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MOUSE SUBMANDIBULAR SALIVARY EPITHELIAL CELL GROWTH AND DIFFERENTIATION IN LONG-TERM CULTURE: INFLUENCE OF THE EXTRACELLULAR MATRIX

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SUMMARY

The adult mouse submandibular salivary gland provides a good model system to study gene regulation during normal and abnormal cell behavior because it synthesizes functionally distinct products ranging from growth factors and digestive enzymes to factors of relevance to homeostatic mechanisms. The present study describes the long-term growth and differentiation of submandibular salivary epithelial cells from adult male mice as a function of the culture substratum. Using a two-step partial dissociation procedure, it was possible to enrich for ductal cells of the granular convoluted tubules, the site of epidermal growth factor synthesis. Long-term cell growth over a period of 2 to 3 mo. with at least 3 serial passages was obtained only within three-dimensional collagen gels. Cells grew as ductal-type structures, many of which generated lumens with time in culture. Electron microscopic analysis in reference to the submandibular gland in vivo revealed enrichment for and maintenance of morphologic features of granular convoluted tubule cells. Reactivity with a keratin-specific monoclonal antibody established the epithelial nature of the cells that grew within collagen. Maintenance of cell differentiation, using immunoreactivity for epidermal growth factor as criterion, was determined by both cytochemical and biochemical approaches and was found to be dependent on the collagen matrix and hormones. Greater than 50% of the cells in primary collagen cultures contained epidermal growth factor only in the presence of testosterone and triiodothyronine. In contrast, cells initially seeded on plastic or cycled to plastic from collagen gels were virtually negative for epidermal growth factor. Biochemical analysis confirmed the presence of a protein with an apparent molecular weight of 6000 which comigrated with purified mouse epidermal growth factor. Epidermal growth factor was also present in detectable levels in Passage 1 cells. This culture system should permit assessment of whether modulation of submandibular gland ductal cell growth can be exerted via a mechanism that in itself includes epidermal growth factor and its receptor and signal transduction pathway.

Key words: submandibular gland; epidermal growth factor; extracellular matrix.

INTRODUCTION

The adult mouse submandibular salivary gland (SSG) is a complex, tubulo-acinar structure that synthesizes and secretes growth factors, homeostatic factors, and digestive enzymes (reviewed in 1). Thus, in addition to its role in the digestive process, the SSG seems to mediate various metabolic processes by virtue of the diversity of its biologically important products. This versatility of functional activities provides an attractive model system to study the regulation of specific gene products during normal cellular processes and abnormal disease states. Such studies require the availability of culture systems that can mimic in vivo behavior. This has been a challenging task for cells of epithelial origin as they readily lose functional activity in culture. Recent advances in the culture of epithelial cells were accomplished by the use of extracellular matrix components as culture substrata (18,37). Thus, it is now possible to obtain sustained growth and expression of the functional cell phenotype for a variety of cell types (2,7,14-16,22-24,28).

Although the culture of functionally active cells from the adult salivary glands has proven to be more difficult than for epithelial cells of other organs, some success in this direction was achieved recently. Using an improved dissociation procedure and increased oxygenation, Quissell et al. (27) demonstrated cell growth and maintenance of mucin synthesis by rat SSG acinar-intercalated duct complexes over a 4-d period of culture on collagen type I-laminin gels. Likewise, short-term growth and secretory activity of adult rat parotid acinar cells was achieved by Oliver et al. (26) with cells plated on Engelbreth-Holm-Swarm tumor-derived basement membrane matrix (19) (EHS-BM). Enhancement of cell growth and maintenance of low levels of epidermal growth factor (EGF) were

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obtained with SSG cells from midpregnant female mice cultured within collagen type I gels (35,36); however, these cultures generated primarily morphologically undifferentiated intercalated duct cells.

The present investigation describes the long-term growth and differentiation of SSG epithelial cells derived from adult male mice maintained as three-dimensional (3D) structures within collagen type I gels, singly or in combination with EHS-BM. Sexual dimorphism of the SSG is apparent in mice with the onset of testosterone secretion in the male (5). Under the influence of testosterone and thyroid hormones (32,33), and possibly adrenal steroids (12), adult male mice develop an extensive granular convoluted tubule (GCT) system and synthesize 10 to 20 times higher levels of EGF. This sex-related difference in tissue histology and functional activity together with a two-step partial dissociation procedure provided the means to enrich for and maintain differentiation of ductal cells of the GCT, the site of EGF synthesis in vivo (13).

Materials and Methods

Materials. Sources for reagents utilized in this study were as follows: Dulbecco's modified Eagle's medium (DMEM), GIBCO, Grand Island, NY; defined horse serum (HS) and fetal bovine serum, Hyclone Laboratories, Logan, UT; insulin, transferrin, selenium, triiodothyronine (T_3) and MATRIGEL, Collaborative Research, Bedford, MA; hydrocortisone (Hc), dihydrotestosterone (DHT), Trasylol, phenylmethylsulfonyl fluoride (PMSF), Dulbecco's phosphate buffered saline (PBS) and piperazine-*N*,N′-bis[2-ethane sulfonic acid](PIPES) buffer, SIGMA Chemical Co., St. Louis, MO; collagenase type II and III and fluorescein conjugated second antibodies, Cooper Biomedical/ORGANON Teknika, Malvern, PA; receptor grade mouse EGF and ^{14}C-labeled molecular weight markers, Bethesda Research Laboratories, Gaithersburg, MD; VectaStain ABC kit, Vector Laboratories, Burlingame, CA; soybean trypsin inhibitor, Boehringer Mannheim, Indianapolis, IN; glutaraldehyde, osmium tetroxide, and Araldite embedding kit (Mollenhauer), Polysciences, Warrington, PA; ^{125}I-labeled protein A, ICN Radiochemicals, Irvine, CA; and X-Omat XAR-5 X-ray film, Southwest and Johnson X-ray, Houston, TX. All reagents for electrophoresis were from BioRad Laboratories, Richmond, CA. Mouse monoclonal antibody LE61 was a generous gift from Dr. Birgitte Lane, Imperial Cancer Research Fund, London, England.

Dissociation of SSG. BALB/c male mice (3 to 5 mo. of age) were housed under conditions controlled for temperature and light and provided with water and commercial chow diet ad libitum. Immediately before removal of the SSGs, they were euthanatized by CO_2 inhalation. The SSGs were quickly dissected out and the adjacent sublingual glands were discarded. The SSGs from 15 to 20 mice were pooled, washed well with tris-buffered saline (pH 7.4) (TBS), and minced finely. SSG pieces were suspended in 25 ml of dissociation solution containing 0.16% collagenase (Collagenase type II and type III, 1:1 vol/vol), 0.5% bovine serum albumin, and 15 m*M* HEPES (pH 7.5) in DMEM. All dissociation steps were carried out at 37° C in a water bath with moderate shaking. Enrichment for GCT cells was determined empirically in preliminary experiments by collecting SSG cell fragments of different sizes after a 45-min period of dissociation and allowing them to settle by gravity at room temperature for different time intervals. It should be noted that the rationale for enriching for specific tissue components on the basis of size was used previously for the mouse mammary gland with the aid of a graded series of filters (17). For enrichment of specific SSG fragments, tissue in the process of dissociation was transferred to sterile conical centrifuge tubes. Fragments that settled within a 2-min period were resuspended in fresh dissociation solution and further dissociated for 30 min. Fragments remaining in the 2-min supernatant were allowed to settle for an additional 5 min; these fragments were also resuspended in fresh dissociation solution and further incubated for 30 min. Fragments remaining in the 7-min supernatant were allowed to settle for an additional 30 min and dissociated further as above. Cells in the last supernatant were collected by centrifugation, washed well in TBS containing 10% HS and maintained on ice for processing by immunofluorescence staining (*see* below) with the rest of the cell fractions. At the end of dissociation, the 2- and 5-min cell fractions consisted primarily of clusters which varied in size (approximately 10 to 20 cells). The 30-min and final supernatant fractions consisted of single cells and very small groups of cells (3–5). The presence of GCT cells in each of the fractions was determined by immunofluorescence staining of cytospin preparations with EGF antiserum (procedure following). The 2-min fraction was found to be most consistently enriched for EGF-positive cells, the proportion of which varied somewhat from experiment to experiment (30 to 50%). A parameter found to be important in the success of this enrichment procedure was the mincing of the tissue before dissociation. Pieces larger than 0.5 mm at the start of dissociation did not generate size subfractions within 45 min of collagenase treatment. Extended periods of collagenase treatment greatly reduced cell viability as estimated by trypan blue exclusion.

Collagen gel cultures. For all experiments in this study only cell pellets generated by further collagenase digestion of the clusters that settled by gravity within 2 min were utilized. These cell pellets were washed 4 times with TBS-10% HS and resuspended in DMEM supplemented with 15% HS, 10 μg/ml insulin, 10 μg/ml transferrin, and 10 ng/ml selenium (standard medium). As mentioned above, this cell fraction contained primarily small clusters. Attempts to dissociate these clusters further, either by digestion with collagenase or trypsin-EDTA solution, yielded a cell suspension with very low viability and growth potential. Therefore further digestion of the cell clusters in the 2-min sedimentation fraction was not undertaken. Estimations of cell numbers were made possible by suspending an aliquot of the cell stock in crystal violet solution, a procedure that releases cell nuclei (*see* below). Unless otherwise stated, cell numbers were standardized among culture dishes by seeding 10, 60-mm collagen gels from a cell pellet with a packed vol-

ume of 0.5 ml (a volume that is normally generated by the dissociation of 20 adult male mice SSGs). Collagen stock solution (0.3%) was prepared from rat-tail collagen fibers as originally described by Michalopoulos and Pitot (25). A neutralized collagen gel mixture was prepared by mixing 4 vol of stock collagen with 1 vol of a 2:1 mixture of 10× Waymouth's medium and 0.34 *N* NaOH; this mixture (pH 7.2-7.5) was kept on ice to prevent gelation. Washed cell pellets (0.5 ml packed volume) were resuspended with 40 ml of cold neutralized collagen solution and spread in 4-ml aliquots onto 60-mm tissue culture dishes. The collagen-cell mixture was allowed to gel at room temperature for approximately 10 min. Cultures were then fed with either standard medium or with hormone-containing medium (standard medium supplemented with DHT, T_3, and Hc; all 1 μg/ml). Collagen gel cultures were consistently fed every other day throughout the duration of any given experiment.

It should be noted that experiments were performed at the beginning of this project to determine optimal conditions for maintenance of long-term growth potential using as a basis the published observations of Yang et al. (35,36) with short-term SSG cell growth in collagen. The use of at least 15% HS was necessary to maintain long-term growth of SSG cells as defined in this study. Insulin, transferrin, and selenium, while not enhancing substantially the growth observed in primary cultures, did promote serial passage. HS could be substituted for fetal bovine serum. Serial passage was not possible in the serum-free medium described by Yang et al. (36) for mouse SSG cells in short-term culture. Addition of DHT, T_3, and Hc did not enhance growth significantly; DHT and T_3 did seem to be necessary for maintenance of detectable levels of EGF.

Recovery of cells from collagen gels. Collagen gels were transferred to sterile centrifuge tubes containing 10 ml of the 0.16% collagenase solution. This was followed by incubation in a 37° C water bath until completion of collagen gel digestion (approximately 15 to 30 min). The cells were washed 3 times in either standard medium (for subculture or cytocentrifugation spreading) or in TBS (for estimation of cell numbers or preparation of cell extracts).

Estimation of cell growth. Cells released from collagen gel cultures by collagenase treatment were diluted 1:10 in 9 vol of 0.1% crystal violet in 0.1 *M* citric acid. This treatment permitted estimations of actual cell numbers as it released individual nuclei from the integrity of the 3D ductal-type outgrowths. Cells growing on plastic were released with trypsin-EDTA and diluted in crystal violet solution. Stained nuclei were counted by standard procedures using a hemacytometer.

Cytocentrifugation of cells and immunofluorescence staining. Freshly dissociated cells before culture or cells released from collagen gels after various periods of culture were spread onto acetone-washed slides with the aid of a cytocentrifuge (Shandon Scientific Co., Inc.). A cushion of 3 drops of HS was placed at the bottom of each cytocentrifuge tube to help preserve cell morphology during cytocentrifugation. Centrifugation was carried out for 5 min at 700 rpm. Smears were air dried and immediately fixed with methanol:acetone (1:1) at −20° C for 5 min, washed in PBS, and incubated overnight at 4° C with rabbit antiserum to EGF diluted in PBS (1:500). Smears were washed extensively with PBS, incubated for 1 h with goat anti rabbit fluorescein conjugated IgG (affinity purified) diluted 1:20 in PBS, washed again in PBS, and mounted in buffered glycerol medium containing 2% n-propyl gallate (10). Cells growing directly on plastic were likewise fixed and processed for immunofluorescence staining.

Histology and detection of EGF by the avidin-biotin-peroxidase complex assay. SSGs or collagen gels containing cells cultured for different times were washed with TBS and fixed overnight at 4° C in Bouin's solution. Samples were dehydrated through increasing concentrations of ethanol, cleared through four changes of Dioxane over a period of 48 h, and paraffin embedded. Paraffin sections cut at 4 μm were deparaffinized, rehydrated, and washed in PBS. Endogenous peroxidase activity was blocked by treatment in a 0.6% (vol/vol) solution of hydrogen peroxide in methanol for 30 min. Sections washed in PBS were incubated with 5% goat serum in PBS for 30 min to prevent nonspecific binding of antibodies. Sections were reacted with EGF antiserum diluted 1:1000 in blocking buffer overnight at 4° C. Control sections were incubated with either preimmune serum or EGF antiserum preabsorbed with purified EGF. After three washes in PBS, sections were incubated for 1 h with 1:200 dilution of goat antirabbit serum conjugated to biotin, washed with PBS, and incubated with avidin and biotinylated horseradish peroxidase complex in blocking buffer (1:100). This was followed by a 10-min incubation in the dark with the substrate solution consisting of 1 mg/ml 3,3-diaminobenzidine in PBS and 0.03% hydrogen peroxide. After a final wash in PBS, sections were counterstained with Harris hematoxilin for 8 min, developed in acid alcohol and ammonia water, dehydrated, and mounted in Permount.

Electron microscopy. SSG fragments or collagen gel cultures were fixed in 2% glutaraldehyde in 0.1 *M* PIPES buffer overnight at 4° C, postfixed in 2% osmium tetroxide in PIPES buffer for 1 h at room temperature, dehydrated, and embedded in Araldite-PolyBed 812. Thin sections were stained with uranyl acetate and alkaline lead citrate (29) and examined in a JEOL 100CX electron microscope.

Biochemical analysis of EGF. Dissociated SSG cells before or after culture were washed in TBS supplemented with protease inhibitors (*see* below). Cells were extracted in buffer consisting of 10 m*M* Tris-HCl (pH 6.8), 8 *M* urea, 2% sodium dodecyl sulfate, and 2% mercaptoethanol. A mixture of protease inhibitors was added to this buffer immediately before extraction (Trasylol: 1%; soybean trypsin inhibitor: 0.1 mg/ml; and phenylmethylsulfonyl fluoride: 1 m*M*). Proteins in cell extracts were analyzed by electrophoresis in 15% polyacrylamide denaturing gels using the discontinuous buffer system of Laemmli (20). Samples in all lanes were normalized on the basis of protein concentration (75 μg/lane). Proteins were electrophoretically transferred to nitrocellulose filters then reacted with EGF antiserum diluted 1:100 with blocking buffer. Antigen-antibody complexes were detected with ^{125}I-labeled protein A (3).

Epidermal growth factor antiserum. Antiserum was prepared in a New Zealand female rabbit; preimmune

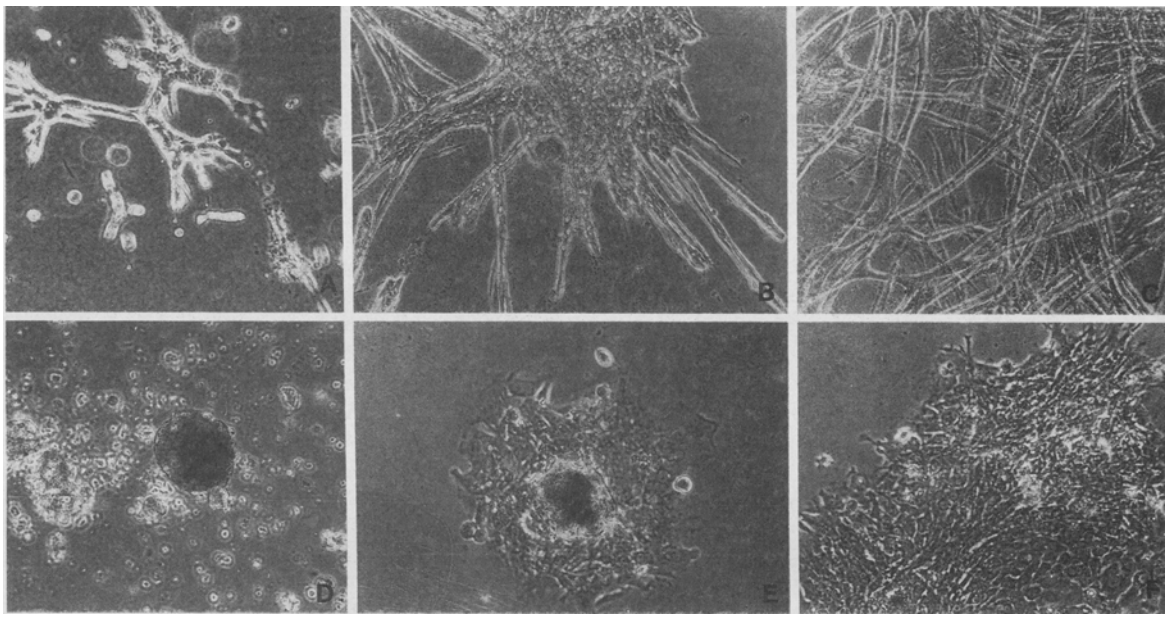

FIG. 1. Normal SSG cells from adult male mice as a function of time in primary culture. Cells at 4 d (*A*), 2 wk (*B*), and 4 wk (*C*) of seeding within collagen gels. Cells were released from the collagen by collagenase treatment after 2 wk of growth and replated on plastic dishes. Their behavior on plastic is shown at 2 d (*D*), 8 d (*E*), and 3 wk (*F*). ×68.

serum was obtained before antigenic challenge. Purified receptor grade EGF (100 μg) in 0.6 ml distilled water was mixed with equal volume of complete Freund's adjuvant and was injected intradermally on the rabbit's back. Five additional injections in incomplete Freund's were given at 2-wk intervals. Blood samples were collected on alternate weeks, and development of EGF-specific antibodies was established by both immunoblotting against purified EGF and SSG cell extracts and immunoperoxidase staining of SSG paraffin sections. The specificity of the antiserum for EGF was established by several criteria. Immunoperoxidase staining of adult SSG sections showed the reaction product only over the GCT cells and not on the surrounding acinar cells. Preabsorption of the antiserum (500 μl of a 1:20 dilution) with 50 μg purified mouse EGF abolished the reactivity over the GCT cells. Last, this antiserum did not react with other mouse tissues by either immunoblotting or immunoperoxidase staining, and preimmune serum did not react with SSG sections.

RESULTS

Growth characteristics in collagen gels and on plastic. Dissociated SSG cells were seeded within collagen gels or directly onto tissue culture plates and fed standard medium (Materials and Methods). With time in culture, cell growth leading to generation of 3D ductal-type structures was obtained (Fig. 1). Small tubular outgrowths were already visible within 4 d of seeding (Fig. 1 *A*). These structures grew quite vigorously, and by 2 wk most outgrowths had extensions radiating in all directions (Fig. 1 *B*). By 4 wk it was no longer possible to discern individual outgrowths, as illustrated by the extensive branching seen in Fig. 1 *C*. Most outgrowths maintained their intercellular contacts and remained as an integral unit after being released from the collagen gels by collagenase treatment (Fig. 1 *D*). These outgrowths attached with low efficiencies when plated onto plastic and only after 5 to 8 d in culture (Fig. 1 *E*). They did generate epithelial-like growth (Fig. 1 *F*) with no

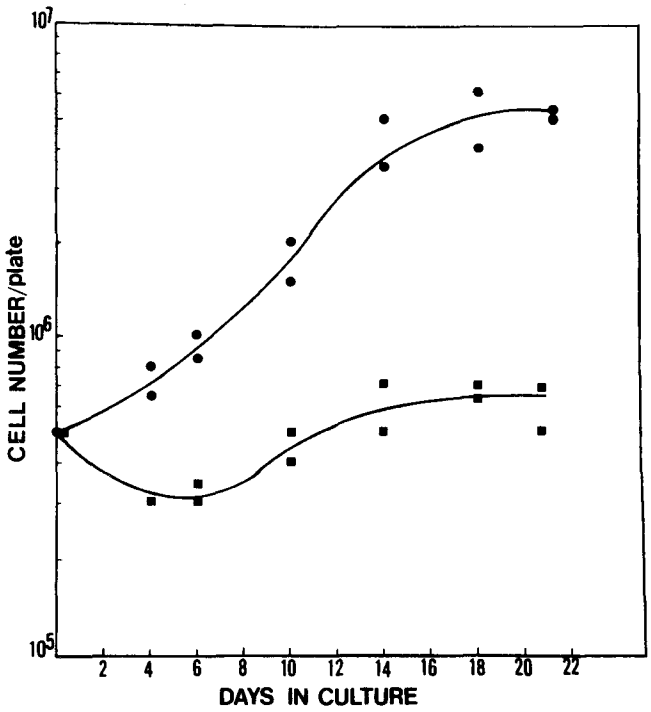

FIG. 2. Growth kinetics of SSG cells within collagen gels (*solid circles*) and on plastic (*solid squares*). Duplicate samples were taken at each time point and all samples were maintained in standard growth medium.

obvious fibroblast contamination. Although the cells on plastic could be maintained for 25 to 30 d with periodic feedings, subculture by standard techniques utilizing mild trypsin-EDTA treatment was not possible. In contrast, serial passage of cells growing within collagen gels was possible (*see* below). The growth kinetics of SSG cells within collagen gels and on plastic is shown in Fig. 2. Approximately 3 to 4 population doublings could be obtained over a 3-wk period when cells were seeded within collagen type I gels. Addition of varying concentrations of EHS tumor-derived matrix to the collagen type I gels did not significantly enhance overall cell growth (data not shown). SSG cells plated directly on plastic as primary cultures attached and grew poorly, i.e. less than 1 population doubling over a 3-wk period, despite repeated feedings. No cell growth was evident in Week 1 of seeding on plastic. This was likely to be the result of very low attachment efficiency (less than 10% at Day 4 of seeding) although cell counts of viable cells in the adherent and floater cell fractions indicated that 30% of the initial cell population remained viable. Plating cells on rather than within collagen type I gels alone or in combination with EHS-BM (10:1 ratio) did not significantly enhance the attachment efficiency. However, cell growth was better on collagen (or collagen:EHS) than on plastic, resulting in approximately 1.5 to 2 population doublings in a 3-wk period (data not shown). Inasmuch as cell growth was much better when cells were plated within collagen matrices, an approach which also avoided the problem of attachment efficiency, all subsequent experiments were carried out with cells growing as three-dimensional structures within collagen gels.

Serial passage within collagen gels. The growth potential of SSG cells within collagen gels as a function of passage number was established. Dissociated SSG cells were plated at 4×10^5 cells/gel (estimated by counting an aliquot of the cell stock with crystal violet solution; *see* Materials and Methods) and allowed to grow for 4 wk. At this time, cells were released from the gels by collagenase treatment and, after estimating total cell numbers, they were seeded once more within collagen at a density of 4×10^5 cells/gel and allowed to grow for 4 wk (Passage 1) (Fig. 3). This process was repeated twice more (Passages 2 and 3). As expected, SSG cells in primary culture

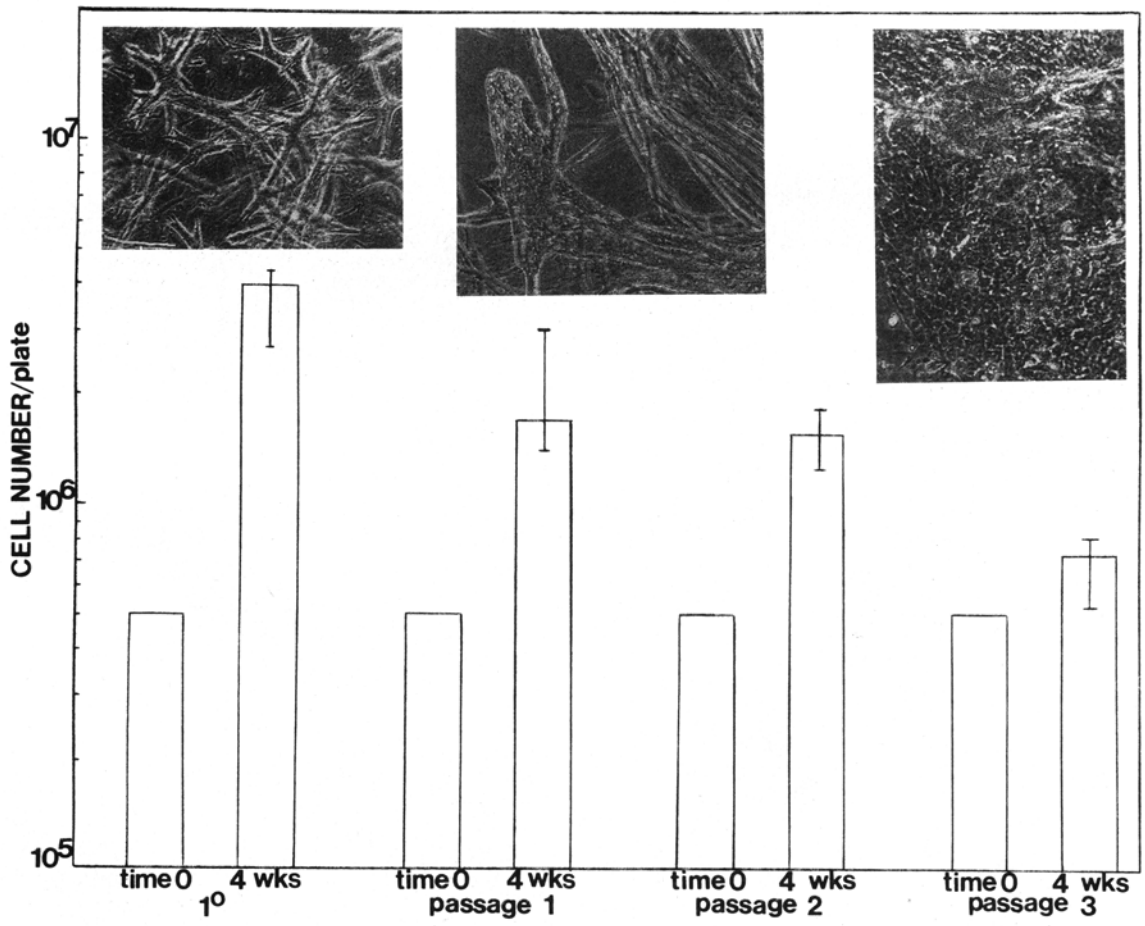

FIG. 3. Serial passage of SSG cells within collagen gel cultures. Dissociated SSG cells were plated at 4×10^5 cells/gel (time 0) and allowed to grow for 4 wk, at which time they were released from the matrix by collagenase treatment. After estimating total cell number per gel, cells were seeded within a new collagen gel at a density of 4×10^5 cells/gel (Passage 1, time 0). This process was repeated twice more (Passages 2 and 3). Cell counts at 4 wk represent the average of triplicate samples; their range is reflected by the *error bar*. Morphologic behavior within collagen at each passage is shown in the photographs above the cell count data.

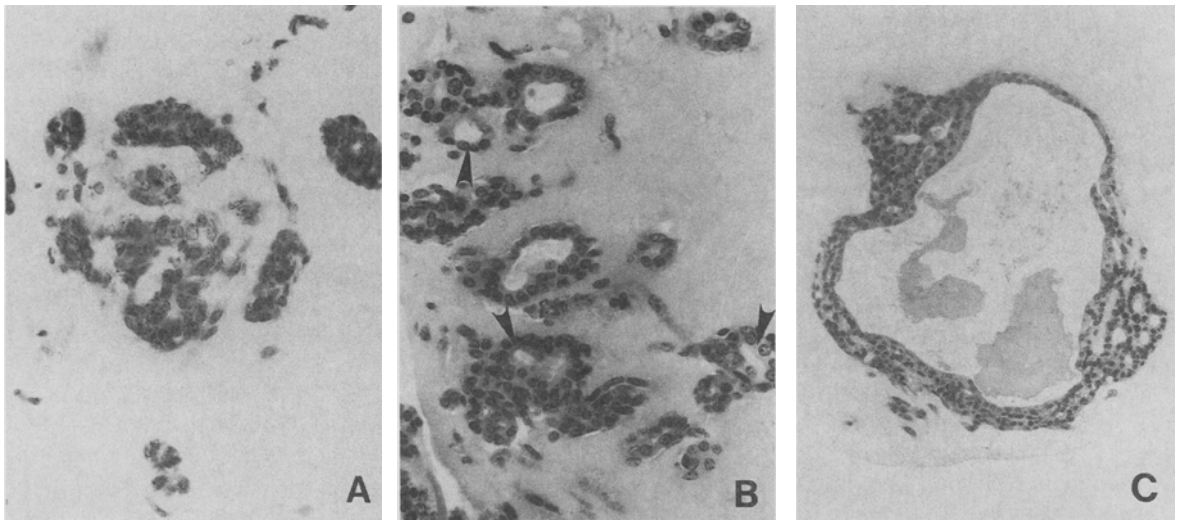

FIG. 4. Generation of 3D ductal-type outgrowths by SSG cells within collagen gels evaluated by histological analysis. Collagen gels with SSG cells at different stages of growth were processed for paraffin embedding and stained with hematoxylin and eosin. Cross sections of cells at 7 d (*A*), 14 d (*B*), and 6 wk (*C*). Formation of lumens is indicated by the *arrows* in (*B*). *A,B* = ×364; *C* = ×182.

underwent approximately 3 population doublings and generated extensive ductal branching (Fig. 3). Passage 1 and 2 cells could undergo approximately 2 population doublings and grew in the characteristic ductal-type branching. By Passage 3, however, cell growth declined. Generation of three-dimensional structures was virtually nonexistent at this passage when cells grew throughout the matrix as small flattened epithelial-like clusters (Fig. 3). Passage beyond this stage was not possible. Thus, the growth potential of normal SSG cells from adult male

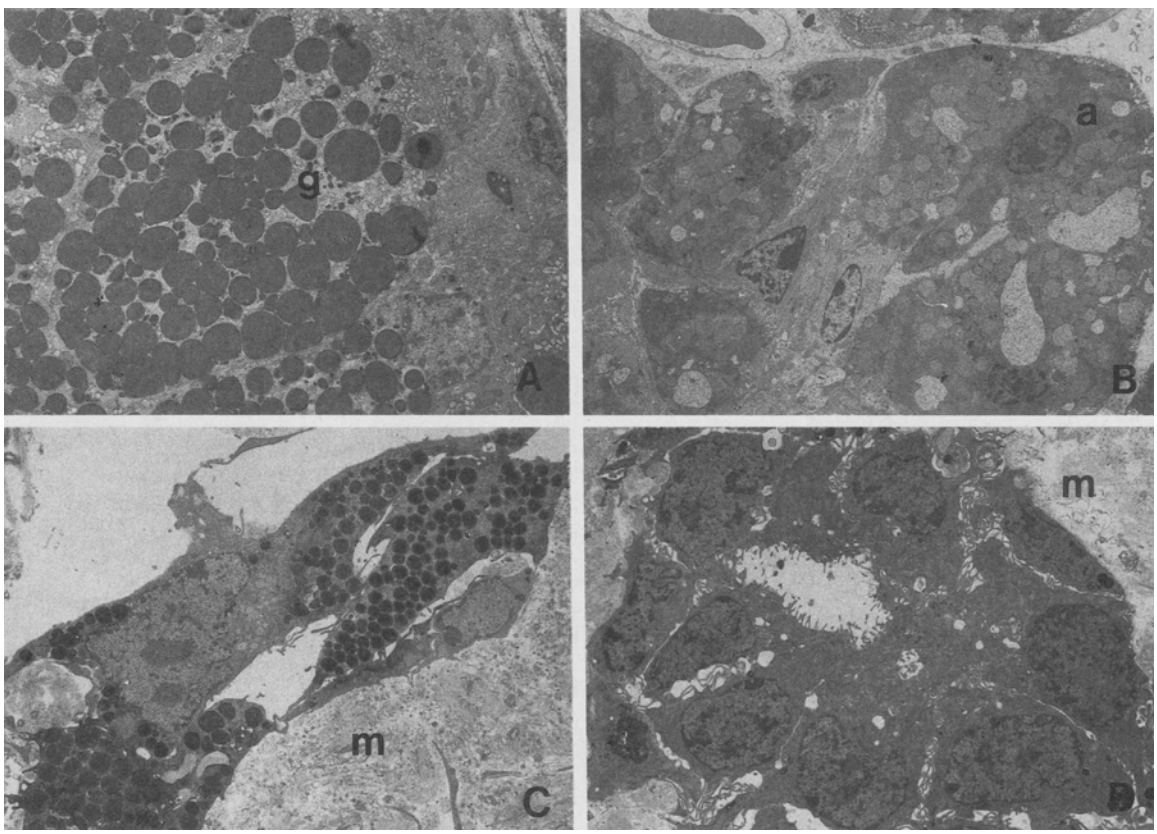

FIG. 5. Electron microscopic evaluation of SSG cells within collagen in reference to the SSG in vivo. GCT (*g*) and acinar (*a*) cells from a SSG of an adult male mouse before dissociation are shown in *A* and *B*, respectively. SSG cells after 2 wk of culture within collagen gels (*m* = collagen matrix) are shown in *C* and *D*. *Note* the high content of electron dense secretory vesicles of GCT in *A* and *C*. *A,C,D* = ×2636; *B* = ×1727.

mice was maintained for a period of 2.5 to 3 mo. within collagen gels. Importantly, no spontaneous immortalized SSG cell clones were obtained despite the fact that cultures from various passages were maintained with repeated feedings, viable yet quiescent, for 5 to 7 mo.

Morphological differentiation and keratin expression. The time course of morphogenesis of the 3D ductal-type structures was examined. Collagen gel cultures were fixed at intervals after seeding, then processed for histology. Cross-sections through the collagen gels indicated that during Week 1 cells grew throughout the matrix as irregular clusters (Fig. 4 *A*). Between Weeks 1 and 2 the formation of lumens was apparent (Fig. 4 *B*). By Week 4 at least 50% of the cell arrangements visible in any given section had lumens. Some of the lumens seemed to enlarge in cultures maintained for extended periods (5 to 7 wk; Fig. 4 *C*). Collagen cultures were also analyzed by electron microscopy. In reference to the adult male mouse SSG (Fig. 5 *A*, *B*), it was estimated that greater than 50% of the cells in 2-wk cultures (Fig. 5 *C*) had morphologic features comparable to those of cells of the GCTs (Fig. 5 *A*). Acinarlike cells resembling those of the adult male mouse SSG (Fig. 5 *B*) could not be detected in these cultures by electron microscopy. About 30% of all ductal arrangements consisted of cells that either lacked or contained few secretory granules (Fig. 5 *D*), more closely resembling cells of the intercalated and/or interlobular ducts (4).

To ascertain that the SSG cells that grow within collagen are indeed epithelial in origin, reactivity with a monoclonal antibody (LE61) that recognizes a keratin component of simple epithelia (21) was established.

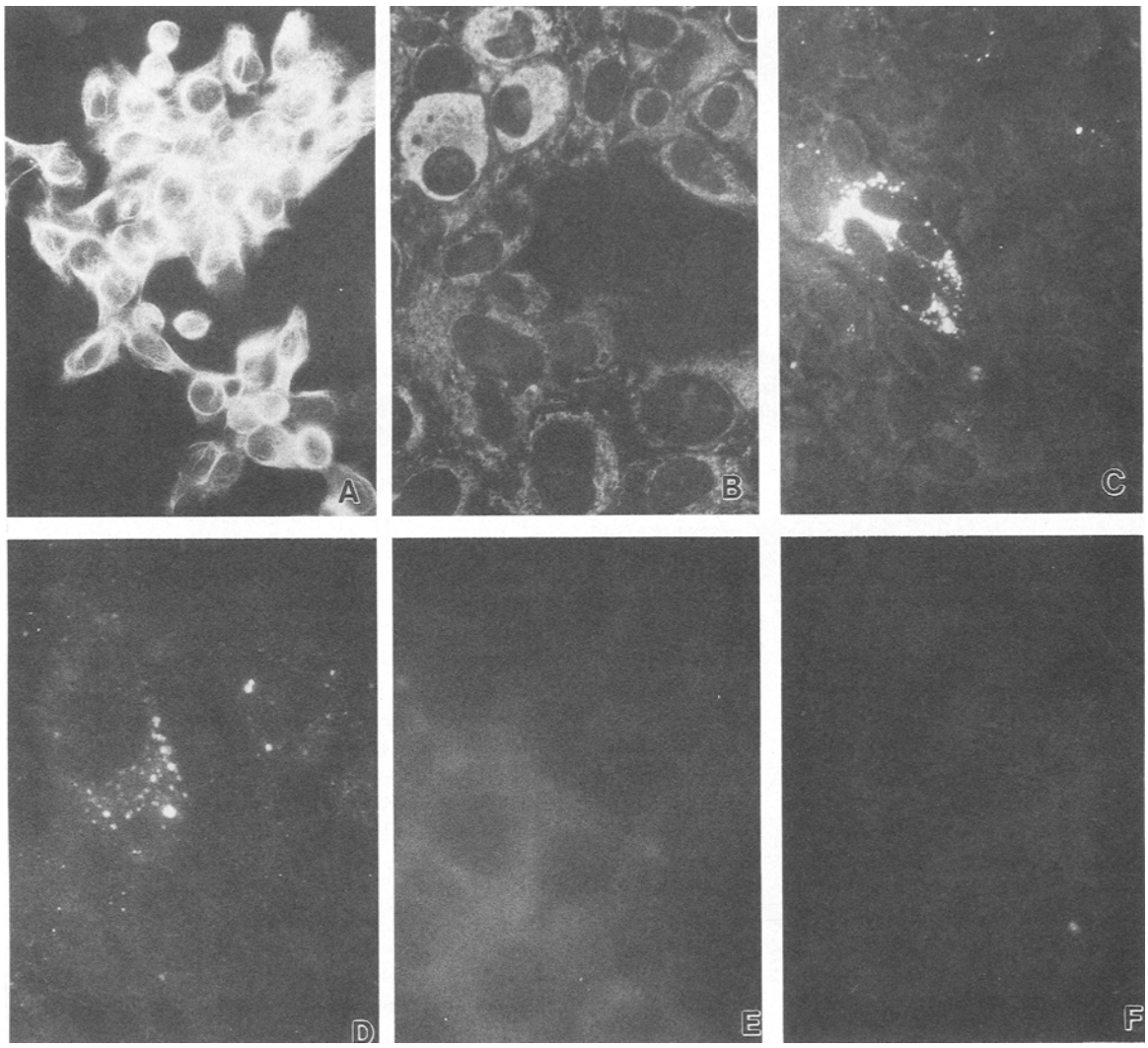

FIG. 6. Characterization of SSG cells in culture by immunofluorescence staining. *A*, cells cultured within collagen gels for 4 wk were released by collagenase treatment, plated onto slide chambers, and allowed to attach and spread for 1 wk. Almost 100% of the cells expressed a keratin component of secretory epithelia as assessed by immunoreactivity with monoclonal antibody LE61. *B-D*, cells cultured within collagen gels for 2 wk in the presence (*B,C*) or absence (*D*) of hormones were released by collagenase treatment, spread onto slides by cytocentrifugation, and reacted with antiserum to EGF. *Note* strong immunoreactivity for EGF in the presence of hormones. *E*, EGF immunoreactivity of cells grown on a plastic substratum for 2 wk. *F*, cells prepared as in *B* but reacted with preimmune serum. *A-C,F* = ×364; *E,D* = ×909.

Almost 100% of the cells recovered from collagen gels after 4 wk of culture showed positive reactivity with the keratin-specific antibody (Fig. 6 *A*). Similar results were obtained with Passage 1 and 2 cells (data not shown).

Expression of a product of cell differentiation. EGF was chosen as the marker of functional differentiation. Analysis of this SSG function was of interest because EGF accumulation in vivo is influenced by testosterone, thyroid, and possibly adrenocorticoid hormones as demonstrated by administration of hormone supplements to castrated, thyrodectomized and/or adrenalectomized mice (5,12). Maintenance and/or accumulation of EGF immunoreactive material was thus examined as a function of the culture substrata and hormones using both immunocytochemical and biochemical approaches and an antiserum specific to EGF (Materials and Methods). The number of EGF immunoreactive SSG cells in a given culture was initially analyzed by immunofluorescence staining of cytospin preparations of cells released from collagen gels. At 2 wk of culture in the presence of the hormones DHT, T_3, and Hc, approximately 50% of the cells were EGF immunoreactive (Fig. 6 *B*). Immunoreactivity was often seen to be compartmentalized to discrete cytoplasmic granules (Fig. 6 *C*). Although EGF immunoreactive cells were detectable in cultures maintained in the absence of hormones, their numbers represented only 10 to 20% of the total cell population (Fig. 6 *D*). On a plastic substratum (Fig. 6 *E*), whether in the presence of hormones or not, EGF immunoreactivity above background levels (Fig. 6 *F*) was minimal. Cells cycled from collagen to plastic lost all reactivity for EGF within 1 wk of plating in the presence of hormones (data not shown).

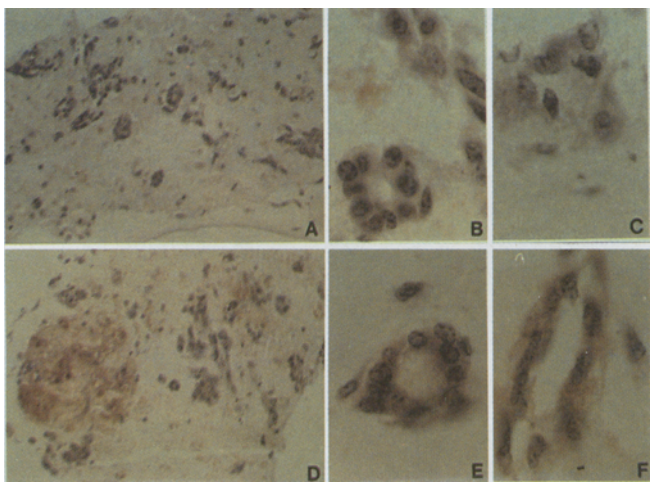

FIG. 7. EGF immunoreactivity of SSG cells in culture in situ by immunoperoxidase staining. Collagen gels with cells at 2 wk of culture were prepared for paraffin embedding, sections were reacted with antiserum to EGF, and processed by the avidin-biotin-peroxidase complex assay. Cells were maintained either in the absence (*A–C*) or the presence (*D–F*) of hormones. Brown precipitate denotes the reaction product at the site of immune complex formation. *Note* accumulation of EGF immunoreactivity in association with cell structures and with the matrix of samples maintained in the presence of hormones. *A,D* = ×154; *B,C,E,F* = ×769.

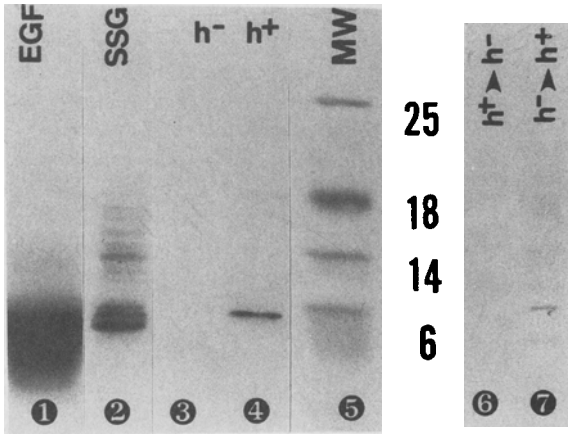

FIG. 8. Immunoblot detection of EGF in SSG cells before and after culture within collagen gels. Cell extracts were separated on 15% SDS-polyacrylamide gels and transferred to nitrocellulose; proteins were detected with antibodies to EGF and ^{125}I-labeled protein A. Control reactivities with purified mouse EGF (receptor grade, 2 μg) and with dissociated SSG cells before culture are shown in *lanes 1* and *2*, respectively. Reactivities of extracts derived from SSG cells in primary collagen gel cultures at 2 wk of seeding are shown in *lanes 3* (h^-; without DHT, T_3, and Hc) and *4* (h^-; with DHT, T_3, and Hc, 1 μg/ml). Reactivity of SSG cells grown within collagen gels for 2 wk in the presence of hormones then cycled for 3 wk to medium lacking hormones ($h^+ \rightarrow h^-$) is shown in *lane 6*. Reactivity of SSG cells cycled instead to medium containing hormones ($h^- \rightarrow h^+$) is shown in *lane 7*. ^{14}C-Labeled molecular weight (MW) markers are shown in *lane 5*.

More extensive analysis of the collagen gel cultures with respect to hormonal effects on EGF immunoreactivity was performed by immunoperoxidase staining of the cells in situ. Of particular interest was to correlate EGF immunoreactivity to the topography of the 3D outgrowths. Representative results at 2 wk of culture are shown in Fig. 7. Little or no EGF immunoreactivity was observed in the collagen gel cultures when maintained in the absence of hormones (Fig. 7 *A–C*). In contrast, EGF immunoreactive cell clusters were detectable throughout the collagen gel in the presence of hormones (Fig. 7 *D–F*). EGF immunoreactivity was often associated with ductal-type cell arrangements (Fig. 7 *E* and *F*) as well as in localized areas of the matrix (Fig. 7 *D*) most likely reflecting secretion of this growth factor. Detection of EGF immunoreactive material was also possible with Passage 1 cells where reactivity varied from 30 to 50% of the cells, depending on the experiment. However, with further passage the number of positive cells greatly decreased, regardless of the presence of hormones. Steady state levels of EGF immunoreactive proteins in primary cultures at 2 wk of seeding were examined by SDS polyacrylamide gel electrophoresis followed by immunoblotting. A protein with an apparent molecular weight (M_r) of 6000 comigrating with either purified EGF (Fig. 8 *lane 1*) or EGF in SSG cell extracts (Fig. 8 *lane 2*) was detected in collagen cultures only in the presence of hormones (Fig. 8 *lanes 3* and *4*). Attempts to modulate the detection of the 6000-M_r protein were undertaken by cycling cells from medium with hormones to medium without them, and vice versa. After 3 wk of removal of hormones, the 6000-M_r band could no longer be detected in

cell extracts of SSG collagen cultures (Fig. 8 *lane 6*). Addition of hormones to cultures initially maintained in their absence resulted in appearance of the 6000-M_r band, albeit only to a level close to the lower limit of detection of the immunoblot assay (Fig. 8 *lane 7*).

Discussion

The results of the present study demonstrate that the proliferative potential of SSG epithelial cells from adult male mice can be maintained in culture for 2 to 3 mo. with at least 2 consecutive passages if the cells are seeded within collagen type I gel matrices. The dissociation procedure used, together with the known sexual dimorphism of this organ, permitted enrichment for ductal cells of the GCT. Importantly, electron microscopic evaluation indicated morphologic features of the differentiated cells of the GCT in at least 50% of the cell population in primary culture and 30 to 50% at Passage 1. Hormonal responsiveness for expression of EGF, a marker of functional activity of this cell lineage, was found to be strictly regulated by interaction with the collagen matrix.

Methods permitting limited growth of mouse SSG epithelial cells in primary explant cultures have been described (34). Although the explants could generate epithelial outgrowths on plastic where they remained in a viable, nonproliferating state for several months (an observation confirmed with dissociated cells in the present study), serial passage was not possible. Hormonal responsiveness of the cells was not demonstrated in this early study, although protease activity was present in the first 2 wk of culture.

Studies on epithelial cell growth and differentiation in culture have been greatly facilitated by the use of collagen type I as culture substratum (37). A vast literature now exists which indicates that many different types of epithelial cells exhibit enhanced proliferative capacity and maintain functional activities when the culture substratum is collagen type I, alone or in combination with other matrix components. Yang et al. (35,36) previously showed that SSG cells from pregnant mice grow well in short-term primary collagen gel cultures maintaining low levels of EGF as measured by a radioimmunoassay. The number of EGF-positive cells was not estimated in that study and the cells seemed largely undifferentiated by electron microscopy. The present study was specifically designed to enrich for cells of the GCT. This may account for the enhanced proliferative activity that we observed as well as for our ability to modulate EGF levels in response to both hormones and the culture substratum.

Rodent cells are often assumed to immortalize spontaneously in culture. An important feature of the behavior of SSG epithelial cells as defined in the present study is that while 2 to 3 consecutive passages through the collagen matrix were possible, the cells did stop generating 3D outgrowths and eventually senesced. This is reminiscent of previous observations with another rodent glandular epithelium, the mammary gland, were intensive efforts to generate "normal" cell lines by spontaneous immortalization events have failed, with one exception (6). The observed strict regulation of SSG epithelial cell normalcy within collagen gel cultures has permitted us more recently to measure alterations in SSG cell growth as a result of introduction of activated oncogenes (9).

It is presently unclear whether mouse SSG acinar cell growth and differentiation could be supported to a significant degree by the 3D culture conditions defined here. The subfraction of dissociated cells utilized in this study selected against acinar cells. Attempts have not been undertaken yet to carefully examine the growth behavior of the other cell fractions generated by the dissociation procedure. Of interest in this context is that in previous studies where acinar cell growth and cell function were maintained in short-term cultures, the use of laminin in combination with collagen type I or other basement membrane components was necessary (26,27). Similarly, mammary epithelial cells derived from virgin mice respond to the addition of EHS-BM to collagen gels not only with enhanced cell growth but with abrogation of their serum requirement for induction of hormone-dependent cell differentiation (Durban, in preparation). In contrast, addition of EHS-BM to collagen type I gels did not enhance SSG ductal cell growth and differentiation beyond what was observed with collagen type I gels alone. It is possible therefore that the specific requirements that epithelial cells have for extracellular matrix components in culture is a reflection of in vivo behavior with respect to interactions between different cell lineages within an organ or the nature of the synthetic activities and functions of each cell lineage, or both. In this context we have recently examined both mammary and SSG-derived 3D cultures by immunocytochemical staining with antiserum to laminin. SSG ductal cells seem to be much more efficient than mammary cells at depositing laminin around the 3D outgrowths generated within collagen type I gel cultures (Barreto and Durban, unpublished observations). Perhaps it is this ability of SSG ductal cells to quickly modify the collagen culture substratum by deposition of laminin, and possible other basement membrane components, that lead to the observed lack of enhanced responsiveness to addition of a more complex extracellular matrix.

The requirement of the collagen matrix for expression of a GCT cell function was clearly illustrated by the inability of cells on plastic to accumulate detectable levels of EGF. That at least some of the EGF detected was actually synthesized de novo in culture is indicated by a) the ability to modulate its accumulation by removal or addition of hormones and b) its detection in cells derived from Passage 1 in culture. In addition, an EGF precursorlike polypeptide (135 000-M_r) as well as the smaller 6000-M_r protein have been detected in [^{35}S]-methionine-cysteine labeled cell extracts from SSG collagen gel cultures treated with hormones for 2 wk (data not shown). Unlike the immunoblotting analyses shown in Fig. 8, the labeling experiments did require a substantial amount of cultured cells, suggesting that maintenance of EGF previously synthesized in vivo as well as new synthesis of EGF are both operative in the

culture system. Because of the large number of cells required for metabolic labeling of EGF in culture we were unable to define the kinetics of EGF synthesis in response to hormones. It is emphasized, however, that the collagen cultures could be easily manipulated for analysis at a one-cell level; thus, further experiments will examine effects on EGF mRNA levels by in situ hybridization. Data from such studies will complement the biochemical observations presented here and will allow determination of the level at which hormones and the extracellular matrix regulate the synthesis of this important growth factor. Sexual dimorphism of GCT cells also exists with respect to other SSG products including nerve growth factor (30), amylase (31), and renin (11). In addition, it will be of interest to determine whether the present culture system supports the maintenance of any other hormonally responsive GCT cell functions.

An important distinction to be made in studies of cell differentiation in culture is that between the maintenance of a function whose expression is initiated in vivo and the induction of a function not yet active at the time of culture. In the course of the present study, cultures were also initiated with immature SSG (3 to 10 d after birth) devoid of GCT. Although epithelial cells from the immature glands grew vigorously within collagen gels, repeated attempts to induce EGF synthesis failed. Thus, other factors in addition to hormones and a collagen matrix must be required to induce differentiation of progenitors to GCT cells, an observation with precedent in the hormone-dependent differentiative behavior of mammary epithelial cells from virgin mice in 3-D collagen gel cultures (7,8).

It is clear that questions concerning normal regulatory mechanisms of cell growth and differentiation cannot be easily addressed experimentally in vivo. Ideally, studies of this nature are best performed with a culture system where cell behavior is representative of the tissue in vivo. The culture system detailed here provides the means to assess growth regulation and/or deregulation, in relation to functional activity of at least one SSG cell lineage. A question being addressed in present experiments with this culture system is whether modulation of SSG ductal cell growth can in fact be exerted via a mechanism that includes EGF itself and the EGF receptor and its signal transduction pathway.

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