Epithelial Bridging of the Primary Palate: I. Characterization of Sub-Cultured Epithelial Cells

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Primary palatogenesis involves an intricate array of events. Cell migration, proliferation, differentiation, programmed death, and fusion occur. Prior to fusion, the morphology of the epithelium undergoes marked changes. Epithelial projections form and extend across the fusion site attaching by filopodia to the opposite prominence. By appearance, the epithelium plays a critical role in facial development. In order to monitor epithelial activities, a study was done to isolate and characterize epithelial cells derived from the primary palate. The primary palate was microdissected from day 13 Sprague-Dawley rat embryos, and the epithelium and mesenchyme were separated by enzymatic digestion with a 3% trypsin-pancreatin solution (3). All explants were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 10% fetal calf serum (FCS) 20 ng/ml epidermal growth factor (EGF) and antibiotics. Explant cells were gathered by trypsin harvesting and sub-cultured. These sub-cultured cells were further characterized. Transmission and scanning electron microscopy showed that the cells retained many morphological features observed in vivo. In passaged cells, type IV collagen, laminin, and cytokeratins were visualized by immunocytochemistry. Gel electrophoresis analysis of the water-insoluble extracts demonstrated major bands of proteins of 50 kD and 44 kD that were synthesized by the epithelial cells but not by the mesenchymal cells. These cytokeratin types are suggestive of a simple undifferentiated embryonic epithelium.

The effect of all-trans retinoic acid (RA) on cell number and [3H]-proline incorporation was assessed. At 10^{-4} M and [10^{-6} M] retinoic acid resulted in significant inhibition in cell proliferation and amount of proline incorporated, with the greater inhibition occurring in the mesenchymal cells. In the concentrations studied, retinoic acid has an inhibitory effect on the two differentially derived cell types.

This study established that sub-cultured epithelial cells maintain their phenotype and can be used to study fusion processes. Part 2 will demonstrate how the morphology of the epithelial cells can be modified to produce the changes that are observed during fusion of the primary palate.

Key words: epithelium, fusion, retinoic acid, immunocytochemistry

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Fig. 1  Scanning electron micrograph of dissected primary palate of a 13-day-old rat embryo showing isolated medial and lateral nasal processes, prior to the enzymatic digestion. Bar  200 μm.

Fig. 2  Scanning electron micrograph of epithelium with underlying mesoderm from the primary palate of a 13-day-old rat embryo. Note the basal lamina (b.l).  Bar  20 μm.
7% sucrose in 0.1 M sodium cacodylate (pH 7.4). The cells were post-fixed for 60 min with 2% osmium tetroxide in the same buffer, and dehydrated in a graded series of alcohols. After dehydration, propylene oxide was added for two washes and the cells were embedded in epon. Ultrathin sections were stained with 0.1% uranyl acetate and lead citrate and photographed using a Joel 1000 transmission electron microscope.

For SEM analysis, the cells were rinsed in PBSa and fixed in 2.25% glutaraldehyde buffered in PBSa, for 24 hours. After fixation, the cells were rinsed several times with cold 0.2 M PBSa, followed with cold distilled water. The cells were transferred and critical-point dried using a DCP01 drying apparatus (Denton Vacuum, Inc.) from liquid CO2. Next, the cells were placed onto aluminum stubs, sputter coated with gold, and examined in a Cambridge stereoscan 250 MK2 scanning electron microscope at 20 kV.

Immunofluorescence Microscopy

Cultured epithelial and mesenchymal cells were plated on 4-well Lab-Tek slides (Lab-Tek Division, Naperville, IL).

Laminin and Type IV Collagen Detection

After 2 days in culture, the medium was aspirated, the cells rinsed twice with PBSa and fixed in 95% ETOH for 5 min. Hyaluronidase in PBSa (40 µg/ml, Cooper Biomedical, Malvern, PA) was added to the cells for 30 min at 37°C. The wells were rinsed with PBSa containing 1% goat serum, 1% bovine serum albumin, and 0.1% Tween-20. Twenty percent goat serum in PBS containing 1% bovine serum albumin, and 0.1% Tween-20 was applied to the cells for 20 min. Rabbit type IV collagen antiserum (donated by S. Dixit, Northwestern University) or rabbit laminin antibody (Collaborative Research, Bedford, MA) in 1/50 dilution in the PBSa mixture was applied for 16 h at 4°C. After several rinses with PBSa, PBSa containing fluorescein isothiocyanate-coupled goat anti-rabbit immunoglobulin (1/20 dilution, Cappel Laboratories, Westchester, PA) was applied for 1 h. After rinsing with PBSa containing 1% goat serum for 20 min, the slides were mounted in 90% glycerol in PBSa and coverslipped. To control for nonspecific immunofluorescence, selected wells were incubated with PBSa containing 1% goat serum and bovine serum albumin.

Keratin Detection

After 2 days in culture, the medium was aspirated and the cells were washed twice with PBSa, and fixed with 3.7% formaldehyde in a PBSa solution for 30 min at room temperature. The wells were rinsed twice with PBSa and made permeable by immersion in a 1:1 solution of methanol/acetone at 20°C. After 2 min, a solution of 0.1% Tween-20 was applied for 10 min at room temperature. After rinsing twice with PBSa, the cells were incubated overnight at 37°C with 1:50 mouse anti-keratin (AE-1 and AE-3, ICN Immunobiologicals, Lilse, IL). After several rinses with PBSa, fluorescein isothiocyanate-coupled goat anti-mouse immunoglobulin was added at a 1:16 dilution in PBSa for 90 min at 37°C. After washing with PBSa for 30 min, the slides were mounted and coverslipped in 90% glycerol in PBSa. To control for nonspecific immunofluorescence, selected wells were incubated with PBSa containing 1% goat serum.
The cells were examined and photographed using a Leitz fluorescing microscope, Dialux 20, with a 450 nm excitation filter

**Effect of Retinoic Acid on Cell Number**

To examine the effects of vitamin A on epithelial and mesenchymal cells, all *trans* retinoic acid (Sigma Chemical Co., St. Louis, MO) at $10^{-4}$ M or $10^{-6}$ M final concentration was added to the medium 12 h after plating. Fresh medium containing retinoic acid was replaced daily. These concentrations were chosen because $10^{-6}$ M concentration represents the dividing point between physiologic and pharmacologic doses of retinoic acid [Roberts and Sporn, 1984] Dimethylsulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) was the vehicle for retinoic acid and did not exceed 0.1% volume of the culture medium. All controls received 0.1% DMSO. Epidermal growth factor was excluded from the culture medium. At day 3, the cells were collected in a known volume of a trypsin/EDTA solution, counted using a cell counter (Coulter Co. Inc., Hialeah, FL). Values were presented as the mean of triplicate wells for each group.

**Effect of Retinoic Acid on Proline Incorporation**

Parallel cultures were set up in a manner as above for both the epithelial and mesenchymal cells. On day 3, five hours prior to the end of the experiment, the cells were washed twice with PBSa, and the medium was replaced with fresh medium containing 0.4% FCS, sodium ascorbate (50 μg/ml, Sigma Chemical Co., St. Louis, MO), 50 U/ml penicillin, and 50 μg/ml streptomycin, and various amounts of RA Two hours later the medium was replaced with the same medium as above plus [2-3-4,5 H]-proline (20 μCi/ml) for an incubation period of 3 h. At the end of 3 h, the medium was removed, and 0.5 ml of cycloheximide (0.05 mg/ml, Sigma Chemical Co., St. Louis, MO) was added to the medium. The medium was gathered and the cells were collected by scraping with a Teflon policeman. An additional 0.5 ml of cycloheximide was added to the culture wells with repeated scraping. This step was repeated twice. Each wash was added to the collected medium, and the total sample consisting of cells and medium was frozen at 20°C.

**Determination of Radiolabel Incorporation**

The combined medium and cells from the radiolabeling experiments were frozen and thawed three times in acetone and dry ice, and then sonicated for 30 s for membrane disruption. Aliquots (0.5 ml) of each sample were combined with 0.5 ml of 20% trichloroacetic acid (TCA, Sigma Chemical Co., St. Louis, MO) containing 1% tannic acid. The solution was allowed to stand for 45 min at 4°C. The resultant precipitates were collected on glass fiber filters presoaked in a proline solution of 5% TCA (GF A, Whatman Co., Clifton, NJ), and washed with 95% ethyl alcohol. The filters were dried, and then suspended in scintillation cocktail. Scintillations were counted with a Beckman Scintillation Counter (Beckman Instruments Inc., Somerset, NJ) and recorded as counts per minute (CPM).

**Keratin Extraction**

Epithelial and mesenchymal cells were collected by trypsin/EDTA digest, and plated on T-75 flasks (Costar, Cambridge, MA). Hydrocortisone was added at a concentration of 10 μg/ml to half the flasks containing epithelial cells to test its ability.
to augment keratin synthesis. After reaching confluence, the cells were rinsed twice with PBSa, scraped off the dish with a Teflon policeman and collected. The extraction procedure of Starger et al. [1978] was followed. Briefly, the collected cells were centrifuged at 780g for 5 min in a tabletop Dynac centrifuge (Clay-Adams, Div., Parsippany, NJ) and the pellet was quickly resuspended in 2.5 ml of lysis buffer. All subsequent steps were performed at 4°C. The lysis buffer consisted of 0.6 M KCl, 1% Triton X 100 (Rohm and Haas Co., Philadelphia, PA), and PBSa with protease inhibitor 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO) Magnesium chloride and DNAase I (Sigma Chemical Co., St. Louis, MO) were added up to a final concentration of 10 mM and 0.5 mg/ml, respectively. The suspension was pelleted by centrifugation at 1,500g for 5 min. The pellet was washed three times in PBSa containing 1 mM PMSF, to remove the excess salts and detergent. The prepared extract was resuspended in diassembly buffer consisting of 8 M urea, 5 mM NaPO₄ (pH 7.4), 1 mM PMSF, 0.2% β-mercaptoethanol, and was stirred for 5 h at room temperature. The sample was centrifuged at 55,000 rpm for 45 min and the supernatant was collected. The supernatant was dialyzed against 5 mM Phosphate buffer (pH 6.6), 0.2 mM PMSF, 0.2% β-mercaptoethanol overnight. The sample was centrifuged for 45 min at 55,000 rpm at 20°C and the supernatant was lyophilized. The resultant sample was weighed and dissolved in Laemmli sample buffer [Laemmli, 1970].

Electrophoretic analysis of the keratin proteins was carried out in 5–10% gradient SDS polyacrylamide vertical slab gels with 4% acrylamide stacking gels. Constant current (15 mA) was applied for 8 h and stopped when the tracking dye reached within 5 mm of the end of the slab according to the method of Laemmli [1970]. Samples were run against keratin standards (ICN Biochemicals) and molecular weight standards (Bio Rad Corp., Richmond, CA). The gel was fixed and then stained with Coomassie brilliant blue (Bio Rad Corp., Richmond, CA).

RESULTS

Two different cell types were isolated from the primary palate of day 13 Sprague-Dawley rat embryos—one type from an ectodermal origin, and the other from a mesodermal origin. These cell types differed in both size, morphology, organization profiles, proliferation rates and constituent proteins. Their responses to retinoic acid were similar but distinctly different.

Isolation of Epithelial and Mesenchymal Cells

The microdissection procedure routinely isolated the primary palate, however, remnants of the maxillary process sometimes remained (Fig 1). Two distinct tissue layers can be seen in the isolated primary palate (Fig 2). After enzymatic digestion, these tissue layers were separated into sheets, and placed in culture. After cell outgrowth reached confluence, the cells were passaged with trypsin-EDTA.

Cell Morphology

One day after passage, the epithelial cells were observed to cluster and form satellite groups. The innermost cells of these groupings were spherical, whereas the outermost cells were elongated, forming contacts with neighboring groupings. In contrast, the mesenchymal cells were fibroblast-like in appearance. Their profiles
were elongated and spindle-shaped. When seeded at low density, the mesenchymal cells spread out in all directions without clustering (Fig 3).

At high density, the epithelial cells were arranged in a cobblestone pattern with numerous intercellular contacts. The epithelial cells were principally arranged as a monolayer, with minimal overlapping. In contrast, the mesenchymal cells formed a loosely packed, multilayered network. Cell to cell contacts among the mesenchymal cells were significantly less. The epithelial cells displayed much greater contact inhibition of growth than the mesenchymal cells (Figs 4–5). The distribution of the two cell types according to their cell diameter is presented in Figure 6.

When plated on a plastic versus a collagen coated dish, the epithelial cells displayed two patterns. On plastic, an intervening layer was seen between the cell and plastic. This layer was absent in the collagen coated cultures. The membrane beneath the epithelial cells plated on plastic was suggestive of a basal lamina (Fig 7).

Detection of Laminin, Type IV Collagen, and Keratin

Immunolocalization techniques confirmed that the epithelial cells retained, after multiple passages, proteins characteristic of their origin. The phenotypic markers for an epithelial cell, laminin, type IV collagen, and cytokeratin were present in the epithelial cells and absent in the mesenchymal cells (Fig 8). Polyacrylamide gel electrophoresis of the cytoskeleton extract from the epithelial cells produced two major bands of proteins with molecular weights of 44 and 50 kDa. Gel electrophoresis of the mesenchymal extract produced only one detectable band with a molecular weight of 45 kDa (Fig 9).

Response to Retinoic Acid

Retinoic acid reduced the number of cells and the amount of proline incorporated in both the mesenchymal and epithelial cells. At $10^{-6}$ M RA, the cell number was reduced by 18% in the epithelial cells and by 35% in the mesenchymal cells. Proline incorporation was reduced by 20% in the epithelial cells and 32% in the mesenchymal cells (Tables I, II).

DISCUSSION

Through a variety of techniques, it was demonstrated that embryonic primary palatal epithelial cells could be obtained as nearly homogenous isolates and maintained in sub-culture. From the cytological, ultrastructural, topographical, biochemical, and immunochemical results, we concluded that the epithelial cells sub-cultured in vitro are clearly different from mesenchymal cells. In addition, the epithelial cells sub-cultured in vitro possess many of the characteristics expected of simple embryonic epithelial cells.

To verify the presence of epithelial cells, and to assure their continued differentiated state, immunofluorescence was performed to detect the presence of various antigens unique to epithelia. In sub-culture, the epithelial cells continued to display reactivity to antibodies to laminin, type IV collagen, and cytokeratins. Cultured epithelial cells maintained this ability even after multiple passages. Since cytokeratins are not found in other cell types [Franke et al., 1978, 1979; Sun et al., 1978, 1979, 1983], they are an important phenotypic marker for epithelial cells.

The composition of keratin filaments in epithelial cells also represents a good
Fig. 3  a: Inverse phase contrast micrograph of epithelial cells one day after passaging. At low density the cells aggregate to form individual clusters displaying maximal intercellular contacts. b: Inverse phase contrast micrograph of mesenchymal cells at one day after passaging. At low density cells are dispersed showing minimal intercellular contacts. Bars, 50 μm.
Fig. 4.  a: Inverse phase contrast micrograph of epithelial cells 3 days after passaging. Note cobblestone appearance with minimal layering at high density  b: Inverse phase contrast micrograph of mesenchymal cells 3 days after passaging. Note cells are arranged in a swirling manner similar to the picture expected of fibroblasts with layering of cells evident. Bars, 100 μm.
Fig 5  a: Scanning electron micrograph of a confluent culture of epithelial cells. Note cells maintain multiple contact with each other and are not arranged in multiple layers  b: Scanning electron micrograph of a confluent culture of mesenchyme cells. Note loosely packed arrangement of cells with layering evident. Bars, 10 μm.
phenotypic indicator for the degree of differentiation of the cell [Banks-Schlegel, 1982, Sun et al., 1983, Shuler and Schwartz, 1986] In general, the smaller keratins are widely distributed, while the larger ones are limited to the more complex epithelium. The results from the gel electrophoresis suggest the presence of cytokeratins that are found in simple epithelial cells during embryogenesis [Lawson and Pedersen, 1987, and Ben-Ze'ev 1984]

The response of epithelial and mesodermal cells to pharmacological doses of retinoic acid was examined. Retinoic acid was chosen as an experimental agent because it is required by tissues for normal growth and differentiation. In addition, retinoic acid administered at higher doses is a potent teratogen and has been reported as an important etiological factor in congenital malformations of the first and second branchial arches [Lammer et al., 1985, Pratt et al., 1987, Sulik et al., 1988, and Sulik et al., 1987] Various cell types react differently to retinoids, but in general, concentrations less than $10^{-6}$ M are physiologic, while concentrations greater than $10^{-6}$ M are pharmacologic [Roberts and Sporn, 1984]. In this study, both the epithelial and mesenchymal cells, grown in cell culture, demonstrated a significant decrease in cell proliferation at $10^{-4}$ M and $10^{-6}$ M retinoic acid administration. These findings are in agreement with other studies [Sporn et al., 1976; Pohl and Christophers, 1981, Watanabe et al., 1988, Yoshikawa et al., 1987] Sulik et al. [1988] and Wiley et al. [1983] reported excessive cell death following the administration of retinoic acid during gestation. Among the cells affected were the neural crest, trigeminal placode, and epibranchial placode cells. Although the viability of the cells was not determined in this study, the cells were inspected and photographed. Visual assessment of the

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Fig. 7 a: Transmission electron micrograph of epithelial cells grown on plastic petri dishes. Arrows indicate the presence of a subcellular interphase between the plastic and the epithelial cells, presumably secreted by the cells. b: Transmission electron micrograph of epithelial cells grown on plastic petri dishes and covered with matrigel (Collaborative Research). Note the absence of a layer between the matrigel and cells. Bars, 200 μm.
Figures 8a, b.
cells that were treated with retinoic acid at $10^{-4}$ M concentration did not reveal any abnormal cytoarchitectture when compared to control cells. Some cells may be more sensitive to retinoic acid and respond with cell death. Other cells may respond to retinoic acid with a generalized inhibition of various activities such as mitosis and synthesis of extracellular proteins. The effect of retinoic acid is not restricted to one cell type. It appears to have a broad adverse effect on many different cell types since both the epithelial and mesenchymal cells were similarly affected.

When retinoic acid was administered at a concentration of $10^{-6}$ M to the cultures (data not shown) the epithelial cells did not display any difference in the amount of fluorescence for cytokeratins using AE-1 and AE-3 antibodies. In contrast, Brown and Benya [1988] found a reduction in intermediate filaments and an increase in actin in chondrocytes after retinoic acid treatment. In the TEM micrographs there was an increase in intracellular filaments as consequence of retinoic acid treatment. Most likely, the filament increase was the actin filament (in preparation).

The development of the primary palate is a dramatic process manifested by numerous morphologic and physiologic events. Cell proliferation, migration, merging, programmed cell death, fusion, and differentiation are all important events of palatal development. This study used a cell culture technique to examine epithelial and mesenchymal cells in more detail. First, it was shown that epithelial and mesenchymal cells could be isolated from the primary palate, and grown in culture. Both of the cultured cell types maintained characteristics observed in vivo. The epithelial cells were still reactive to antibodies to type IV collagen, laminin and cytokeratins.

This study established that sub-cultured epithelial cells maintain their phenotype.
Fig. 9  Coomassie blue staining of SDS-PAGE analysis of epithelial and mesenchymal cell extracts for water insoluble proteins. Equal amounts of proteins were loaded on a 5–10% gradient gel with a 4.5% stacking gel and run 8 h at constant current of 15 mA. **Lane a:** Molecular weight standards (β-galactosidase, 200 kDa; phosphorylase b, 116.3 kDa; bovine serum albumin, 66.2 kDa; and ovalbumin, 42.7 kDa). **Lane b:** Epithelial cell extract from cells grown without hydrocortisone. **Lane c:** Epithelial cell extract from cells grown with 10 µg/ml hydrocortisone. **Lane d:** Mesenchymal cell extract from cells grown without hydrocortisone. **Lane e:** Cytokeratin standards.

| TABLE I. The Effect of Retinoic Acid on Cell Number After 3 Days in Culture^1 |
|---------------------------------|-----------------|-----------------|
| **Agent**                      | **Epithelial cells** | **Mesodermal cells** |
| Control                        | 6.39 ± 0.96      | 30.37 ± 0.80     |
| RA (10^-6 M)                   | 5.24 ± 0.58      | 19.83 ± 1.40     |
| RA (10^-4 M)                   | 3.74 ± 0.19      | 14.52 ± 0.84     |

^1 All cells were seeded at a density of 1 × 10^4 cells per cm^2^ were harvested 3 days later by trypsin/EDTA digest, and counted in a Coulter counter. Results are reported as mean cell number ± standard deviation × 10^-4 for four replicate cultures.

All values were statistically significant from the control values at a P < 0.01. RA = retinoic acid.
TABLE II. The Effect of Retinoic Acid on Tritiated Proline Incorporation After 3 Days in Culture

<table>
<thead>
<tr>
<th>Agent</th>
<th>Epithelial cells</th>
<th>Mesodermal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.09 ± 0.43</td>
<td>2.45 ± 0.15</td>
</tr>
<tr>
<td>RA [10^{-6} M]</td>
<td>1.68 ± 0.17</td>
<td>1.67 ± 0.17</td>
</tr>
<tr>
<td>RA [10^{-4} M]</td>
<td>0.78 ± 0.09</td>
<td>1.20 ± 0.14</td>
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All cells were seeded at a density of 1 × 10^4 cells per cm² and were labeled with 20 µCi/ml [2,3,4,5] tritiated proline. Results are reported as mean counts per minute ± standard deviation × 10 per cell for four replicate cultures. All values were statistically significant from the control values at a P < 0.01 RA = retinoic acid.

and can be used to study fusion process Part 2 of this study will demonstrate how the morphology of the epithelial cells can be modified to produce changes observed during fusion of the primary palate

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