Suprabasal 40 kd Keratin (K19) Expression as an Immunohistologic Marker of Premalignancy in Oral Epithelium

Kristina Lindberg*† and James G. Rheinwald‡†
From the Department of Oral Pathology and Oral Medicine, Harvard School of Dental Medicine,* the Division of Cell Growth and Regulation, Dana-Farber Cancer Institute,‡ and the Department of Cellular and Molecular Physiology, Harvard Medical School,¶ Boston, Massachusetts

The authors have studied the expression of keratin 19 in normal oral mucosa and in oral lesions exhibiting a range of histopathologic changes that are thought to precede squamous cell carcinoma. Formalin-fixed, paraffin-embedded sections were pre-treated with pronase and stained with a K19-specific antibody by the avidin-biotin immunoperoxidase method. In nonkeratinized mucosa, whether normal or benign hyperplastic, K19 was detectable in the basal cell layer. In keratinized mucosa, whether normal or benign hyperplastic, there was no detectable K19. All lesions from any oral site that exhibited atypia diagnosed from hematoxylin and eosin stained sections as moderate-to-severe dysplasia or carcinoma in situ, whether hyperkeratotic or not, stained strongly for K19 in the basal and suprabasal cell layers. The number of cell layers that were K19-positive correlated with the level in the epithelium to which dysplasia persisted. Suprabasal K19 staining tended to occur in regions of the epithelium in which expression of the terminal differentiation protein involucrin was delayed or absent. Thus, K19 expression may be linked to the retention of stem cell character or a state otherwise uncommitted to terminal squamous differentiation. Suprabasal K19 staining is clearly correlated with premalignant change in oral epithelium and therefore promises to be a useful tool in oral histopathologic diagnosis. (Am J Pathol 1989, 134: 89–98)

Oral cancers comprise 3–5% of all malignant tumors and about 90% of these are squamous cell carcinomas (SCC). These are thought to arise from lesions of the oral epithelium, termed leukoplakia and erythroplakia. Five-year survival rates in patients with oral SCC have been found to be enhanced by early diagnosis, but this has been limited by a lack of sufficiently sensitive and objective histopathologic methods for detecting premalignant changes in these oral lesions.

The keratins comprise one of five families of cytoplasmic intermediate filament proteins. Keratins are expressed exclusively by epithelial cells and cancers derived from them. Two to ten different keratins are expressed by each epithelial tissue. Keratin filaments are formed from tetrameric building blocks composed of at least two different types of M, 40–67 kd protein subunits from among a family of about 25 members, each of which appears to be encoded by a separate gene. Antibodies recognizing a broad range of keratin subunits are used routinely in immunohistopathology for determining the epithelial origin of undifferentiated malignant neoplasms and monoclonal antibodies specific for individual keratins are now available, enabling more detailed characterization of normal and pathologic epithelial differentiation.

Several years ago this laboratory reported that about one third of squamous cell carcinoma (SCC) cell lines we had cultured from oral tumor biopsies express elevated levels of an M, 40 kd keratin now known as keratin 19 (K19). Here we report the pattern of K19 expression in the oral cavity, comparing normal mucosa, premalignant lesions, and invasive carcinoma. We find that expression of K19 in the suprabasal cell layers of oral stratified squamous epithelia, disclosed immunohistochemically, is strongly correlated with premalignancy. The inverse relation between cellular content of K19 and the terminal differentiation protein involucrin in normal and dysplastic tissue suggests that K19 expression is...
linked to retention of stem cell or undifferentiated character.

**Materials and Methods**

**Tissue and Tumors**

Biopsy specimens of oral and epidermal lesions were obtained from the pathology files of the Harvard School of Dental Medicine, Tufts Dental School, Framingham Union Hospital, Brigham and Women's Hospital, and the New England Deaconess Hospital. All specimens had been fixed in formalin, processed routinely, and embedded in paraffin. Most of the samples were from nonkeratinizing mucosa, as they represent high risk sites for oral squamous cell carcinoma. These included floor of the mouth (nine cases) and ventral tongue (four cases). Six specimens of keratinizing mucosa (three of retromolar pad and three of gingiva), and three specimens of epidermal squamous cell carcinoma also were studied. Normal control nonkeratinizing and keratinizing mucosa was obtained from healthy, nonsmoking adults 25-40 years of age, and fixed in methanol/Carnoy's fixative (a mixture of methanol, chloroform and acetic acid 6:3:1 vol/vol) because this fixative was reported not to destroy keratin 19 antigenicity.

**Immunohistochemistry**

The keratin 19-specific mouse monoclonal antibody A53-B/A2, purchased from IgM Immunobiotics as K19.1, was used in most experiments. The monoclonal antibody LP2K17 or a conventional rabbit antiserum specific for K1911 was sometimes used for immunostaining of methanol/Carnoy's-fixed tissue, and yielded the same results as A53-B/A2. A rabbit anti-human involucrin antiserum18 was provided by Dr. R. Rice. The avidin-biotin-peroxidase (ABC) technique (Vector Laboratories, Burlingame, CA) or indirect immunofluorescence performed as described elsewhere19 was used for K19 and involucrin staining.

We found that formalin fixation masks the antigenicity of keratin 19 for the three antibodies we tested. Treatment of sections with 0.1% pronase (Sigma Chemical Co., St. Louis, MO) for 1-3 hours20 exposed the antigen and permitted adequate staining with antibody A53-B/A2 for about half of the samples tested. The two other K19 antibodies were unreactive with pronase-treated sections. Three-hour pronase treatment resulted in severe tissue destruction of some specimens; these were excluded from consideration. Salivary glands or basal cells of normal nonkeratinizing mucosa present in the specimen served as internal positive controls for K19 staining in each section. If a lesion appeared K19-negative but normal, K19-containing structures were either absent or did not stain, the sample was excluded from consideration. Formalin-fixation did not block staining with the anti-involucrin antibody.

Tissue sections were successively treated for 30-minute incubations (interspersed by brief rinses with PBS) with normal horse serum (1:70), anti-K19 antibody (1:20 dilution), and biotinylated horse anti-mouse immunoglobulin IgG (1:200 dilution). Avidin-biotin-peroxidase complex (1:50) (Vector Laboratories) was then applied for 60 minutes. The sections were then rinsed and incubated for 2-7 minutes in a solution of the peroxidase substrate diaminobenzidine tetrahydrochloride. The stained sections were finally dehydrated and mounted with Permount.

**Tissue Analysis**

K19 staining pattern and conventional hematoxylin and eosin (H & E) staining characteristics of each case were studied in parallel using light microscopy. The histologic evaluation of H & E stained sections was performed by two independent observers (K.L. and Dr. George Gallagher, a pathologist from Harvard School of Dental Medicine). The epithelium was classified as exhibiting one or more of the following histologic patterns: normal, hyperplasia, hyperkeratosis, mild dysplasia, moderate-to-severe dysplasia, carcinoma in situ, or invasive squamous cell carcinoma, according to standard criteria.21 K19 staining was recorded for its presence or absence in basal and suprabasal cells in epithelia and for its overall distribution in tumors.

**Results**

**Nonkeratinized Mucosa**

Normal mucosa from floor of the mouth and ventral tongue stained strongly for K19 in virtually all basal cells (Figure 1a, b) as recently reported for nonkeratinized oral epithelia.22 In biopsies exhibiting benign reactive basal cell hyperplasia, K19 staining was similarly restricted to the basal cell layer. However, when such hyperplastic areas were also hyperkeratotic, the basal cells were K19-negative (Figure 1c, d). Epithelium with mild dysplasia showed K19 staining of the basal cells and, in most cases, also of the atypical cells in the suprabasal layers. When hyperkeratosis was present, however, regions that were mildly...
dysplastic were sometimes completely K19-negative (Figure 1e, f).

Epithelium with moderate-to-severe dysplasia, with or without hyperkeratosis, always showed suprabasal K19 staining involving all strata in which dysplastic cells were evident (Figure 2a, b). Sections displaying full thickness dysplasia (so-called carcinoma in situ) were K19-positive in all cell strata (Figure 2c, d). Six of eight cases of squamous cell carcinoma (SCC) showed a patchy distribution of predominantly K19-positive cells (Figure 3b). Two cases of squamous cell carcinoma failed to show K19 staining, while the adjacent hyperplastic epithelium showed normal, basal cell staining (Figure 3d). These results are summarized in Table 1.

Keratinized Mucosa

Normal gingiva and retromolar pad were negative for K19 in all cell layers (Figure 4b) as reported previously. No
example of hyperplastic or mildly dysplastic epithelium was found among the cases included in this study. Lesions containing moderately-to-severely dysplastic areas showed a patchy, suprabasal distribution of K19-positive cells (Figure 4d). Areas of carcinoma in situ contained K19-positive cells throughout the epithelium (Figure 4f). A case of early invasive SCC of the retromolar pad (Figure 3e, f) had K19-positive basal cells. The lower spinous cells and invasive cells were also K19-positive. H & E-stained sections of the same case disclosed that the surface cell layers were well organized and had no dysplastic features; these cells were K19-negative (Figure 3f). Two of four SCCs examined were K19-negative or contained only occasional positive cells (Figure 3h). The K19-negative tumors were well-differentiated, whereas the K19-positive tumors were poorly or poorly-to-moderately differentiated. These results are summarized in Table 1.

**Epidermis**

Normal adult epidermis was K19-negative (data not shown). Three epidermal SCCs were studied. Only one case disclosed substantial expression of K19 in the invasive tumor nests. This tumor was graded as moderately differentiated. Rare, weakly positive basal cells, and occasional positive cells in the invasive nests were detected in the two other cases, both of which were well-differentiated (data not shown).

**Involucrin Expression**

Normal nonkeratinizing and keratinizing oral mucosa expressed involucrin in a homogeneous suprabasal pattern (Figure 5c), essentially identical to that described previously for ectocervical epithelium. Involucrin was both delayed in expression to more superficial cell layers and decreased in amount with increasing degree of dysplasia (Figure 5f). Moderate-to-severely dysplastic lesions stained for involucrin only in the most superficial cell layer (not shown). Involucrin expression was greatly reduced or was absent in invasive oral SCCs. When present it was confined to well-differentiated keratotic areas with squamous pearl formation (Figure 5i).
Figure 3. Keratin 19 expression in oral squamous cell carcinomas. Panels a, c, e, and g show H & E-stained tissue sections with nearby sections immunostained for K19 at right in panels b, d, f, and h. a, b: SCC of floor of mouth. Note that most cells in invasive nests are K19 positive, and the connective tissue stroma is negative. c, d: SCC of ventral tongue. Note that hyperplastic epithelium on the left side of the field shows a normal K19 staining pattern with positive basal cells while invasive cell nests at right (arrow) are K19 negative. e, f: Early invasive SCC of retromolar pad. Intense, chronic inflammatory cell infiltration in the lamina propria obscures the image of the invading cells in e. Note that the well-organized surface cell layers do not express K19 whereas the invasive cells are K19 positive (f). g, h: Well differentiated SCC of gingiva. Note that the tumor contains only rare K19 positive cells.
Table 1. Keratin 19 Distribution in Lesions of Nonkeratinized and Keratinized Oral Mucosa

<table>
<thead>
<tr>
<th>Histopathologic classification</th>
<th>Regions examined</th>
<th>Nonkeratinized</th>
<th>Keratinized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Basal cell hyperplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonkeratinized</td>
<td>5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hyperkeratinized</td>
<td>5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>2</td>
<td>+</td>
<td>+,-</td>
</tr>
<tr>
<td>Nonkeratinized</td>
<td>4</td>
<td>+,-</td>
<td>+,-</td>
</tr>
<tr>
<td>Hyperkeratinized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate-to-severe dysplasia</td>
<td>4</td>
<td>+</td>
<td>+,-</td>
</tr>
<tr>
<td>Nonkeratinized</td>
<td>4</td>
<td>+</td>
<td>+,-</td>
</tr>
<tr>
<td>Hyperkeratinized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>12</td>
<td>+,-</td>
<td>+,-</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+, K19 staining; 
-, No K19 staining; 
+,-, Variability of K19 staining among specimens with the same histopathologic classification. 
ND, Not done.

Discussion

Our results indicate that the basal cells of normal and benign hyperplastic regions of nonkeratinizing mucosa of the oral cavity express keratin 19, but that this keratin is not detectable in the suprabasal cell layers. Normal or benign, hyperplastic regions of keratinizing oral epithelia do not contain detectable K19 in any cell layer. In contrast, moderate-to-severe dysplasias and carcinomas in situ of all oral epithelia invariably contain K19 in the suprabasal as well as the basal cells, providing a simple immunohistologic stain for premalignant change. The ten benign hyperplastic lesions studied had a K19 distribution identical to that of normal oral epithelium. Thus, suprabasal K19 expression is not a simple reflection of a hyperproliferative state, but rather is a marker of cellular atypia associated with premalignancy. It would be interesting to examine the distribution of K19-positive cells in tissue that is hyperproliferative as a result of wound healing so as to generalize the conclusion that suprabasal K19 never occurs in nonmalignant tissue.

The first indication that a change in keratin expression, specifically induction of keratin 19 synthesis, might be useful in assessing malignant change in the oral epithelium began with this laboratory's report that cell lines cultured from two of six oral and one of three epidermal squamous cell carcinomas expressed high levels of keratin 19, whereas cells cultured from normal oral and epidermal tissue did not.11 We have now examined a total of nine oral SCC cell lines and have found that four of them express high levels of K19 in culture (unpublished results). The present study has identified K19 in some oral carcinomas in vivo, and has consistently detected abnormal (suprabasal) expression in premalignant oral lesions.

The presence of K19 in basal cells of normal, nonkeratinizing oral mucosa was also reported by Morgan et al22 as our study was nearing completion. An earlier report23 did not find K19 in extracts of total keratins from oral epithelium analyzed by 2D gel electrophoresis. Most of the keratin protein of stratified squamous epithelia is in the suprabasal cell layers, however. Thus, basal cell keratins represent only a very small proportion of the keratins seen on two-dimensional gels. This small amount is, nevertheless, readily detectable by antibody staining in tissue sections.

We reported earlier that normal oral nonkeratinizing mucosal keratinocytes synthesize low to undetectable levels of K19 in culture,12 even though most of the cells are actively proliferating and might be expected to possess certain features of basal cells in vivo. In a recent electrophoretic analysis of keratin expression in adult keratinocyte strains cultured from ventral tongue, buccal, and floor of mouth, we found variable levels of K19 expression ranging from trace amounts to easily detectable, but low, levels.25 These results indicate that culture conditions do not always favor K19 synthesis and also that K19 expression is not functionally involved with the proliferative behavior of the cell. We have also cultured keratinocytes from 21 week fetal oral lining mucosa, and find that these cells express high levels of K19 in vitro.22,25 Clearly, there are developmental as well as regional differences in the oral epithelium with respect to mechanisms regulating K19 expression. The nature of these mechanisms and how they are modulated by exogenous signals such as vitamin A25 remain to be elucidated, but seem amenable to study in culture.

Not all oral squamous carcinoma cell lines express K19 in culture. In the present study, we found that K19 was not expressed in vivo by 4 of 12 invasive squamous cell carcinomas that could be scored with confidence for presence or absence of this keratin. We interpret this as indicating that, despite the consistent retention of K19 ex-
pression by suprabasal cells during early stages of malig-
nant progression, the K19 gene is sometimes turned off by cells at later stages of the disease. The more well-
differentiated of the tumors we examined were K19-nega-
tive, although the number of samples in our study was
small and the evaluation of the grade of differentiation is somewhat subjective.

We found in several samples an inverse relation be-
tween K19 and involucrin expression in normal and pre-
malignant oral epithelial tissue. K19 is restricted to the

Figure 4. Keratin 19 expression in keratinized oral mucosa. Panels a, c, and e show H & E stained tissue sections with nearby sections immunostained for K19 in panels b, d, and f. a, b: Methanol/Carnoy’s-fixed normal gingiva (keratinized epithelium). Note that the entire epithelium is K19 negative. c, d: Formalin-fixed biopsy from retromolar pad showing moderate-to-severe dysplasia. Note that the distribution of K19-staining cells correlates with cellular atypia and that the well-differentiated surface layers lacking cellular atypia (arrowheads), are K19 negative. e, f: Formalin-fixed biopsy from retromolar pad. Carcinoma in situ. Virtually all cells are K19 positive.
basal layer of normal lining mucosa while involucrin expression begins in the first suprabasal cell layer (Figure 5c). K19 expression in suprabasal cells was accompanied by a delay of involucrin expression until even higher cell layers of the epithelium (Figure 5e, f). This suggests that retention of K19 by suprabasal cells in oral lining epithelium is a consequence of a delay in commitment to terminal differentiation and may indicate retention of proliferative (stem cell) potential by K19-positive suprabasal cells. Expression of K19 may, therefore, be coupled with long-term replicative potential, in contrast to a delay of overt expression of differentiation by committed cells that have lost stem potential, as occurs in benign hyperplastic lesions. Further studies are needed to define more precisely the correlation between stem character and K19 expression in the oral non-keratinized mucosa, and to determine the mechanism behind this association. Certainly K19 is unlikely to be causally related to epithelial stem cell
character, K19 is not expressed by stem cells in keratinized epithelia, and the pattern of K19 expression in the mammary gland has been interpreted as indicating that mammary stem cells are K19-negative.16

General application of K19 immunostaining for routine histopathologic evaluation is impeded by the fact that formalin fixation damages the keratin 19 antigenic sites recognized by three different K19-specific antibodies: two mouse monoclonal antibodies and a rabbit polyclonal antiserum. Protease treatment of formalin-fixed sections succeeded in revealing this antigen to one antibody (A53B/A2) without excessive damage in half of approximately 40 cases we examined. Clearly methanol/Carnoy’s fixative would be recommended for tissue specimens that might be examined immunohistochemically.

Immunohistochemical assessment of K19 expression should be extended to lesions of other tissues similar to oral mucosa, such as the esophageal and cervical epithelia. Small amounts of keratin 19 are expressed by these epithelia in culture,13,28 and in vivo22,29 where K19 has been found to be restricted to the basal cell layer.30,31 An increase in K19 content has been detected electrophoretically in extracts of some esophageal and cervical carcinomas,13,27,32 suggesting that neoplastic lesions of these tissues may display aberrations in K19 distribution similar to those reported here.

References


Acknowledgment

The authors thank Dr. George Gallagher for his help in conventional histopathologic diagnosis of oral specimens, Dr. R. Rice, Harvard School of Public Health, and Dr. B. Lane, Imperial Cancer Research Fund Laboratories, London, for their gifts of involucrin antisera and LP2X antibody, respectively. Drs. Ramzi Co-Turan and Howard Green made helpful suggestions about the manuscript. Maria Brown sectioned embedded tissue samples and Virginia Ribeiro and Kelly Havican typed the manuscript.