Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts

Kristina Lindberg1,2* and James G. Rheinwald1,3† ‡

1 Division of Cell Growth and Regulation, Dana-Farber Cancer Institute,
2 Department of Oral Medicine and Oral Pathology, Harvard School of Dental Medicine, 177 Longwood Ave, Boston, MA 02115, USA
3 Department of Molecular and Cellular Physiology, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA

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Abstract. We have characterized the cells that form the human oral epithelia by analyzing their patterns of keratin expression in culture and in transplants. Keratinocytes of all oral regions synthesized high levels of keratins K5/K14 and K6/K16.K17, as expressed by cells of all stratified squamous epithelia in culture. However, cells from different regions varied in their expression in culture of retinoid-inducible (K19 and K13) and simple epithelial (K7, K8 and K18) keratins. By these criteria, all oral cells could be classified as belonging to one of three intrinsically distinct subtypes: “keratinizing” (gingiva, hard palate), “typical nonkeratinizing” (inner cheek, floor of mouth, ventral tongue) and “special non-keratinizing” (soft palate), all of which differed from the epidermal keratinocyte subtype. Cells from fetal floor of mouth expressed a pattern of keratins in culture markedly different from that of adult floor of mouth cells but identical to that of the adult “special nonkeratinizing” subtype and similar to that of several oral squamous cell carcinoma lines. When cultures of oral keratinocytes were grafted to the dermis of nude mice, they formed stratified epithelial structures after 10 days. In some areas of the stratified structures, the basal layer recapitulated the K19 expression pattern of the oral region from which they had originated. Thus, regional differentiation of the oral epithelium is based on an intrinsic specialization of regional keratinocyte stem cells. Additionally, oral cell transformation either frequently involves reversion to the fetal keratin program or else oral cells that express this keratin program are especially susceptible to transformation.

Introduction

The mechanism by which site-specific histology in epithelia is established and maintained is poorly under-

stood. Mesenchymal-epithelial interactions during early embryogenesis are thought to play essential instructive roles in establishing the location and boundaries of specialized epithelia and epithelial-derived structures [17, 44, 51]. The extent to which connective tissue continues to provide instructive signals directing the local differentiation pattern of epithelia in the adult remains uncertain, however.

The various anatomical regions of the oral cavity exhibit histological differences in both the epithelium and the connective tissue. Most oral surfaces are covered by nonkeratinized stratified squamous epithelium, which is supported by soft, flexible connective tissue [6]. In contrast, the gingiva, hard palate, and retromolar pads are covered by keratinized (i.e., containing an outermost multilayer of flattened cornified cells) epithelium, which is attached by dense, rigid connective tissue to deeper structures. This histological variability across small distances makes the oral epithelium particularly interesting and important for the study of intrinsic epithelial cell characteristics versus mesenchymal instruction in determining epithelial differentiation.

Keratin protein expression is a very sensitive and specific marker for assessing differentiation in epithelial cells and tissues [32]. The keratins are a family of more than 20 different proteins, a subset of 2–10 of which is expressed by each epithelial cell type and assembled into its intermediate filament network (reviewed in [9]). Our interest in oral epithelial differentiation and keratin expression originated with an early finding by this laboratory that the 40-kDa keratin, K19, and other members of the subfamily of keratins that we originally called “mesothelial keratins” [55] but, by virtue of their distribution, are more accurately termed “simple epithelial keratins” [28], are expressed abnormally by some human oral squamous cell carcinomas (SCCs) in culture [40, 54]. K19 is normally not expressed by keratinized stratified squamous epithelia such as the epidermis and the gingival and hard palatal regions of the oral epithelium in adults, and it is expressed at very low levels, restricted to the basal cell layer, in nonkeratinized oral epithelia [2, 24, 29, 33, 45].
We wished to determine definitively whether regional variation in keratin expression and suprabasal differentiation in the oral epithelium is primarily based upon intrinsically determined programs of the epithelial cells in each region and the extent to which specialized connective tissue plays an instructive or permissive role in oral epithelial differentiation. Oral keratinocytes have been cultured previously (see [47] and references cited therein), but cells from the different oral regions have not been compared to permit determination of the extent to which intrinsically distinct subtypes exist. We have cultured pure populations of keratinocytes derived from the various regions of human oral epithelium and have compared their patterns of keratin expression in culture and in reconstructed epithelia formed by transplanting cultured cell sheets to the subdermal connective tissue of nude mice. These experimental conditions prevent exposure of the epithelial cells to connective tissue signals specific to any oral region. Thus any differences observed in epithelial differentiation can be attributed to intrinsic cell specialization, and not to site-specific mesenchymal influence. A similar experimental approach to studying the basis of epithelial differentiation was originally described by Doran et al. [13], who compared the histogenesis and keratin expression pattern of cultured rabbit esophageal, corneal, and epidermal cells inoculated subcutaneously as cell suspensions into nude mice.

Extending our studies of normal oral cells, we also wished to determine whether apparently abnormal keratin expression exhibited in culture by many oral squamous cell carcinomas (SCCs) might be explained by their possible origin from a particular regional oral keratinocyte subtype. We report here that at least three intrinsically distinct keratinocyte subtypes form the oral epithelial surfaces. Surprisingly, keratinocytes of the soft palatal epithelium differ from those of other nonkeratinized oral regions; they resemble the developmentally immature keratinocyte subtype that populates most or all of the oral epithelial surface during fetal development. This subtype shares features of keratin expression with some oral SCC lines.

**Methods**

*Cells and culture media.* Normal human keratinocyte cell strains from inner cheek (buccal mucosa; OK-B2, OK-B3 and OK-B4), ventral tongue (OK-T4), anterior soft palate (OK-P4), floor of mouth (OK-F4), gingiva (OK-G4), and hard palate (OK-HP1) were cultured from biopsies of healthy, nonsmoking volunteers 28–38 years of age. Cells (strains B4-OKF2 and B4-OKF3) were also cultured from fetal (20 weeks gestational age) floor of mouth. Keratinocytes were also cultured from benign hyperplastic, hyperkeratotic lesions (leukoplasias) of the anterior soft palate (OK-P2) and gingiva (OK-G1). Normal epidermal keratinocytes were cultured from adult reduction mammaryplasty skin (N4-Ep, P1-Ep, and D2-Ep) [1], newborn foreskin (strain N) [54], and trunk skin from two fetuses that were of 20 weeks gestational age (B4-Ep2 and B4-Ep3). OK-B2, OK-P4, and OK-F4 were all cultured from a single individual. OK-G4 and OK-HP1 were cultured from a different individual. B4-Ep2 was cultured from the same individual as B4-OKF2, and B4-Ep3 from the same individual as B4-OKF3. Epithelial cell populations free from human stromal fibroblasts were obtained by selectively removing fibroblasts from early passage cultures as described earlier [36]. Preconfluent cultures were incubated briefly with 0.02% EDTA followed by vigorous pipetting, rinsing to remove detached fibroblasts, and finally adding back fresh 3T3 feeder cells. This procedure was repeated several times during the primary and secondary passages of each keratinocyte strain. Absence of any human stromal fibroblasts from the keratinocyte populations used for keratin analysis and grafting was confirmed by plating the cells in the absence of 3T3 feeder cells in a medium optimal for clonal growth of human fibroblasts (M199/MCD105 +7% fetal calf serum (see [26]) and examining these cultures for 2 weeks for the appearance of fibroblast colonies. Established cell lines lines were described previously [39, 40, 54]. SCC-4 was cultured from a tumor of the floor of mouth, SCC-9, SCC-15, and SCC-25 from tumors of the ventral tongue (which is contiguous with and identical histologically to floor of mouth), SCC-40 from a tumor of the soft palate, and SCC-12F.2 and SCC-13 from tumors of the facial skin. Culture media and conditions were as described previously [1, 36, 37, 39]: normal keratinocytes were cultured with mitomycin-treated or gamma-irradiated 3T3 feeder cells in Dulbecco's minimal essential (DME)/F12 medium supplemented with 5% fetal calf serum (FCS), 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10^-7 M cholera toxin, 5 μg/ml insulin, and 24 μg/ml adenine. SCC cells were grown with 3T3 feeder cells in DME medium supplemented with 10% FCS and 0.4 μg/ml hydrocortisone.

**Analysis of keratin expression in culture.** Cell proteins were metabolically radiolabelled for 2–4 h with 3H-methionine when cultures were about 80% confluent. The keratin filament protein fraction was then selectively extracted as the Triton/high-salt-insoluble precipitate. The 8 M urea/dithiothreitol-solubilized keratin protein subunits were separated electrophoretically by two-dimensional, nonequilibrium-pH-gradient electrophoresis and identified as described previously [41, 55]. Gels were loaded with equivalent amounts of trichloroacetic acid-precipitable counts and the resulting autoradiograms exposed so as to permit assessment and comparison of simple epithelial keratin (K7, K8, and K18), K13, and K19 expression relative to that of the stratified squamous epithelial keratins (K5, K6, K14, and K16/17), which are expressed by all keratinocyte subtypes. Keratin nomenclature is as suggested by Moll et al. [28].

**Transplantation of cultured keratinocytes to the subdermal connective tissue of athymic (nude) mice.** Confluent cultured cell sheets were grafted to the underside of the dermis of nu/nu mice using a minor modification of the inverted flap method described by Barrandon et al. [5]. Female NIH Swiss nu/nu mice (Taconic Farms, New York) or male Balb C nu/nu mice bred at the Dana-Farber Cancer Institute were used at about 6–9 weeks of age. They were anesthetized by intraperitoneal injection of avertin [19] followed by methoxyflurane inhalation, or by subcutaneous injection of pentobarbital and xylazine [5]. Second- to fourth-passage cultures (20–30 cell divisions) were grafted. Cells were plated at 5 x 10^3–35 mm dish or 2 x 10^3–60 mm dish and were grown to a confluent epithelial sheet. As described earlier [37], in this culture system the keratinocytes attach directly to the plastic culture vessel surface and the 3T3 feeder cells are displaced as the colonies expand laterally. When the keratinocyte population neared confluence, any residual feeder cells were selectively removed by brief incubation with an 0.02% EDTA solution in order to aid the complete merging of colonies. One day after reaching confluence the culture was rinsed and incubated with Dispase II (Boehringer-Mannheim; 2.5 mg/ml in culture medium) to detach the cells as a coherent sheet [16]. The cell sheet was carefully rinsed with medium without disturbing its orientation and a sterile circular or rectangular piece of silicone membrane (Silastic; Dow Corning, New Jersey) was placed on top of it. A semicircular or three-sided rectangular incision was made through the dorsal skin of the anaesthetized mouse and the flap was opened, leaving the muscle fascia exposed. The Silastic membrane and cell sheet were turned over and stuck to the muscle fascia such that the basal layer of the cell sheet faced upward and the Silastic membrane contacted the muscle fascia. The skin flap was then folded back to its normal position, placing...
the basal surface of the cultured cell sheet in contact with the underside of the mouse dermis, and the incision was closed with surgical staples (Ligaclips, Ethicon, Inc., New Jersey). The tissue formed by the grafted cell sheet was removed after 7–10 days, fixed in 100% ethanol or in methanol/Carnoy's fixative (see [24]), embedded in paraffin, and sectioned for hematoxylin-eosin (H&E) staining and immunostaining.

Immunostaining of tissue sections. Five-micron sections of tissue biopsies and grafts were stained by the avidin-biotin complex (ABC) peroxidase technique (Vector Laboratories, Burlingame, Ca) or by indirect immunofluorescence, as described elsewhere [24], using the following antibodies:

* A53-B/A2, a mouse monoclonal antibody specific for keratin 19 [21], purchased as antibody Kc19.1 from ICN Biomedicals, Lisle, Illinois.
* A2E2, a mouse monoclonal antibody specific for keratins 1, 2, and 10 [53].
* A6E8, a mouse monoclonal antibody specific for keratin 13 [12].
* CEP, a rabbit antiserum specific for human involucrin [42, 49].

Fig. 1a-f. Morphology of human oral and epidermal keratinocyte subtypes in culture. Colonies of normal human keratinocytes are shown after three passages (approx. 20–30 cell generations) in culture, growing with 3T3 feeder cells. Bars in a and b indicate 100 μm. Magnification of c-f is the same as in b. a Seven-day-old colony of OK-P2 (soft palate), showing the well-ordered, unstratified epithelioid morphology that is characteristic of all keratinocyte subtypes during the first week of growth after each subculture. b Seventeen-day-old colony of OK-P4 (soft palate) showing the reduced degree of cell-cell adhesion and stratification typically exhibited by soft palatal keratinocytes in culture. c Ten-day-old colony of OK-F4 (floor of mouth), showing tighter cell-cell adhesion and modest stratification characteristic of the typical nonkeratinizing oral keratinocyte subtype. d Fourteen-day-old colony of OK-B2 (buccal mucosal), showing characteristic morphology and stratification of a large colony. e Nine-day-old colony of B4-OKF2 (fetal floor of mouth), showing morphology similar to that of the adult “typical nonkeratinizing” oral keratinocyte subtype. f Ten-day-old colony of B4-Ep2 (fetal epidermal), showing tight cell packing and stratification beginning early in colony growth, similar to the behavior of adult epidermal keratinocytes. Arrows indicate obvious regions of stratification. (Adult gingival and hard palatal keratinocytes, which are not shown, exhibited a morphology intermediate between that of typical nonkeratinizing oral epithelial cells and epidermal cells)
Table 1. Keratin expression in native oral epithelium and epidermis and in xenografted epithelium formed by cultured cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Antibody staining</th>
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<tr>
<td></td>
<td></td>
<td>K19</td>
<td>K13</td>
<td>K1/K10</td>
<td>Involucrin</td>
</tr>
<tr>
<td></td>
<td>Native</td>
<td>Graft</td>
<td>Native</td>
<td>Graft</td>
<td>Native</td>
</tr>
<tr>
<td>Nonkeratinized</td>
<td>b+</td>
<td>b+</td>
<td>s+</td>
<td>s+</td>
<td>s+</td>
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<tr>
<td>oral</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Keratinized</td>
<td>–</td>
<td>–</td>
<td>s+</td>
<td>s+</td>
<td>–</td>
</tr>
<tr>
<td>oral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Epidermis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>s+</td>
<td>s+</td>
</tr>
</tbody>
</table>

+, positive staining; -, no detectable staining; b, basal cells; s, suprabasal cells. Results indicated for grafts refer to well-organized areas, as described in Methods.

* Some areas contained K19-positive basal cells; b patchy distribution of K13-positive cells.

Results

Colony morphology of keratinocytes cultured from different oral sites

Epithelial cell cultures were initiated from biopsies of the keratinized oral surfaces of gingiva and hard palate, from the nonkeratinized oral surfaces of the inner cheek, floor of mouth, ventral tongue, and anterior soft palate, and from the epidermis using the 3T3 fibroblast feeder layer method, as described in Methods. The culture method ensured that human stromal fibroblasts from the biopsy did not contaminate the keratinocyte cell populations (see [36]). Cells from benign, hyperplastic lesions (leukoplakias) of keratinized and nonkeratinized oral regions were also cultured. In addition, floor of mouth epithelial cells and epidermal cells were cultured from two fetuses of approximately 20 weeks gestational age. The epithelial cell populations cultured for all of these tissues exhibited general morphological features (Fig. 1) and mitogen requirements (data not shown) of normal keratinocytes ([13, 37, 55]; reviewed by Watt [50], and Rheinwald [36]). All cell strains could be cultured for 30–80 cell generations (3–10 passages over approximately 3–10 weeks of serial culture) before senescence. Cells cultured from the nonkeratinizing oral regions formed colonies that were less extensively stratified than those formed by epidermal cells (Fig. 1). Soft palatal cells usually formed looser, less stratified colonies than did keratinocytes cultured from other oral regions. Colonies formed by gingival and hard palatal cells (not shown) appeared nearly as tightly packed and stratified as the colonies of epidermal cells. Keratinocytes cultured from benign hyperplastic, hyperkeratotic oral lesions were indistinguishable from those cultured from histologically normal tissue. Cells cultured from 20-week-fetal floor of mouth and epidermis were also indistinguishable from those cultured from the respective adult tissues. The morphological characteristics of each cell strain persisted throughout its culture lifespan.

Fig. 2a, b. K19 expression in poorly organized area of a graft formed by cultured oral keratinocytes. a Seven-day graft of strain OK-F4 (hematoxylin and eosin; H&E stain). Note the disorganized multilayer and signs of cell degeneration. b K19 immunostaining of the same area as in a. Note that staining is not restricted to basal cells as in native tissue or in well-formed areas of the same graft (see Fig. 4d) but, instead, is variegated in basal and suprabasal cells.

Transplantation of cultured oral and epidermal keratinocytes to the dermis of athymic mice

Keratinocyte cultures were grown to confluence, and the cell sheet detached and grafted to the subdermal connective tissue of nude mice [5], as described in Methods.
The epithelium formed by the grafts was compared to that of the native epithelium from which the respective cell strains had been cultured. The results are summarized in Table 1. Grafts of cultured epidermal keratinocytes consistently stratified and formed a well-defined granular layer and stratum corneum by 7–10 days after transplantation (Fig. 3), as previously described [5, 18]. Like native epidermis [9, 10, 14, 28, 29], grafts formed by cultured epidermal keratinocytes consistently expressed K1/K10 suprabasally and were negative for K19 (Fig. 3) and K13. Most areas of oral keratinocyte grafts were disorganized and poorly stratified (Fig. 2). Nevertheless, some areas, representing about 10% of the linear dimension of sections taken through the centers of these grafts, exhibited at least some specific markers of the corresponding native oral epithelium (Figs. 3, 4). In well-organized regions of grafted floor of mouth and buccal keratinocytes (Fig. 4), K19 was expressed in the basal layer, as in the normal native tissue [24, 29, 45]. Suprabasal cells were K1/K10-negative and K13-positive. Attempts to graft the poorly stratified, less cohesive monolayers of soft palate keratinocytes were unsuccessful; a stratified epithelium did not form. Well-organized areas of grafts of cultured gingival and hard palatal cells were generally K19-negative, except for occasional basal cells in some areas, which were K19-positive (Fig. 3). Grafts of cultured gingival or hard palatal cells did not express K1/K10, and most suprabasal cells stained for K13 (data not shown); thus they did not recapitulate the suprabasal keratin expression pattern of native oral parakeratinized epithelium — namely, homogeneous K1/K10 expression with occasional clusters of K13-positive suprabasal cells [20, 29, 33, 35, 45]. Nevertheless, their pattern of K19 expression in grafts clearly distinguished them both from epidermal cells and from nonkeratinizing oral epithelial cells.

Xenografts of cells cultured from all oral regions expressed K13 and the cornified envelope protein involucrin suprabasally, as did the native epithelia (Fig. 4). Even poorly organized areas of oral keratinocyte xenografts expressed K13 and involucrin suprabasally (data not shown), but showed a variegated basal and suprabasal staining pattern with antibodies to keratin 19 (Fig. 2). We do not know of any technical reason for the variable and generally modest differentiation of cultured oral keratinocytes in grafts as compared with epidermal kera-
Fig. 4a-h. Keratin and involucrin expression in native, nonkeratinized oral epithelium and in graft formed by cells cultured from this tissue. a, c, e, and g show a biopsy of normal, adult floor of mouth epithelium. b, d, f, and h show the xenograft epithelium formed from cells cultured from floor of mouth, strain OK-F4. a, b: H&E stain; c, d: K19 immunostain; e, f: K13 immunostain; g, h: involucrin immunostain. (Arrows indicate the epithelial-connective tissue junction.) Note the correct compartmental expression of K19 (basal) and of K13 and involucrin (suprabasal) in the ectopic, dermal graft of cultured oral nonkeratinizing epithelial cells. This oral keratinocyte graft was K1/K10 (AE2 antibody)-negative (data not shown). (Absence of K13 and involucrin staining in some of the outermost regions of grafts was sometimes seen associated with cell degeneration, where cell protein may have been lost before fixation)
Table 2. Keratins expressed by normal oral and epidermal cells in culture

<table>
<thead>
<tr>
<th>Subtype and site</th>
<th>K5</th>
<th>K6</th>
<th>K13</th>
<th>K14</th>
<th>K16/17</th>
<th>K7</th>
<th>K8</th>
<th>K18</th>
<th>K19</th>
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<tbody>
<tr>
<td>Gingiva</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hard palate</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
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<tr>
<td>Oral typical nonkeratinizing</td>
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<td></td>
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<tr>
<td>Floor of mouth</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
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<tr>
<td>Ventral tongue</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
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<tr>
<td>Oral special nonkeratinizing</td>
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<td></td>
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<tr>
<td>Soft palate</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
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<td>--</td>
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<tr>
<td>Fetal oral</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
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<tr>
<td>Epidermis</td>
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<tr>
<td>Interfollicular foreskin, newborn</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fetal epidermis</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>--</td>
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++, moderate to high levels; +, moderate to low levels; (+), low levels; --, undetectable

tinocytes. Recent results suggest that first or second passage cultures of adult keratinocytes yield much better grafts than those of later passage, yet epidermal keratinocytes consistently form a stratum corneum, even in a graft of otherwise poor morphology.

Electrophoretic identification
of distinguishing patterns of keratin expression
in culture by two-dimensional SDS-PAGE

We compared the keratin patterns expressed in culture by cells from the various oral regions in order to detect with greater sensitivity any intrinsic differences in their differentiation characteristics. The results are shown in Fig. 5 and summarized in Table 2. Hard palatal and gingival cells expressed high levels of the four "stratified squamous epithelial keratins" (K5, K6, K14, and K16/17), which are synthesized by all normal human keratinocytes in culture [14, 30, 55], as well as low levels of K13. No K19 synthesis was detected. This keratin pattern was similar to that of cultured epidermal keratinocytes, with the exception that the adult skin keratinocytes we examined differed from gingival and hard palatal keratinocytes (and also from newborn foreskin epidermal keratinocytes) by the synthesis of low levels of K1 and the absence, or synthesis of no more than trace levels, of K13.

Cells cultured from most of the nonkeratinized oral mucosal regions (floor of mouth, buccal mucosa, and ventral tongue) synthesized keratins K5, K6, K14, and K16/17 at high levels, similar to those of hard palatal and epidermal cells, but they expressed moderate levels of K13 and low but detectable levels of K19. The cells cultured from soft palate were notably different from those of the other nonkeratinized oral regions in that they expressed K13 and K19 at high levels and also

Fig. 5 a-f. Electrophoretic separation of keratins synthesized in culture by oral and epidermal keratinocyte subtypes. Two-dimensional, nonequilibrium-pH-gradient gels, autoradiographed, of 35S-methionine-labelled, Triton/high-salt-insoluble proteins extracted from cultured keratinocytes. a Adult hard palatal (OK-HP1). b Adult soft palatal (OK-P4). c Adult epidermal (N4-Ep). d Adult floor of mouth (OK-F4). e Fetal epidermal (B4-Ep2). f Fetal floor of mouth (B4-OK-F2). Keratins are indicated by their Moll/Franke numerical designations; arrows indicate β-actin. K16, which is also expressed by all keratinocytes in culture, is not resolved from K17 in these gels. Note the distinctive patterns of expression of K7, K8, K18, K19, and K13 by the various keratinocyte subtypes when cultured in an identical environment without any possible specific mesenchymal influence.
expressed moderate levels of the simple epithelial keratins K7, K8, and K18.

**Similarities between fetal oral and adult “special nonkeratinizing” oral keratinocytes and a comparison with squamous carcinoma cells**

We wished to determine whether cells exhibiting a different, “immature” intrinsic program regulating the expression of differentiation-related keratins could be identified in fetal oral epithelium and epidermis. At 20 weeks of development, these epithelia display an immature histology: they are stratified and nonkeratinized in appearance and, whereas they express the appropriate suprabasal keratins (e.g., K13 in oral mucosa and K1/K10 in epidermis), they very notably express elevated levels of basal layer K19 as compared with that of the respective adult tissues ([2, 31]; our unpublished observations). Thus, we cultured 20-week-fetal floor of mouth and epidermal keratinocytes and examined their patterns of keratin synthesis (Fig. 5 and Table 2).

Fetal floor of mouth cells differed from adult cells cultured from the same site by the expression of simple epithelial keratins and high levels of K19 and K13. In contrast, fetal epidermal keratinocytes of the same gestational age expressed the adult epidermal keratin pattern in culture; most interestingly, they did not synthesize elevated levels of K19 under our conditions of culture, despite their expression of basal layer K19 in vivo.

In view of the surprising result that in the adult a keratinocyte subtype (namely, the “special nonkeratinizing” oral epithelial cell) expresses the same unusual combination of simple and stratified squamous epithelial keratins as seen both in fetal and in some malignantly transformed oral cells, we analyzed the keratins expressed by cells cultured from histologically abnormal oral lesions. Cells cultured from benign leukoplakias (i.e., hyperkeratinized lesions) of soft palate and gingiva expressed keratin patterns indistinguishable from those of cells cultured from histologically normal epithelia of the respective region (data summarized in Table 3). We had previously found that these abnormally keratinized areas within the boundaries of nonkeratinized oral epithelial regions were K19-negative in vivo [24]. That cells cultured from a soft palate leukoplakia synthesized the abundant K19 that characterizes cells of histologically normal soft palate epithelium suggests that the abnormal differentiation displayed by such lesions is a consequence of an altered environment sensed by cells that maintain an intrinsically normal differentiation program. This result is consistent with the clinical observation that most leukoplakias are not premalignant and can revert to a normal histology [27].

**Table 3. Keratin expression patterns of cells cultured from benign and malignant lesions as compared with normal adult and fetal oral and epidermal keratinocytes**

<table>
<thead>
<tr>
<th>Keratinocyte subtype</th>
<th>Str. Sq. K's(^a)</th>
<th>Sim. Ep. K's(^b)</th>
<th>K13</th>
<th>K19</th>
</tr>
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<tbody>
<tr>
<td>Epidermal, interfollicular</td>
<td></td>
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<td></td>
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<tr>
<td>Normal</td>
<td>++</td>
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<tr>
<td>Carcinoma</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SCC-12F.2</td>
<td>+</td>
<td></td>
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<td>+</td>
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<tr>
<td>SCC-13</td>
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<tr>
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<tr>
<td>Normal</td>
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<tr>
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<td>Oral, typical nonkeratinizing</td>
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<tr>
<td>Fetal</td>
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<tr>
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<tr>
<td>SCC-4</td>
<td>+</td>
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<td>(+)</td>
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<tr>
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<td>SCC-15</td>
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<tr>
<td>SCC-40</td>
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\(^a\) Stratified squamous keratins: K5 and K14 (expressed by all keratinocytes in native tissue and in culture) and K6 and K16/K17 (expressed by all hyperplastic keratinocytes in native tissue and by all keratinocytes in culture)

\(^b\) Simple epithelial keratins: K7, K8, and K18
We also reexamined the pattern of keratins expressed in culture by seven oral and epidermal squamous cell carcinoma (SCC) lines [39, 40, 54, 55]. As we had reported earlier, some of these lines expressed higher levels of K19 and/or the other simple epithelial keratins than did normal keratinocytes cultured from the suspected site of tumor origin. However, three of the lines, including two which were thought to originate from keratinocyte subtypes that normally express K19 in culture, did not express K19. Thus, the pattern of keratins synthesized by the SCC lines did not typically correspond to the site of origin of the tumor. Instead, the pattern expressed by three of the lines (SCC-4, SCC-15, and SCC-12.F2) resembled that of fetal oral or adult special nonkeratinizing cells (Table 3).

**Discussion**

Our experiments provide strong evidence that regional specialization of the oral epithelium (i.e., keratinized vs. nonkeratinized type of stratified squamous differentiation) is based on intrinsic differences in the keratinocyte cell type that forms the tissue in each region. Several investigators had attempted to answer this question previously by making transplants recombining anatomically alien epithelium and connective tissue. The results of some of these experiments were interpreted as suggesting that the oral cavity is lined by a single, multipotent epithelium that differentiates according to instruction from underlying connective tissue (reviewed in [7, 8]). However, more recent analyses have recognized the inherent difficulties in interpreting the results of recombining native mouse tissues [25, 43]. Furthermore, direct attempts at inducing nonkeratinized human oral epithelium to keratinize by transplanting hard palatal or gingival connective tissue beneath it have proven unsuccessful [34].

Recombination of native epithelial and connective tissue components does not lend itself to definitive interpretation, because residual epithelial stem cells may remain in the connective tissue after mechanical or enzymatic removal of the epithelium and may, therefore, be the source of the epithelium that ultimately is observed in the “recombinant” graft. We avoided this problem by growing pure populations of keratinocytes from each oral region in culture for several passages, devoid of native environmental influences, including tissue-specific stromal fibroblasts. The epithelial cells could then be studied in culture or in vivo, the latter in contact with a common, ectopic connective tissue under conditions that topographically preclude displacement of the transplanted epithelial cells by host epithelial cells in the vicinity of the graft bed.

The stability of phenotypic differences between cells from keratinized and nonkeratinized oral epithelium maintained during long-term growth in culture indicates that these oral regions are formed by different epithelial cell types. They are properly considered as distinct subtypes within the keratinocyte family, all of which are clearly different from the interfollicular epidermal keratinoctye subtype. In addition to those we have described here, it is likely that dorsal tongue epithelium, which contains structurally complex papillae and expresses an unusual set of keratins in vivo [12], is formed by a fourth oral keratinocyte subtype or, perhaps, by a combination of several, which maintain strict topological organization to form the tongue papilla structure.

The soft palatal epithelium is indistinguishable histologically and in its basal-cell-restricted K19 expression from the other nonkeratinized regions of the mouth, yet this epithelium is characterized by a distinctive keratinocyte subtype. We do not know any functional reason for this regional subspecialization of the nonkeratinizing oral epithelia. The soft palatal epithelium is contiguous with the hypopharyngeal epithelium, which covers the uvula and palatine tonsils and which abuts the nasopharyngeal and the esophageal epithelia. Hypopharyngeal epithelial cells have not been studied in culture but esophageal epithelial cells have been, and their pattern of keratin expression [4, 55] is essentially identical to that of the typical nonkeratinizing oral epithelial cell subtype, and not the soft palatal subtype. Thus the soft palatal surface does not represent a transition within the oral cavity to the esophageal keratinocyte subtype.

The gingival and hard palatal epithelia are formed by a keratinocyte subtype that is intrinsically different from either of those that form the nonkeratinizing oral surfaces, and which also is distinct from the “orthokeratinizing” interfollicular epidermal keratinocyte subtype. Interestingly, the absence of suprabasal keratin K1/K10 expression by gingival and hard palatal keratinocyte grafts in the nude mouse subdermal transplant system suggests that extrinsic factors may determine the extent to which this subtype’s intrinsically favored “parakeratinization” program (i.e., formation of a stratified corneum without dekeratinization or presence of a granular layer, in contrast to the “orthokeratinization” of epidermis) is expressed in vivo. Intrinsic differences among the various keratinocyte subtypes clearly favor expression of a particular program of stratified squamous differentiation, but this favored program may, as in the case of the keratinizing oral subtype, require a particular connective tissue environment in order to be fully expressed. Such an environment apparently is not achieved in the graft bed formed within 10 days on subdermal granulation tissue of the nude mouse. This result is consistent with the very recent report by DeLuca et al. [11] that cultured human hard palatal keratinocytes autografted to connective tissue underlying the mucogingival junction of patients suffering from severe gingival recession stratify quickly but do not keratinize until several months after grafting. Our typical result using the short-term mouse graft system, that oral keratinocytes did not differentiate as completely or as consistently as did epidermal keratinocytes, is consistent with the notion that some keratinocyte subtypes remodel their underlying connective tissue over time into a state that permits them to differentiate more completely according to their particular, intrinsically determined, program.

Some features of a usually benign pathological condition of oral epithelial regions, the so-called “white lesion”
or leukoplakia, indicate that extrinsic signals can influence oral epithelial differentiation. These lesions are hyperkeratinized areas at sites of chronic irritation within the boundaries of regions that are normally nonkeratinized [46]. The reversibility of most of these lesions to a normal histology after the source of irritation is removed demonstrates the ability of pathologic external influences such as inflammation to modulate the differentiation program of normally nonkeratinizing oral epithelial cells.

Keratinocytes cultured from 20-week-fetal floor of mouth epithelium were different from cells cultured from developmentally mature epithelium of the same oral region. They displayed some properties of the adult “typical nonkeratinizing” subtype (i.e., colony morphology and cell-cell adhesiveness) and some properties of the adult “special nonkeratinizing” subtype (i.e., expression of simple epithelial keratins and high levels of K19) in culture. After this manuscript was submitted, Oda et al. [31] reported their findings that cells cultured from fetal alveolar ridge (e.g., presumptive keratinizing oral epithelium) express substantially higher levels of K13 and K19 in culture than do adult gingival cells. Although their study did not look for expression of the other simple epithelial keratins, it is likely that even the presumptive keratinizing regions of the oral cavity are covered by the same keratinocyte subtype that we cultured from fetal floor of mouth and ventral tongue and have described here. It is remarkable that the fetal oral keratinocytes cultured in these two studies were stable during serial culture, and did not convert to the mature program of keratin expression. This result is consistent with one of the two possible scenarios of oral epithelial development: either all of the fetal cells respond irreversibly to a developmental signal to convert to a mature program of differentiation, or else rare cells possessing the mature program arise and displace the immature ones from the epithelium as development proceeds.

Either way, the distinctive fetal pattern of keratin expression and its apparent retention in only one part of the adult oral cavity may provide an important clue toward understanding the significance of apparent aberrant keratin synthesis by many squamous cell carcinomas and preneoplastic lesions of adult oral epithelium [24, 40, 54, 55]. Rare cells of the fetal and adult “special nonkeratinizing” subtype may be present at low frequency in all regions of the adult oral epithelium as rare “holdovers” from fetal development; these cells may be more susceptible to malignant transformation than the majority, “typical nonkeratinizing” epithelial cell population, which covers most of the oral cavity. Alternatively, a “switch” that shuts off the fetal program of keratin expression at a later stage of development may occasionally become defective and reverse during the course of malignant transformation, thus causing the cells to return to the fetal differentiation program. However, we cannot explain why two of the SCCs we studied (i.e., SCC-9 and SCC-40), for which clinical histories supported their origin from nonkeratinizing oral regions far from a border with a keratinizing epithelium, displayed reduced levels of K19 and other simple epithelial keratins compared with their normal cell counterparts. Perhaps a switch to a more keratinized differentiation program can also occur during malignant transformation.

A relatively simple master mechanism may determine the specific patterns of keratin gene expression and the suprabasal differentiation programs of the various keratinocyte subtypes and their malignant variants we have described here. A good candidate for such a mechanism would be one that is subject to regulation by retinoids. Dietary vitamin A deficiency in animals and humans [48, 52] causes nonkeratinizing stratified epithelia to undergo epidermoid differentiation. High levels of retinoic acid in culture suppresses K1/K10 expression and induces K19 and K13 expression in epidermal cells [15, 23]. Recent experiments (Crowe, Hu, Gudas, and Rheinwald, in preparation) have revealed that differential expression of members of the retinoid receptor family [56] is correlated with the particular keratin expression pattern in culture and in vivo of fetal and adult oral and epidermal keratinocyte subtypes. We are currently following this lead toward understanding the mechanism behind the intrinsic differences in keratinocytes we have described here.

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