Protein and mRNA Expression of Simple Epithelial Keratins in Normal, Dysplastic, and Malignant Oral Epithelia

Lan Su,* Peter R. Morgan,* and E. Birgitte Lane†

From the Department of Oral Medicine and Pathology,* United Medical and Dental Schools of Guy’s and St. Thomas’s Hospitals, London, United Kingdom, and the †Cancer Research Campaign Laboratories, Department of Anatomy and Physiology, Medical Sciences Institute, University of Dundee, Dundee, United Kingdom

Simple epithelial keratins K7, K8, and K18 are present in no more than trace amounts in normal stratified squamous epithelia but have been reported in squamous cell carcinomas. With the aim of determining the level at which keratin synthesis is regulated in vivo, we have compared the expression of mRNA by in situ hybridization and protein by immunohistochemistry for K7, K8, and K18 in a series of normal, dysplastic, and malignant oral epithelia. In normal epithelia mRNAs for K7, K8, and K18 were present in basal and lower spinous cells but adjacent sections were generally negative for the respective proteins. In severe dysplasia there was irregular suprabasal extension of K8 and K18 mRNAs in all cases and their proteins were expressed in more than half of the cases. The carcinomas expressed K8 and K18 mRNAs homogeneously and were strongly reactive for these keratin proteins but K7 expression appeared reduced in malignancy. These results are consistent with the post-transcriptional regulation of K7, K8, and K18 expression in normal epithelia and the presence of their proteins in dysplastic and malignant epithelia suggests the release of these epithelial cells from a post-transcriptional block on K8 and K18 translation. Alternatively, rapid degradation of K8 and K18 protein might be occurring in normal epithelia but be suppressed in dysplasia and malignancy. (Am J Pathol 1994, 145:1349–1357)

Previous biochemical and immunohistochemical studies have indicated that squamous cell carcinomas (SCCs) express a wider range of keratin expression than would be expected from the pattern shown by their normal parent epithelium. A striking feature of altered keratin expression in SCC is the anomalous expression of simple epithelial keratins K8, K18, and sometimes K7. Normally, simple epithelial keratins are expressed in luminal cells of glandular ducts and none of these keratins are major constituents of stratified squamous epithelia.

The means by which these changes are brought about are not clear, indicating the need for a better understanding of the regulation of gene expression of K7, K8, and K18 in normal and neoplastic epithelia. An in vitro study has suggested that the expression of K8 and K18 can be controlled at different levels in transformed non-epithelial cells. One of our recent studies has demonstrated that the expression of K7, K8, and K18 in simple epithelium of normal salivary glands is regulated at the transcriptional level but this may not necessarily be true of keratinocytes. We have mapped the distribution of these keratin transcripts for comparison with their protein distribution to provide insight into the molecular basis of normal human keratin expression and the changes that occur in dysplasia and SCC.

If tissue morphology is to be preserved, analysis of keratin gene expression at the transcript as well as protein levels involves combining in situ hybridization (ISH) and immunohistochemistry (IHC). By using riboprobes constructed for K7, K8, and K18 specific for ISH and monoclonal antibodies (MAbs) to these individual keratins for IHC, the distribution of the mRNAs and proteins of simple epithelial keratins in tis-

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Dr. Su’s current address: Emory University School of Medicine, Division of Oral, Head and Neck Pathology, 160 Clairemont Avenue, Suite 400, Decatur, GA 30030.

Address reprint requests to Dr. P. R. Morgan, Department of Oral Medicine and Pathology, UMDS, Floor 28, Guy’s Tower, Guy’s Hospital, London SE1 9RT, UK.
sues can be compared directly. Oral mucosa is especially useful for mapping keratin gene expression as it exhibits a variety of epithelial types in close proximity and accessibility of routine mucosal biopsies from the oral cavity enables the easy collection of fresh tissues required for mRNA ISH.

Materials and Methods

Tissue Samples

Twenty-seven specimens of fresh human oral mucosa were obtained. Each was rapidly frozen on to cork in OCT cryoprotectant, quenched in melting isopentane, and stored in liquid nitrogen within 30 to 60 minutes of removal. Serial cryostat sections (6 μ) were mounted on thoroughly clean, 3-aminopropyl-trimethoxysilane-coated microscope slides.

Normal oral mucosa (10 specimens) from buccal, labial, floor of mouth, hard palate, dorsum of tongue, and attached gingival sites, was obtained from patients undergoing surgery for odontogenic or benign salivary gland tumors or from biopsies subsequently reported as normal. The diagnoses of moderate to severe dysplasia (nine cases) and SCC (eight specimens) were confirmed by conventional histological analysis. Specimens of these lesions were obtained from intra-oral sites normally lined by noncornified epithelium.

Designing Keratin Riboprobes

From published data on gene sequences of human K7, K8, and K18 stretches unique to each keratin were identified within 3' coding and non-coding areas of the mRNA. Each pair of oligonucleotide primers for these keratins was designed to define each desired fragment and were synthesized with the incorporation of EcoRI or HindIII sites (Table 1). Whole cDNA clones of human K7, K8, and K18 were used as templates for polymerase chain reactions and the products were subcloned into the pGEM4 transcription vector. The dideoxy chain termination method was used to confirm the fidelity of the desired sequence.

Synthesis and Labeling of Riboprobes

Recombinant plasmids (pGEM4) containing cDNA sequences specific for K7, K8, and K18 were used as the templates of riboprobes and their specificity has been confirmed both by Northern blot and ISH.

Transcription in vitro was carried out in a transcription buffer (Promega, Southampton, UK), 10 mmol/L dithiothreitol, 1 unit/μl ribonuclease inhibitor, 160 μmol/L ribonucleotides (rNTP), 120 μmol/L [35S]UTP (200 μCi; Amersham International, Amersham, UK), 1 μg of the linearized template DNA, and T7 RNA polymerase for antisense RNA (cRNA) probe or SP6 RNA polymerase for sense RNA (sRNA) probe at 37 C for 60 minutes. Nensorb 20 cartridge (Du Pont, Stevenage, UK) was used to remove unincorporated [35S]UTP and the efficiency of the labeling was determined by trichloroacetic acid precipitation. The quality of labeled riboprobes was monitored by polyacrylamide-urea (sequencing) gel electrophoresis.

In Situ Hybridization

ISH was carried out as described previously. In brief, the tissue sections were fixed in 4% paraformaldehyde and incubated in a hybridization mixture containing approximately 4 × 10⁶ cpn/μl [35S]labeled cRNA probe for each keratin. The hybridization reaction was carried out at 45 C for 16 hours. After a series of washes of increasing stringency (approximately 10 C below the calculated dissociation temperature of the probe-target duplexes formed) and post-RNAse digestion, the slides were dipped in Ifford K-5 nuclear emulsion and exposed for 20 to 35 days at −20 C. Sections were examined by bright field, dark field, or tandem scanning confocal microscopy (TSM). The TSM was configured in combined

<table>
<thead>
<tr>
<th>Keratins</th>
<th>Primers</th>
<th>Nucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>K7</td>
<td>P1</td>
<td>5’ (1318)AGC AGT GAA TTC TGG CTT ATG ACC 3’</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>5’ (1495) GTG GCT GGA AGC TGG TGG TGA GAG GC 3’</td>
</tr>
<tr>
<td>K8</td>
<td>P3</td>
<td>5’ (1557) GAG CCT GAA TTC GAG GCC GCT ATG C 3’</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>5’ (1697) AAT TGT AAG CTT GCT GAA GGC ATG GCC 3’</td>
</tr>
<tr>
<td>K18</td>
<td>P5</td>
<td>5’ (1224) ATC TGA GAA TTC CCT TGG ACA GCA A 3’</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>5’ (1386) TTA TGT GCA AGC TTC TCC CCA AAC GGT ACC 3’</td>
</tr>
</tbody>
</table>

* The number in parentheses indicates the site of nucleotide sequences on the full length of keratin cDNAs.

1 The sequences for the restriction enzyme sites of EcoRI (GAA TTC) or HindIII (AAG CTT) are underlined.
conventional and confocal modes\textsuperscript{2,1} to maximize grain visualization and tissue localization.

**Immunohistochemistry**

IHC was performed on selected acetone-fixed sections adjacent to each series of those collected for ISH. The primary layer consisted of a MAb specific for the appropriate individual keratins: RCK105\textsuperscript{22} and LP5K\textsuperscript{23} for K7, M20\textsuperscript{24} and CAM 5.2\textsuperscript{23} for K8, and RCK106\textsuperscript{25} and LE61\textsuperscript{26} for K18. A conventional method of biotin-streptavidin-peroxidase complex was used (Dako, High Wycombe, UK).

**Controls for ISH and IHC**

Minor salivary glands or ducts underlying normal oral epithelia in most specimens were examined as internal positive controls for K7, K8, and K18 mRNA and protein.\textsuperscript{11} Three types of negative control for ISH were included: 1), \textsuperscript{35}S-labeled mRNA probe for each keratin with specific activities; 2), RNase predigestion; and 3), prehybridization with cold cRNA probes. The negative control for IHC consisted of the substitution of the primary antibody with phosphate-buffered saline or an irrelevant antibody.

**Quantitation of Results**

A semi-quantitative 4-point scale was employed (see Table 2) for assessment of both ISH (0 to 3) and IHC (0 to ++ +) reaction products. A score of 0 represents a distribution of silver grains indistinguishable from that of background whereas 3 denotes dense labeling. A score of 1 indicates labeling just above background levels and 2 indicates an intermediate level. Similarly, a minus (−) represents an epithelium with no IHC reaction product and + to +++ indicates levels from low to high, respectively. To indicate the extent of heterogeneous expression, oc denoted positivity in an estimated 30% or less of cells whereas h indicated that positive cells made up between 40 and 70% of the total.

**Results**

**Normal Oral Mucosal Epithelia**

K7 mRNA was detected consistently in the basal and spinous cells of both cornified and noncornified epithelium (Figure 1). Immunohistochemical staining with K7 MAb RCK105 and LP5K showed no staining in any cells of the mucosal epithelium except for Merkel cells in cornified epithelium.

Similar labeling to that of K7 was seen in normal epithelia with K8 and K18 cRNA probes, distributed mainly in the basal and lower spinous cells (Figure 2) and in rete processes where these were present. Staining with K8 MAbs M20 and CAM5.2 was absent from most keratinocytes, but occasional weak staining was present in basal cells of noncornified epithelium. MAbs RCK106 and LE61 (to K18) failed to stain keratinocytes but, like M20 and CAM5.2, revealed Merkel cells in specimens from attached gingiva and hard palate.

There were no significant differences in the mRNA expression of K7, K8, and K18 between cornified and noncornified oral epithelia (Table 2).

**Table 2. The Distribution of mRNAs and Proteins of K7, K8, and K18**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Protein</th>
<th>mRNA</th>
<th>Protein</th>
<th>mRNA</th>
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</tr>
</thead>
<tbody>
<tr>
<td>K7</td>
<td></td>
<td>K8</td>
<td></td>
<td>K18</td>
<td></td>
</tr>
<tr>
<td>Cornified epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rete processes</td>
<td>1−2</td>
<td>−</td>
<td>2</td>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td>Noncornified</td>
<td>0−2</td>
<td>−</td>
<td>1−2</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>Basal cells</td>
<td>1−2</td>
<td>+h</td>
<td>2</td>
<td>++h</td>
<td>2</td>
</tr>
<tr>
<td>Spinous cells</td>
<td>0−2</td>
<td>−</td>
<td>1−2</td>
<td>−</td>
<td>1−2</td>
</tr>
<tr>
<td>Dystasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal layer</td>
<td>2</td>
<td>+h (1/9)</td>
<td>2−3 (5/9)</td>
<td>++h (5/9)</td>
<td>2−3 (5/9)</td>
</tr>
<tr>
<td>Suprabasal layer</td>
<td>2</td>
<td>+h (5/9)</td>
<td>2−3 (5/9)</td>
<td>− (4/9)</td>
<td>1−2 (4/9)</td>
</tr>
<tr>
<td>SCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>1</td>
<td>−</td>
<td>2</td>
<td>−</td>
<td>2−3</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>2</td>
<td>+ + to + + +</td>
<td>2−3</td>
<td>+++ +h</td>
<td>2</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>0</td>
<td>−</td>
<td>2−3</td>
<td>+ +</td>
<td>1−2</td>
</tr>
</tbody>
</table>

\* A four-level scale represents the density of silver grains by ISH (0 to 3) and the staining intensity by IHC (− to ++ +). Where expression is heterogeneous, h indicates that positive cells comprise an estimated 40 to 70% of the total whereas oc (occasional) indicates that they make up 30% or less. See text for further details.

\* Numbers in parentheses indicate the ratio of positive cases to the total if it is not 100%.
Figure 1. In situ hybridization with the K7 cRNA probe in oral noncornified epithelium. The labeling is located in the basal and some spinous cells. Figures 1 to 4 were photographed with the TSM under combined confocal and bright field conditions. Magnification, ×540.

Figure 2. Expression of K18 mRNA in oral noncornified epithelium showing moderate labeling in the basal and lower spinous cells.

Figure 3. Expression of K18 mRNA in oral dysplasia. Abundant signal is homogeneously distributed in the rete process.

Figure 4. Expression of K8 mRNA in a moderately differentiated SCC. A substantial amount of K8 mRNA is present in the tumor cells.
**Dysplasia**

A trend for increased labeling of K7 mRNA was observed in all cases of dysplasia, whereas a weak, heterogeneous staining for K7 protein was present suprabasally in only five cases. There was inconsistent expression of K8 and K18 in different cases (Table 2). Of these, five showed stronger labeling of K8 and K18 mRNAs in both basal and suprabasal layers (Figures 3, 5, and 6) than in normal epithelia, and positive staining for K8 and K18 proteins was distributed focally in the lower layers of the same cases. The remaining four showed similar labeling to that of normal epithelia and there were no detectable proteins except for one case that gave a moderate staining in some cells of the lower layers with K18 MAbs. The cells that were positive for K8 and K18 proteins were located heterogeneously in the deep rete ridges of dysplastic epithelium (Figure 7).

**Squamous Cell Carcinomas**

The intensity of labeling with the K7 cRNA probe in well and moderately differentiated SCCs was similar to that in basal cells of normal epithelia. No signal for K7 mRNA could be detected in the single poorly differentiated SCC examined. Immunohistochemically, a moderate reaction to K7 MAbs was observed in a few sporadic tumor cells from only one moderately differentiated SCC.

The expression of K8 mRNA in well differentiated SCCs was similar to that in basal cells of normal epithelia but was greater in moderately differentiated SCCs (Figure 4) and the poorly differentiated case. Labeling for K18 mRNA in well differentiated SCCs was more intense than that in normal epithelia but moderately differentiated SCCs and the poorly differentiated case showed a similar level of mRNA expression to that in basal and lower spinous cells of normal epithelia.

K8 and K18 proteins were expressed similarly to each other in the SCCs studied (Figure 8). In well to moderately differentiated SCCs, the strongest staining for K8 and K18 was present in the basal and less differentiated cells whereas cells with a spinous morphology showed a trace of, or moderate, reaction. Uniform staining for these two keratins was observed in the poorly differentiated SCC (Table 2).

**Discussion**

**General Considerations of ISH and IHC**

It is well accepted that mRNA ISH with radiolabeled riboprobes can give a higher sensitivity than when non-radioactive probes are used but the presence of background often reduces its sensitivity. To overcome this obstacle was a challenge in the present study because the mRNAs of simple epithelial keratins in stratified epithelia are not always as abundant as that of K14,11,19,29 It is of prime importance to ascertain that background is not due to the widespread low-level expression of the mRNA in question. To achieve a satisfactory signal-to-noise ratio, adequate stringency of post-hybridization washing was determined empirically for each keratin probe used for both Northern blot and ISH,11,12 and this washing appeared to suppress most nonspecific binding (Figures 5 and 6). Careful autoradiography helped to control the internal background of the emulsion. Thus, with sufficient reduction of background, a low level of mRNA expression could be distinguished from nonspecific signals.

There are certain advantages in using TSM, which gives sharp resolution of silver grains and showed a low density of hybridized signals better than other approaches (compare Figures 3 and 5). By combining conventional and confocal modes, silver grains and tissue structure were displayed more readily than with dark field microscopy.20,21

IHC was the method by which the distribution of keratin protein was demonstrated for close comparison with mRNA. For this it was important that the MAbs could be relied upon to detect any keratin protein present. Therefore, MAbs were selected that had a good record in use for specificity and sensitivity in their laboratories of origin. All have been used in several relevant IHC studies in a variety of tissues and were consistent in their tissue-specific localization. A further precaution against laboratory error or unexpected masking of an epitope was the use of multiple antibodies for the same keratin.

**Simple Epithelial Keratin mRNAs Are More Widely Expressed in Stratified Epithelia than Their Proteins**

Previous studies with MAbs to each simple epithelial keratin employing immunoblotting and IHC analysis did not record any of these keratins in oral stratified epithelia except for a few sporadic Merkel cells in the basal layer of cornified epithelium.13,36 Results from IHC in the present study were in general agreement with those of previous studies and an additional observation was a weak positive reaction to K8 with M20 and CAM5.2 in some basal cells of noncornified epithelium.
Figure 5. Expression of K18 mRNA in oral dysplasia. The signal is distributed similarly in dysplastic epithelia to that in Figure 3 but the lower magnification emphasizes the satisfactory signal-to-noise ratio. Photographed under bright field conditions; magnification, ×240.

Figure 6. Negative control for K18 mRNA expression in oral dysplasia. This serial section (close to that illustrated in Figure 5) was incubated with the K18 sRNA (sense) probe and shows a low level of randomly distributed silver grains in both dysplastic epithelium and underlying connective tissue. Photographed under bright field conditions; magnification, ×240.

Figure 7. Expression of K18 protein in oral dysplasia. IHC with MAb RCK106 shows heterogeneous staining in a rete process of dysplastic epithelium in an adjacent section to that illustrated in Figure 5. Photographed under bright field conditions; magnification, ×120.

Figure 8. Expression of K8 protein in a squamous cell carcinoma. Moderate staining with MAb M20 is seen in a field corresponding to Figure 4 in an adjacent section. Photographed under bright field conditions; magnification, ×240.
Messenger RNAs for these simple epithelial keratins in oral stratified epithelia have not been investigated previously. In addition, no evidence of K7 mRNA in any stratified squamous epithelia has been published before. It is of interest to note that the mRNAs of simple epithelial K7, K8, and K18 were consistently present in normal stratified oral epithelia and that the labeling intensity was no different between the protein-positive and protein-negative areas (Table 2). A search in esophageal epithelium for K8 and K18 revealed similar findings to ours.36

That keratin mRNA is expressed more widely than its respective protein has been reported also in normal epidermis in respect of K6 and K16.37,38 As in the case of K6 and K16, post-transcriptional control of K7, K8, and K18 expression might be operating in normal stratified oral epithelia.

The reason why these keratins should be controlled post-transcriptionally in normal stratified epithelia is not clear. K8 and K18 are known to be expressed in early embryonic epithelia but to disappear from stratified epithelia after birth.6,39 There may be a program during the development of epithelial stratification to downregulate the expression of K8 and K18 genes and meanwhile to induce differentiation-specific keratins, such as K4 and K13 in noncornified and K1 and K10 in cornified epithelia. Alternatively, these genes may never, or only rarely, be completely silenced. The low level of K8 protein detected in some sites suggests that translation is sometimes implemented, the product probably being degraded rapidly in the absence of a partner keratin of the complementary type.

The reason for the presence of K7 mRNA in stratified squamous epithelia remains obscure but our study has revealed a similar distribution of mRNA between K7 and K8 in that tissue. Protein expression of both K7 and K8 is often found in the same cells, although a range of dissimilarities in expression confirmed that these two genes are nevertheless controlled differently. Expression of these two keratins in basal cells of noncornified epithelium suggests that they might serve as polymerization partners of the type I K19. In addition, sequence analysis of K7 has shown close homology between coding portions of the K7 and K6b genes,15 i.e., evidence that a simple epithelial keratin gene has some structure in common with one of the epidermal keratin genes. The fact that K7 protein is expressed in transitional stratified epithelium22,31 also implies a linkage of K7 to stratified epithelium.

Altered Levels of Keratin Expression in Premalignant Lesions

It is generally accepted that SCC may develop from both oral and cervical dysplasia, particularly if the changes are severe.32,40–42 As the premalignant lesions progress towards invasion, multiple changes may take place in gene expression. The study of these lesions in respect of K7, K8, and K18 may advance our understanding of how keratin gene control is altered before malignant transformation. The findings in this group of oral lesions have shown clearly that the mRNAs of simple epithelial keratins, which are not translated into stable protein in normal stratified epithelia, do produce stable keratin protein to varying degrees in dysplasia, a moderate staining for K8 and K18 and weak staining for K7 being detected in most dysplasias (Table 2).

The evidence for the expression of K8 and K18 protein in dysplasias is consistent with previous IHC studies in oral5 and cervical dysplasias.32,41 It seems that the translation of simple epithelial keratins might gradually become functional in dysplasia, a clue that may indicate some linkage with the pattern of K8 and K18 protein expression in malignancy.

The Pattern of Simple Keratin Expression in SCCs

Proteins of simple epithelial keratins, especially K8 and K18, have been detected by IHC in both mucosa5–7,43 and epidermal2 SCCs, with results similar to those of the present study. The mechanism for this phenotypic shift is unclear as is its significance. However, ISH has revealed more information on the level of gene activity, especially when this is integrated with similar data on normal and dysplastic epithelia. The finding that the expression of K8 and K18 transcripts paralleled that of their proteins in oral SCCs contrasts with normal mucosa in which mRNAs only were present. It seems that the control normally suppressing translation of K8 and K18 is lost after malignant transformation and thus, the regulation of expression of these two keratins in SCCs is switched to the transcriptional level.

The reason why the expression of K8 and K18 is regulated differently between normal and malignant epithelia remains to be explored further. It is tempting to postulate that the development of malignant lesions may involve the removal of a block on K8 and K18 translation resulting in the synthesis of corresponding proteins. It is possible that oncogenes such
as v-ras\textsuperscript{44} and the c-myc family\textsuperscript{45} might be involved in such activities. An alternative explanation, not testable with the present methods, is that immediate degradation of these keratin proteins occurs in normal epithelia,\textsuperscript{46-48} whereas they are stabilized in dysplasia and malignancy.

In contrast to the findings with K8 and K18, K7 transcription appeared to be suppressed in malignancy. The expression of K7 mRNA, present in normal and dysplastic epithelia was reduced in SCCs and that of its protein was only focally expressed. The fact that a weak protein staining was present suprabasally in some dysplasias and lichen planus (personal observations not presented here), and sporadically in some SCCs, may imply that the expression of K7 protein is not linked specifically to malignancy.

In summary, the rules of keratin expression were first based on a knowledge of protein expression\textsuperscript{8,49} and the present studies have helped to extend these to the mRNA level and also to suggest that simple epithelial keratins can be transcribed in normal stratified squamous epithelia. The increased expression of their proteins in SCC and dysplasia suggests either a release of these epithelial cells from the normal post-transcriptional block on K8 and K18 translation or the formation of a stabilized intermediate filament between these two keratins in malignant cells.

Acknowledgments

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