Role of laminin-1 and TGF-β3 in acinar differentiation of a human submandibular gland cell line (HSG)

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SUMMARY
Previous studies show that culturing an immortalized human submandibular gland cell line (HSG) on Matrigel, a basement membrane extract, induces cytodifferentiation. We have further defined this model system and identified factors involved in HSG cell acinar development and cytodifferentiation. Acinar development is marked by cell migration into multi-cellular spherical structures, cell proliferation and apoptosis of the centrally localized cells. In addition, functional differentiation was determined by indirect immunofluorescence and immunoblot analysis for cystatin, a salivary gland acinar cell-specific protein found to be produced by differentiated HSG cells. Matrigel contains multiple extracellular matrix proteins, however, laminin-1 was identified as the major matrix component that induced HSG cell acinar development and cytodifferentiation. Antibodies against specific components of Matrigel and against cell surface adhesion molecules were added to cells in culture to identify components important for HSG cell acinar differentiation. Immunostaining of HSG cell acini identified TGF-β2 and β3 as the predominant isoforms within the cells. Neutralizing antibodies directed against TGF-β3 significantly decreased (P≤0.0002) the size of acini formed. These results indicate that multiple components, including laminin-1 and TGF-β3, contribute to HSG cell acinar development. This model system will be useful to study acinar differentiation and salivary gland-specific protein expression in vitro.

Key words: HSG cell, Acinar differentiation, Matrigel, Laminin-1, Basement membrane, TGF-β3, Salivary gland

INTRODUCTION
There are no well established cell models to study salivary acinar cell differentiation or function and little is known about the factors responsible for the initiation and maintenance of these processes despite the number of immortalized salivary gland cell lines described (Prasad, 1994; Patton and Wellner, 1993). Maintenance of the acinar phenotype in primary salivary gland cell cultures has also proved difficult (Redman and Quissell, 1993). A critical factor required for maintaining an acinar phenotype in primary cells is the presence of an extracellular matrix (Oliver et al., 1987; Durban, 1990). Production of acinar cell-specific salivary proteins, such as salivary cystatin, have been used to identify acinar cells in vitro (Ball, 1993). Salivary cystatins are a family of cysteine proteinase inhibitors with 5 isoforms, approximately 14 kDa in size, secreted primarily by the acinar cells of submandibular glands (Dickensen et al., 1993; Bobek et al., 1991; Kousvelari and Tabak, 1991).

The immortalized human submandibular gland cell line (HSG) is derived from intercalated ductal cells (Shirasuna et al., 1981). During salivary gland development, intercalated ductal cells are believed to be the pluripotent stem cells that give rise to acinar and myoepithelial cells (Eversole, 1971). When HSG cells are cultured on a basement membrane extract, morphologic changes and cytodifferentiation occur (Royce et al., 1993).

Basement membranes are thin extracellular matrix layers separating epithelial cells from the underlying connective tissue and are found directly adjacent to salivary acinar cells in vivo (Skalova and Leivo, 1992). Many of the components in basement membranes are biologically active (Kleinman et al., 1982, 1986). Laminin-1, an 800 kDa glycoprotein, is one of the major constituents of basement membranes and of Matrigel, a tumor derived basement membrane extract (Timpl et al., 1979; Timpl and Brown, 1994). Laminin-1 polymerizes to form a three-dimensional matrix within which other basement membrane components bind or interact (Yurchenco et al., 1992). In a variety of biological systems, laminin-1 promotes cellular adhesion, migration, differentiation, proliferation, neurite outgrowth, and tumor growth (Hoffman and Kleinman, 1996; Malinda and Kleinman, 1996). Studies of mammary epithelial cell differentiation on either laminin-1 or Matrigel, have determined that laminin-1 in cooperation with lactogenic hormones regulates tissue-specific gene expression (Streuli and Bissel, 1990; Streuli et al., 1995, 1991).

Basement membranes and Matrigel also contain various growth factors and TGF-β (Vukicevic et al., 1992). Three mammalian TGF-β isoforms, TGF-β1, β2 and β3, have been identified (Kingsley, 1994; Hogan et al., 1994) and have regu-
The growth of HSG cells cultured on plastic, Matrigel or laminin-1 was studied using a non-radioactive method to determine cell proliferation (Cell Titer 96™, Promega, Madison, WI), according to the manufacturer’s instructions. In a separate assay, cells were aliquoted into two tubes and separately labeled with either a green or a red fluorescent vital dye (Cell linker PKH26 or PKH2, Sigma, St Louis, MO). Labeled cells were plated on opposite sides of a culture dish containing laminin-1 and incubated at 37°C for 48 hours during which acini formed. At this low initial cell density (5x10^3 cells of each color/0.32 cm^2 well) individual cells were not touching each other. Double exposure photographs were taken with a fluorescence microscope to visualize both labels and determine whether acinar formation involved cell migration.

**Immunohistochemistry and staining**

Cells were fixed in 3.7% formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Apoptosis was detected using a peroxidase ApopTag™ kit (Oncon, Gaithersburg, MD) according to the manufacturer’s instructions. The acinar cell-specific proteins, salivary cystatin and α-amylase, were detected in cells cultured for 24-48 hours. Acini were collected and immobilized on microscope slides using a Cytospin centrifuge, fixed with 3.7% formaldehyde for 10 minutes and permeabilized by treatment with ice-cold methanol for 10 minutes. Cells were blocked by overnight incubation at 4°C in blocking buffer (10% goat serum, 0.2% BSA and 0.075% saponin) and stained with either a polyclonal anti-cystatin antibody (GS2), or a pre-immune control (pre-immune GS2), provided by Dr A. Aguirre, SUNY Buffalo, NY, both at a 1:400 dilution in blocking buffer for 2 hours at room temperature. Cells were incubated for 1 hour with a 1:1,000 dilution of fluorescein-labeled secondary antibodies in blocking buffer, washed and immunofluorescence was detected using a confocal laser scanning microscope (Leica, Heidelberg, Germany). Paraffin embedded sections of the HSG cells cultured on Matrigel were immunostained with isofrom specific antibodies directed against TGF-β1 (Flanders et al., 1989), TGF-β2 (Flanders et al., 1990) and TGF-β3 (Flanders et al., 1991) followed by peroxidase staining as previously described (Flanders et al., 1989). All TGF-β antisera were gifts from Dr A. B. Roberts, NCI, NIH, Bethesda MD.

**Inhibition of acinar formation**

The effect of antibodies against laminin-1 (polyclonal rabbit anti-serum #72, described by Grant et al., 1989), pan-specific TGF-β (R and D Systems, Minneapolis, MN), TGF-β1, TGF-β2 (Danielpour et al., 1989) and TGF-β3 (Flanders et al., 1991) on acinar formation was determined. ICAM-1 (clone 8H10), α5 integrin (clone GoH3, both AMAC Inc., Westbrook, ME), human E-cadherin (clone AE1, a gift from Dr W. Kim, NIDR, NIH), and the integrin subunits β1 (clone m13), α2 (clone m13, gifts from Dr S. K. Akiyama, NIDR, NIH), αc (clone P1E6), αc (clone P1B5), αc (clone P4G9), αc,β1 (clone P1F6, all from Life Technologies Inc.) and αV (clone L230, ATCC, Rockville, MD), were used. Non-immun rat and mouse ascites and non-immune turkey and rabbit serum were used as negative controls. Antibodies were used at a range of dilutions (1:10, 1:20, 1:100, 1:200). Recombinant latency-associated peptide (LAP, 1.6-16 μg/ml) which binds and inactivates all three TGF-β isoforms was also added (R and D Systems). Cytochalasin D (1-20 μg/ml) and cycloheximide (0.02-20 μg/ml ICN Biomedicals, Inc., Irvine, CA) were added to the culture medium to determine whether microtubule reorganization or protein synthesis were required for acinar formation. After 24 hours cells that were not forming acini were replated in fresh medium to determine whether the cytochalasins D or cycloheximide concentrations were cytotoxic. The minimum cycloheximide concentration to inhibit more than 90% of protein synthesis was 2 μg/ml, which inhibited acini formation but was not cytotoxic. Three fields/well were assayed, in triplicate wells (70-150 acini total) and all experiments were performed at least three times.

**Immunoblot analysis**

Whole cell lysates of cells cultured on plastic or a laminin-1 gel were
prepared by sonicating the cells briefly in a hypotonic lysis buffer containing: phosphate buffer (pH 7.0), 20 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM ABESF and 0.36 mM pepstatin. The lysate was clarified by centrifugation in a microfuge (Eppendorf 5415C) at 14,000 rpm for 10 minutes at 4°C. The protein concentration of the cleared lysate was measured using the microplate BCA protein assay (Pierce, Rockford, IL). Equivalent protein samples (20 μg) were separated on a 16% polyacrylamide gel containing SDS. Proteins were transferred to nitrocellulose filters which were blocked by incubation in 5% skim milk in PBS containing 0.1% Tween-20 (SM-PBS-T) for 1 hour at room temperature. Identical blots were incubated with either the anti-cystatin antibody (GS2) or the pre-immune GS2 antibody at a dilution of 1:7,500 in SM-PBS-T for 2 hours at room temperature. The primary antibodies were detected with a peroxidase-labeled goat anti-rabbit secondary antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD), at a dilution of 1:1,500 in SM-PBS-T for 1 hour at room temperature. Identical blots were incubated with either the anti-cystatin antibody (GS2) or the pre-immune GS2 antibody at a dilution of 1:7,500 in SM-PBS-T for 2 hours at room temperature. The primary antibodies were detected with a peroxidase-labeled goat anti-rabbit secondary antibody (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD), at a dilution of 1:1,500 in SM-PBS-T for 1 hour at room temperature. Identical blots were incubated with either the anti-cystatin antibody (GS2) or the pre-immune GS2 antibody at a dilution of 1:7,500 in SM-PBS-T for 2 hours at room temperature.

The antibodies were used at dilutions ranging from 1:20 to 1:200 and were added to the culture medium when the cells were plated on laminin-1 gels or Matrigel. The effect of the antibodies on the size of acini formed at 48 hours was determined and compared with serum or antibody negative controls.

![Image](image_url)

Fig. 1. HSG cells form discrete spherical structures when cultured on either Matrigel or a laminin-1 gel. (A) Cells cultured on plastic grow as an epithelial monolayer. (B) Cells cultured on either a laminin-1 gel (shown here) or Matrigel form three-dimensional structures within 24 hours. (C) Acinar formation on laminin and Matrigel involved cell proliferation at a similar rate to cells cultured on plastic. Cells were photographed with Hoffman Optics. Bar, 50 µm.

### RESULTS

**HSG cells form acinar structures on Matrigel**

We first tested the effects of various substrates including Matrigel, laminin-1, type I collagen and plastic on HSG cell acinar differentiation. HSG cells cultured on plastic form a cobblestone-like epithelial monolayer (Fig. 1A). HSG cells form discrete spherical structures when cultured on either Matrigel or a laminin-1 gel (Fig. 1B), with acinar formation observed within 24 hours. Acinar formation on laminin and Matrigel involved cell proliferation at a similar rate to cells cultured on plastic (Fig. 1C). Discrete spherical acini did not form on type I collagen gels, but rather cells grew as a monolayer on top of the gel. In loosely formed or floating type I collagen gels, HSG cells spread along the collagen fibrils (data not shown).

We next studied how the HSG cells form acini on either Matrigel or laminin-1 gels. As acini develop (Fig. 2A), the cell nuclei become polarized, a central lumen forms and there is an increase in the amount of cytoplasm relative to the nuclear area. The initial cell plating density affected the size and morphology of acini that formed. Cells plated at higher cell densities (>1.5×10^4 cells/0.32 cm^2) formed large multi-acinar aggregates within 24 hours. These multi-acinar structures contain polarized cells, multiple lumens and duct-like structures. When lower cell densities (10^4 cells/0.32 cm^2) were used, more uniform sized acini developed in a reproducible manner that could be easily quantitated. The use of an acinar size of 800 µm^2 was chosen because at 48 hours cells plated at lower cell densities (10^4 cells/0.32 cm^2) formed acini with a size comparable to those formed at higher cell densities.

**Table 1. Factors that alter HSG acinar formation**

<table>
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<tr>
<th>Antibodies added to culture medium directed against:</th>
<th>Result</th>
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<tr>
<td>TGFβ-pan-specific</td>
<td>Decreases size of acini*</td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>No effect†</td>
</tr>
<tr>
<td>TGFβ-2</td>
<td>No effect ‡</td>
</tr>
<tr>
<td>TGFβ-3</td>
<td>Decreases size of acini</td>
</tr>
<tr>
<td>Laminin-1 (#72)</td>
<td>Alters acini formation‡</td>
</tr>
<tr>
<td>β1 Integrin (clone m13)</td>
<td>Decreases size of acini</td>
</tr>
<tr>
<td>α2 Integrin (clone P1E6)</td>
<td>No effect</td>
</tr>
<tr>
<td>α3 Integrin (clone P1B5)</td>
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</tr>
<tr>
<td>α4 Integrin (clone P4G9)</td>
<td>No effect</td>
</tr>
<tr>
<td>α6 Integrin (clone m16)</td>
<td>No effect</td>
</tr>
<tr>
<td>α6 Integrin (clone GoH3)</td>
<td>Decreases size of acini</td>
</tr>
<tr>
<td>αv Integrin (clone L230)</td>
<td>No effect</td>
</tr>
<tr>
<td>αvβ5 (clone P1F6)</td>
<td>No effect</td>
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<tr>
<td>E-cadherin (clone AE1)</td>
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</tr>
<tr>
<td>ICAM-1 (clone 84H10)</td>
<td>No effect</td>
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<tr>
<td>Other factors added to culture medium:</td>
<td></td>
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<tr>
<td>Cyclheximide ≥2.0 µg/ml§</td>
<td>Decreases size of acini</td>
</tr>
<tr>
<td>Cytochalasin D ≥2.5 µg/ml</td>
<td>Decreases size of acini</td>
</tr>
<tr>
<td>Latency associated peptide (LAP) ≥8 µg/ml</td>
<td>Decreases size of acini</td>
</tr>
</tbody>
</table>

*Decreases size of acini - a decrease in the number of acini >800 µm^2 (see Fig. 7C).
†No effect - similar to the serum or antibody control (see Fig. 7B).
‡Alters acini formation, the antiserum resulted in large cell aggregations.
§2.0 µg/ml of cycloheximide inhibited protein synthesis by 90% as determined by [35S]methionine uptake.

The antibodies were used at dilutions ranging from 1:20 to 1:200 and were added to the culture medium when the cells were plated on laminin-1 gels or Matrigel. The effect of the antibodies on the size of acini formed at 48 hours was determined and compared with serum or antibody negative controls.
contain about 50% of the acini either smaller or larger than 800 µm². This allowed us to determine whether agents added to the assay resulted in either a decrease or an increase in acinar size. Three examples of acini with an area of approximately 800 µm² are indicated by arrows in the 48 hour time point. An acinus containing a cell within its lumen with a condensed, fragmented nuclei is marked, with an asterisk (+) above it, in the 72 hour time point. Bar, 50 µm. (B) HSG cell acini increase in size with time. Cells cultured on Matrigel were fixed and stained with Diff-Quik™. The area of individual acini measured as described in Materials and Methods. The number of acini greater (■) or less (□) than 800 µm² are shown in a bar graph. Each bar represents 3 fields measured/well, and 3 wells for each time point (70-150 acini). The s.e.m. are indicated.

Cycloheximide was added to the culture medium to determine whether acinar formation on Matrigel involves protein synthesis. At concentrations greater than 2 µg/ml, cycloheximide inhibited acinar formation (Table 1). Cytochalasin D also inhibited acinar formation at concentrations greater than 2 µg/ml indicating that cell division and/or microfilament reorganization was required (Table 1). Treatment of HSG cells with concentrations of 2 µg/ml of either cytochalasin D or cycloheximide were not cytotoxic as cells were still viable after these treatments.

Acinar formation was not observed if cells were cultured on a matrix coated dish that did not form a three-dimensional gel, or if cells were cultured under a layer of matrix. Therefore, induction of three-dimensional acinar structures requires cells

**Fig. 2.** HSG cells form acinar structures which enlarge with time when cultured on either a laminin-1 gel or Matrigel (shown here). (A) HSG cells were cultured on plastic (time 0 hours) or Matrigel for 24, 48 and 72 hours. Cells were fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Acini with an area of approximately 800 µm² are indicated by arrows in the 48 hour time point. An acinus containing a cell within its lumen with a condensed, fragmented nuclei is marked, with an asterisk (+) above it, in the 72 hour time point. Bar, 50 µm. (B) HSG cell acini increase in size with time. Cells cultured on Matrigel were fixed and stained with Diff-Quik™. The area of individual acini measured as described in Materials and Methods. The number of acini greater (■) or less (□) than 800 µm² are shown in a bar graph. Each bar represents 3 fields measured/well, and 3 wells for each time point (70-150 acini). The s.e.m. are indicated.

**Fig. 3.** Apoptosis occurs to cells within the lumen of developing acini in vitro. Cells undergoing apoptosis were identified with a peroxidase ApopTag™ kit, and counterstained with methyl green. (A) Negative control, no TdT enzyme. (B) Apoptotic cells appear dark brown after staining.

**Fig. 4.** Acinar formation includes cell migration. (A) HSG cells cultured as a monolayer on plastic were independently labeled with either a green (cell linker PKH26) or red (PKH2) fluorescent vital stain. Labeled cells were plated on opposite sides of a culture dish containing laminin-1 and incubated at 37°C for 48 hours during which acini formed. At the initial low cell density individual cells were not touching each other. (B) Cells on either side of the dish formed all red or all green acini (shown in the first two examples), whereas cells in the center of the dish had migrated together forming acini made up of both red and green cells (shown in the second two examples).
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Plated on a three-dimensional matrix of either Matrigel or laminin-1; however, a three-dimensional type I collagen gel did not induce acinar formation.

Apoptosis occurs to cells within the lumen

Cross sections of HSG cell acini revealed that some cells forming the acini were undergoing mitosis and some cells located within the lumen had small condensed fragmented nuclei, suggesting apoptosis was occurring (Fig. 2A, 72 hour time point). Staining with ApopTag™, an in situ apoptosis detection kit, revealed that cells within the lumen were undergoing apoptosis (Fig. 3). These apoptotic cells were generally not in contact with the basement membrane and their loss may contribute to lumen formation.

Acini formation involves migration and proliferation

In order to determine whether acini formation involved cell migration and/or cell proliferation, we added two independently labeled cell populations to opposite sides of a culture dish containing laminin-1. Cells on opposite sides of the dish formed entirely red or green acini. In the middle of the dish, however, cells migrated together to form acini made up of both green and red cells (Fig. 4). Time lapse video microscopy also confirmed that cells migrated on the matrix to form acini (data

Fig. 5. HSG cell acini produce salivary cystatin, a marker of functional acinar differentiation. (A) Cells cultured on plastic or a laminin-1 gel were immunostained with a salivary cystatin antiserum (upper panels), or a pre-immune serum control (lower panels). Immunostaining was detected by taking 1 μm optical sections with a laser scanning confocal microscope. Bar, 10 μm. (B) Immunoblot analysis of salivary cystatin protein production by HSG cells cultured on a laminin-1 gel. HSG whole cell lysates (20 μg protein/lane) were separated by SDS-PAGE, transferred to a filter and incubated with a salivary cystatin antiserum. Lane 1, whole human saliva, a positive control for cystatin which contains multiple isoforms of similar size. Lane 2, HSG cells cultured on plastic. Lane 3, HSG cells cultured on laminin-1 for 2 hours. Lane 4, HSG cells cultured on laminin-1 for 6 hours. Lane 5, HSG cells cultured on laminin-1 for 24 hours. The predicted molecular mass for cystatin is 14 kDa. Markers in kDa are shown on right.

Fig. 6. Immunostaining for TGF-β isoforms reveals TGF-β2 and TGF-β3 are the predominant isoforms present within differentiated HSG cells. TGF-β1 immunostaining (shown) is similar to background serum controls (not shown). HSG cells were cultured on plastic (time 0) and either Matrigel (shown here) or a laminin-1 gel, for 24, 48 and 72 hours. Cells were fixed, paraffin embedded, sectioned, immunostained for the indicated TGF-β isoforms with peroxidase detection and counterstained with methyl green. Bar, 50 μm.
not shown). Histologic sections of the acini revealed that cells in the acini were undergoing mitosis, which was confirmed by cell proliferation assays with an MTS cell proliferation assay (Fig. 1C). Cells growing on either Matrigel or a laminin-1 gel proliferate at a similar rate than cells on plastic. We conclude that HSG cell acinar formation on either Matrigel or laminin-1 involves both cell migration and proliferation.

**HSG cells produce cystatin, a marker of functional differentiation**

Cells were analyzed by indirect immunofluorescence for amylase and cystatin proteins to determine whether cells in the acini are functionally differentiated, i.e. producing salivary proteins. HSG are derived from the submandibular gland, and although amylase is not an abundant secreted protein from the submandibular gland, it has been reported previously (Royce et al., 1993) that HSG cells produce amylase when cultured on Matrigel. We made a similar observation using indirect immunofluorescence to reveal amylase protein in HSG cell acini (data not shown). The salivary gland cysteine protease inhibitor, cystatin, is a salivary gland-specific protein abundant in submandibular gland saliva and was therefore selected as another potential marker of functional differentiation. Indirect immunofluorescence revealed cystatin proteins were present in the cytoplasm of cells within acini, but were not detected in HSG cells cultured on plastic (Fig. 5A). Positive immunostaining with the cystatin antibody was a novel finding, as no other salivary gland cell line has been reported to produce cystatin. Immunoblot analysis of cell lysates with the cystatin antiserum identified a 14 kDa protein band, the expected molecular mass of cystatin, which was induced when the cells differentiated on laminin-1 (Fig. 5B). Cross reactive bands of a slightly higher molecular mass were seen in the lysate of cells cultured on plastic (Fig. 5B, lane 2). The identity of these cross reactive species is not known. They may be an unprocessed form of the cystatin protein.

**Inhibition of acinar formation**

Antibodies to known Matrigel components and to cell surface adhesion molecules were added to the culture medium in order to identify components of Matrigel and their cellular receptors which may be involved in acinar formation (Table 1). Anti-functional antibodies against the β1 and α6 integrin subunits, E-cadherin and TGF-β all decreased the size of the acini formed (a representative example is shown in Fig. 7A and C) whereas anti-functional antibodies against α2, α3, α4, α5, αv and αvβ5 integrin subunits and ICAM-1 had little or no effect on acinar size compared with the serum or antibody controls (a representative example is shown in Fig. 7A and B). Polyclonal antiserum to laminin-1 resulted in what appeared to be increased cell-cell interactions with large cell aggregates forming. We conclude that both cell-cell and cell-matrix interactions are involved in acinar formation and that TGF-β3 may also have an important role.

**TGF-β in acinar differentiation**

The role of TGF-β in acinar formation was further investigated. Histological sections of HSG acini were immunostained for TGF-β isoforms (Fig. 6). The predominant isoforms present within both differentiated and undifferentiated cells are TGF-β2 and β3. TGF-β1 immunostaining (Fig. 6) was similar to the background control which was therefore not shown. Staining for TGF-β2 in the cytoplasm of HSG cells occurred at all time points shown, whereas TGF-β3 staining appears to increase over the first 24 hours when cells are cultured on Matrigel, and is maintained over the 72 hours. The intensity of immunostaining within cells on Matrigel decreases for both TGF-β2 and β3 at 48 and 72 hours as compared with 24 hours. This decreased staining may be a result of the increased cytoplasm area visible in the acinar sections as cells polarize.

When neutralizing TGF-β antibodies were added to the culture medium (Table 1 and Fig. 7), the pan-specific polyclonal antibody and the anti-TGF-β3 antibody resulted in a significant decrease (*P<0.0001 and *P<0.0002, respectively) in the size of acini formed when compared to controls. Antibodies to TGF-β1 and β2 had no effect on acinar size and were identical to serum controls. LAP binds and inactivates all three TGF-β isoforms. The addition of LAP to the culture medium had an effect identical to the pan-specific TGF-β antibody (Fig. 7) and inhibited acinar formation, thus confirming the role of TGF-β in acinar formation. We conclude that while both TGF-β2 and
β3 are present within HSG cells during acinar differentiation only TGF-β3 is necessary for acinar differentiation.

**DISCUSSION**

The purpose of this study is to develop an in vitro model system using HSG cells cultured on growth factor-reduced Matrigel, to study salivary acinar cell differentiation and to identify factors important in HSG cell acinar cell differentiation. The process of salivary gland acinar cell differentiation is not understood. Suitable cell model systems have not been available to study salivary acinar cell differentiation; therefore, efforts to characterize salivary specific gene expression and protein secretion have been difficult. Attempts to dissociate and maintain primary cultures of ductal and acinar cells from salivary glands has met with some success (Oliver et al., 1987; Durban, 1990) but long term culture of acinar cells has not been successful. Acinar cells de-differentiate from a secretory phenotype and lose their characteristic morphology when placed in culture.

In humans, serous acinar differentiation begins in utero while mucous acinar differentiation occurs postnatally (El-Mohandes et al., 1987). During gland development, acinar cells are thought to arise from the intercalated ductal cells. The HSG cell line was derived from intercalated ductal cells of an irradiated submandibular salivary gland. HSG was initially described as a pluripotent cell type that could be chemically induced to express markers of a myoepithelial or acinar cell type (Sato et al., 1987). The cell line did not express secreted salivary proteins when cultured on plastic, but when cultured on basement membrane Matrigel, HSG cells displayed morphological changes and cytodifferentiation (Royce et al., 1993). We have further developed and characterized this model system to study acinar cell differentiation.

Our study has focused on growth factor-reduced Matrigel and laminin-1, which we initially observed were more effective than regular Matrigel at promoting acinar formation. Regular Matrigel as used by Royce et al. (1993), provides a firm substrate on which HSG cells form networks, then ducts, and within 72 hours, formed acinar-like structures which produced amylase. At the concentrations used in our studies growth factor-reduced Matrigel (2.7 mg/ml) and laminin-1 (2.4 mg/ml) form soft gels on which HSG cells form discrete acinar structures within 24 hours that develop to a size similar to normal human submandibular gland acini (El-Mohandes et al., 1987). Diluting regular Matrigel to the same protein concentration as growth factor-reduced Matrigel and laminin-1 results in some acinar formation. However, diluted regular Matrigel is not as effective as the latter substrates at promoting acinar formation and many cells grow in a monolayer. Although the physical properties of the matrix may promote acinar differentiation, the presence or absence of certain factors may also be important.

We used time lapse video microscopy to characterize the HSG cell acinar formation and preliminary observations revealed cells migrating together to form acini. Cell migration was demonstrated by combining two fluorescently labeled cell populations. Acinar formation also involved cell proliferation which is consistent with salivary glands where acinar cells continue to divide (Redman, 1987). With prolonged culture (>48 hours), individual acini appear to form buds creating multi-lobular acini, making quantitation of acinar size difficult. Individual acinar formation was also dependent on the initial cell density. For simplicity of quantitation we selected a cell density (10,000 cells/0.32 cm² well), that promoted single acini to form.

Apoptosis occurred in cells within the lumen of the developing acini. Apoptosis has not been reported in the developing salivary gland. We are presently investigating whether apoptosis occurs within the developing mouse submandibular gland. The apoptosis observed in this experimental system may be a result of the cells losing contact with the matrix when they become internalized in the developing acini. Apoptosis as a result of loss of contact with the extracellular matrix has been reported in other epithelial cell systems (Boudreau et al., 1995; Dirami et al., 1995).

The significant finding that HSG cells produce salivary cystatins when they differentiate on either Matrigel or a laminin-1 gel was confirmed by immunostaining and by immunoblot analysis. Submandibular gland acinar cells produce saliva which contains abundant cystatin proteins (Bobek et al., 1991). HSG cells produce amylase when cultured on Matrigel. Amylase is also produced by other tissues, is an abundant serum protein and is also present in Matrigel, whereas salivary cystatins are a salivary gland-specific family of proteins not found in serum or Matrigel. Antibodies to salivary amylase cross react with serum amylase, making interpretation of experimental results difficult because there is serum in our culture system. The cystatin antibodies used did not cross react with serum, Matrigel or laminin-1 (data not shown).

Neutralizing antibodies against the β1 and α6 integrin subunits and E-cadherin all decreased the size of acini formed (Table 1) in a similar way to the anti-TGF-β3 antibodies shown in Fig. 7A and B, suggesting that both cell-cell and cell-matrix interactions are involved in HSG cell acinar differentiation on laminin-1. For the purpose of this report we focused on the role of TGF-β in acinar differentiation. Based on embryonic gene expression patterns, TGF-β isoforms have different developmental functions (Millan et al., 1991) but the role of TGF-β isoforms in salivary gland acinar differentiation has not been determined. In situ and northern hybridization of RNA from various tissues has shown that all three isoforms are expressed in the developing murine salivary glands (Miller et al., 1987; Millan et al., 1991). Our own studies of TGF-β mRNA expression in the developing mouse submandibular gland by in situ analysis showed an increase in staining in newborn mouse glands (Lee et al., 1995). We have also confirmed the observations that TGF-β2 and β3 are the predominant TGF-β isoforms present in the developing mouse submandibular glands (Hoffman et al., 1996).

Since Matrigel has been previously shown to contain TGF-β1 (Vukicevic, 1992), neutralizing antibodies to TGF-β isoforms were added to HSG cells on Matrigel to see whether TGF-β isoforms played a role in acinar differentiation. Interestingly TGF-β3 antibodies significantly decreased the number of acini >800 µm², however, the mechanism for this decrease is not understood. TGF-β1 was reported to be present in Matrigel (4.5-6.0 ng/ml) and in growth factor-reduced Matrigel (2.6-4.0 ng/ml) (Vukicevic et al., 1992), both within physiological ranges, but quantitation of other TGF-β isoforms in Matrigel.
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