). These studies have shown that adhesion of cells to Matrigel or collagen I was mediated by β1 integrin family adhesion molecules and that these β1 integrin-dependent interactions were critical for the expression of the differentiation markers (Roskelley et al., 1994). In the HSG cell model, the α2β1 integrin partially mediated adhesion to the differentiating substrates, collagen I and Matrigel, suggesting α2β1 integrin may induce the signals that lead to differentiation. We have previously shown that adhesion of HSG cells to fibronectin or collagen I gel can induce different genes suggesting that each integrin can promote different signaling pathways (Lafrenie et al., 1998). However, HSG cells adherent to gelatin (denatured collagen I)-coated substrates, which was also mediated by the α2β1 integrin, did not promote differentiation. This suggests that factors in addition to α2β1 are involved. One of the possibilities is the nature of the substrate. It has been suggested that culturing cells on the relatively pliable collagen I gels or Matrigel allows the cell to undergo changes in cell shape, such as cell rounding, that are required for adhesion-dependent changes in cellular differentiation (Roskelley et al., 1994; Cukierman et al., 2002). In contrast, cells cultured on rigid gelatin- or fibronectin-coated substrates cannot undergo these required changes in cell shape and therefore cannot differentiate. For example, mammary epithelial cells, cultured on Matrigel or in suspension, became rounded, did not proliferate, and were able to express differentiation markers even as single cells, while cells cultured on dried extracellular matrix substrates spread, remained flat, and did not express differentiation markers (Close et al., 1997).

Culturing HSG cells on Matrigel-, collagen I gel-, or fibronectin-coated substrates also upregulated the expression of the polyamine biosynthetic enzymes, SAM-DC and ODC. Treatment of cells with growth factors or other stimuli that activate the Ras, PKC, or MAP kinase pathways enhances the expression of SAM-DC and ODC (Hurta et al., 1993, 1996; Soininen et al., 1996; Bielecki and Hurta, 2000). Since integrin-mediated adhesion can also activate the Ras, PKC, or MAP kinase signaling pathways (Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Lafrenie and Yamada, 1998; Giancotti and Ruoslahti, 1999; Lam et al., 2001; Hynes, 2002), it is not surprising that adhesion also enhances SAM-DC and ODC expression. We have previously shown that the upregulated expression of SAM-DC (RL10) following adhesion of HSG cells to fibronectin- or collagen I gel-coated substrates requires adhesion-dependent activation of the PKC (Lam et al., 2001) and MAP kinase (Lam et al., in preparation[Q4]) signaling pathways. Interestingly, the expression of the salivary gland differentiation marker, α-amylase, during HSG differentiation also requires both the PKC and MAP kinase signaling pathways (Zheng et al., 1998; Jung et al., 2000).

Since polyamine biosynthesis is usually related to changes in cellular proliferation (Pegg, 1988; Heby and Persson, 1990; Marton and Pegg, 1995), the ability of cells adherent to different substrates to alter SAM-DC and ODC expression suggests that the culture substrate may alter the growth rate of the cells. In fact, culturing HSG cells on the various extracellular matrix substrates did have effects on cellular proliferation. HSG cells cultured on fibronectin-coated substrates grew faster than cells on non-precoated substrates. Interestingly, HSG cells cultured on Matrigel- and collagen I gel-coated substrates grew more slowly than cells grown on non-precoated substrates. However, closer examination showed that HSG cells cultured on collagen I- or Matrigel-coated substrates showed an early increase in growth rate and earlier entry of cells into cell cycle in comparison to cells on non-precoated substrates. These early differences in cell growth rates were also shown by alterations in DNA replication since incorporation of ^{3}H-thymidine into replicating DNA was earlier in cells cultured on extracellular matrix-coated substrates. To determine if the changes in growth rate involved polyamine biosynthesis, the growth of HSG cells cultured in the absence or presence of pharmacological inhibitors of SAM-DC or ODC were determined. MGBG and DFMO, specific inhibitors of SAM-DC and ODC, respectively, are potent inhibitors of cell growth and can inhibit malignant behavior in several cultured cancer cells (Pegg, 1988; Gawel-Thompson and Greene, 1989; Hurta et al., 1993, 1996; Soininen et al., 1996; Desiderio et al., 1998; Bielecki and Hurta, 2000). Inclusion of DFMO or MGBG inhibited cell growth and ^{3}H-thymidine incorporation by HSG cells cultured on all of the substrates suggesting adhesion-induced polyamine biosynthetic enzymes contributed to changes in cell growth. Further, these results are consistent with previous results that showed that polyamine biosynthesis is a critical component of cellular proliferation and indicate that the polyamine biosynthesis inhibitors are active in HSG cells.

Since adhesion of HSG cells to extracellular matrices can induce the expression of SAM-DC and ODC and promote cellular differentiation, the impact of polyamine synthetic enzyme expression on cellular differentiation was determined using polyamine biosynthesis inhibitors. Treatment of HSG cells cultured on collagen I gel- or Matrigel-coated substrates with inhibitors of polyamine biosynthesis, DFMO or MGBG, was able to inhibit, or delay, HSG cell differentiation as determined by changes in culture morphology and the expression of the salivary gland differentiation markers. This

supports the idea that polyamine biosynthesis is functionally involved in matrix-dependent differentiation of HSG cells. However, HSG cells adherent to fibronectin can induce SAM-DC and ODC expression but do not differentiate indicating that alterations in the polyamine synthetic enzymes are not sufficient for adhesion-dependent differentiation. Thus, differences in signals, in addition to polyamine synthesis, induced in cells adherent to fibronectin versus cells adherent to collagen I gel or Matrigel are likely involved.

The increased expression of ODC and SAM-DC has been associated with cell proliferation, progression through the cell cycle, and carcinogenesis. Overexpression of ODC also promotes cell growth and neoplastic transformation in a variety of cell types. Since differentiation is usually associated with a decrease in cellular proliferation, it seems paradoxical that Matrigel-induced differentiation of HSG cells is associated with elevated levels of ODC and SAM-DC and that inhibitors of ODC or SAM-DC can inhibit/delay differentiation. In some experimental systems, inhibition of ODC (by addition of DFMO) can promote differentiation. For example, DFMO treatment induces differentiation of erythroleukemia (MEL) cells as measured by their ability to synthesized hemoglobin (Choudhary et al., 1999). However, in HSG cells, adhesion to Matrigel (and collagen I gel)-coated substrates promoted a rapid rise (2–8 h) in ODC and SAM-DC activity and a rapid entry into the cell cycle although continued culture on Matrigel (or collagen I) resulted in a marked slowing of growth rate and acquisition of differentiated characteristics. Inhibition of ODC or SAM-DC was shown to delay entry into the cell cycle and to inhibit/delay differentiation. These data suggest that Matrigel-dependent differentiation of HSG cells may require an early round of cell division and suggest that entry into the cell cycle might be important for differentiation. It is possible that the transient signals initiated by adhesion collaborate with an early entry into the cell cycle to promote differentiation. Thus, the ability of treatment with DFMO or MGBG to delay cell-cycle entry is sufficient to inhibit differentiation.

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