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Effect of snuff on cytokeratin expression in oral vestibular sulcus epithelium

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Differences in the expression of cytokeratins (CK) in specimens obtained from snuff-affected oral epithelium of the maxillary vestibular sulcus and clinically normal sulcular epithelium were studied by indirect immunofluorescence staining with a panel of monoclonal antibodies (MAbs). CK 14, a marker of stratified squamous epithelium, was not seen expressed in 3/11 of the snuff user's specimens. Terminal differentiation markers, typical of cornified epithelia (CK 1, 9, 10 and 11), were detected suprabasally in the snuff user's keratosis but not in the normal control epithelium. The use of snuff seemed to change the CK staining pattern of the mucosa so that it resembles more that of a cornified type of epithelium. Simple epithelial-type CK were included in the study in order to establish the CK profile of the snuff-induced keratosis, for comparison with normal and dysplastic lesions. MAb to CK 7 and 19 showed reactivity in the basal cells and suprabasally, whereas the monospecific MAb anti-CK 7 showed suprabasal staining both in the control and affected epithelia. By using MAbs, we found no immunoreactivity against CK 18 either in normal or affected epithelia, whereas we found suprabasal reaction (5/11) against CK 8 in the snuff user's epithelia. The two MAbs demonstrating the expression of CK 19, normally confined to the basal cells of the stratified squamous epithelium, showed variable patterns of expression both in basal cells and suprabasally in the snuff lesions. The results show that use of oral snuff causes some alterations in the CK expression pattern of the affected epithelium. Whether the alterations are indicative of a premalignant change is, however, uncertain. The results encourage further studies on the subject.

Key words: cytokeratins; oral epithelium; snuff

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Oral vestibular sulcus epithelium comprises non-cornified stratified epithelium. Characteristically the epithelial cells synthesize a sequence of different cytokeratins (CKs), the pattern of which is related to the state of cellular maturation and to location in the oral cavity (1). CKs constitute the major component of the cytoskeleton that provides mechanical support for the cells and their nuclei in the epithelia (2).

Twenty different CK polypeptides have been identified in epithelial cells. Their classification is defined by their immunoreactivity, molecular weight and isoelectric point. CKs are generally divided into two subfamilies: type I (acidic) and type II (basic or neutral) (3). The acidic CKs are relatively small (40–56.5 kDa) and the basic and neutral CKs are somewhat larger (53–67 kDa) (2, 3). These subfamilies appear normally in pairs so that each CK type I is usually associated with a type II (4). CKs can also be classified as stratification-related (numbers 1, 2, 6, 9, 10, 11, 13, 16) and as simple epithelial CKs (numbers 7, 8, 18, 19) (1).

Oral mucosal lesions, both benign and dysplastic, are often characterized by an increase or decrease in the degree of cornification. It is well established that the CKs expressed by epithelial cells reflect the state of cell differentiation (5, 6). Studies have been published that show changes in CK composition of premalignant and malignant oral lesions (2, 5, 7). Antibodies to CKs might therefore be useful in revealing any changes in tissue profile undergoing preneoplastic or neoplastic alterations.

Use of smokeless tobacco in the form of snuff results in clinical and histological changes of the oral mucosa (8–11). Hyperplasia of epithelium, koilocytosis, chevron-type keratinization and epithelial dysplasia have been reported (12–15), whereas in the connective tissue variable degrees of inflammation, hyalinization, sialoadenitis and other degenerative alterations, for example in lip salivary glands, have been observed (11–15). Snuff dipping has been suggested to be one of the predisposing fac-

tors in oral squamous cell carcinoma (16, 17).

The aim of the present study was to examine whether the use of snuff exerts an effect on CK expression in oral sulcular epithelium by assessing biopsies from snuff-users and non-snuff users.

Material and methods

Patients

Eleven oral snuff users with oral epithelial lesions consistent with snuff user's keratosis were included in the study. All patients were men (mean age of 25.7 years; range 21–37 years). They had continued to use oral snuff for 2 to 15 years (mean 6.1 years) with a mean frequency of 8 times per day (range 4 to 10 times per day). The following snuff products were used by the patients: Ettan (6 patients), Grovsnus (3 patients) and Tre Ankare (2 patients) (Swedish Tobacco Co., Sweden). The oral lesions were clinically classified according to the criteria given by GREER & POULSON (12) as degree one, two and three in 4, 6, and 1 patient, respectively.

Tissue specimens

The biopsy specimens were obtained from snuff-affected oral epithelium of the maxillary vestibular sulcus in the incisor area. Biopsies of two healthy men who did not use oral snuff or other tobacco products served as controls. The specimens were quick-frozen in liquid nitrogen and stored at −70°C until used.

For immunofluorescence microscopy, 5 μm sections were cut and fixed in −20°C methanol for 10 min and thereafter air-dried. The sections were overlaid by MAbs for 60 min, washed, and incubated with fluorescein isothiocyanate- (FITC-) labelled goat antimouse IgG (Jackson Immunoresearch Laboratories Inc., West Chester, PA, USA) at room temperature for 30 min.

After washing the specimens thoroughly with PBS, the sections were embedded in Veronal glycerol buffer (1:1, pH 8.4), coverslipped and studied in a Leitz Aristoplan microscope equipped with an epi-illuminator IIIRS and appropriate filters for FITC-fluorescence.

Immunostaining reactions were controlled by omission of the primary antibody or by replacing the primary antibody by non-immune mouse serum or irrelevant hybridoma supernatant of the same Ig subtype as the primary MAbs.

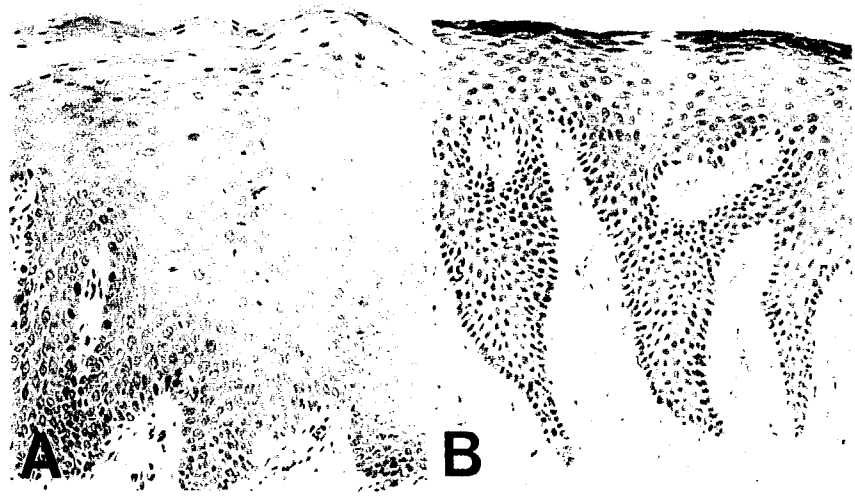

Fig. 1. A) Epithelial hyperplasia accompanied by hyperparakeratosis and mild chronic inflammation in the connective tissue following 3 years of snuff use (hematoxylin-eosin stain). Corresponding frozen section in Fig. 3 (×250). B) Degree 1 lesion showing orthokeratosis, mild chronic inflammation and increased stainability of cellular nuclei after 15 years of snuff use. Corresponding frozen section is shown in Fig. 5. (Hematoxylin-eosin; ×250).

Monoclonal antibodies

The following MAbs were used in order to study CK expression (see Table 1): The MAb KA1, binding to CK 4, 5 and 6 (1:300; 18, 19), stains the keratin filaments in all layers of stratified non-keratinizing epithelium. CK 4 is normally expressed in the suprabasal cell layer, CK 5 in the basal layer and CK 6 in hyperproliferative oral epithelium (1). The MAb KA5 reacts with cornified epithelia only (1:400; 19). The MAb 6B10 (Eurodiagnostics BV, Apeldoorn, The Netherlands) (1:5; 20) reacts normally with suprabasal cells of non-cornified mucosa (1). The MAb anti-CK 14 (1:200, 21) (Sigma Chemical Co., St. Louis, Mo, USA) is expressed in the basal cells of stratified epithelia. The MAb 4F5 (undiluted; 22) reacts with basal cells of stratified epithelia in normal tissue, the MAb 2A4 (undiluted; 22) stains various simple epithelial cells, and the MAb anti-CK 7 (1:200; 23) is typically expressed in simple epithelium (Sigma Chemical Co.).

The MAb K4.62 (Sigma Chemical Co.) (1:30; 24) and the MAb K19.1 (1:5; 25) react with basal cells in non-cornified epithelia and with various simple epithelial cells. The MAbs K18.174 (1:5; 26) and K8.17.2 (1:5; 27) are both found in simple epithelia (1).

The evaluation of the immunostained sections was performed by two indepen-

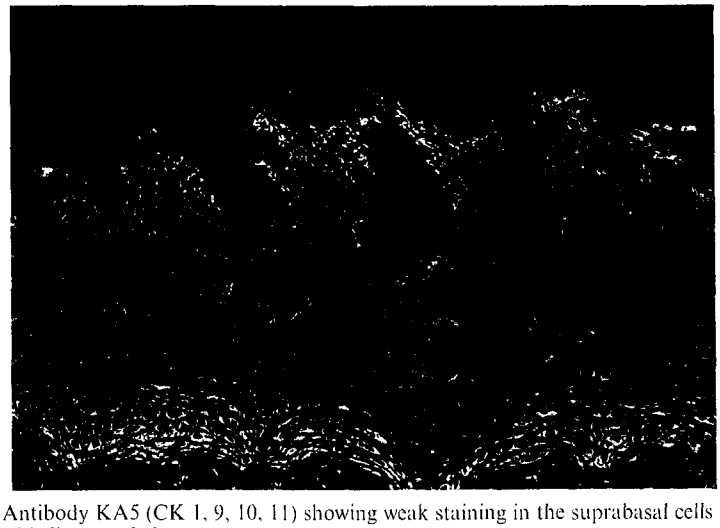

Fig. 2. Antibody KA5 (CK 1, 9, 10, 11) showing weak staining in the suprabasal cells of snuff user's epithelium. (×250)

Table 1. Cytokeratin expression in snuff user's keratosis and in control epithelium

Antibody	Reference	Reactive polypeptide	Normal epithelium	Snuff user's keratosis
Stratification-related cytokeratins				
KA1	18, 19	4, 5, 6	All layers +	All layers +
KA5	19	1, 9, 10, 11	All layers -	Basal - Suprabasal +
6B10	20	4	Basal - Suprabasal ++	10/11 basal - Suprabasal ++ 1/11 basal - Suprabasal -
Anti-CK 14	21	14	Basal + Suprabasal -	8/11 basal + Suprabasal - 3/11 all layers -
Simple epithelial cytokeratins				
4F5	22	7, 19	Basal ++ Suprabasal +	9/11 basal + Suprabasal + 2/11 all layers -
2A4	22	8, 18, 19	Basal ++ Suprabasal -	10/11 basal + Suprabasal - 1/11 all layers -
Anti-CK 7	23	7	Basal -/+ Suprabasal +	Basal -/+ Suprabasal +
K 4.62	24	19	Basal ++ Suprabasal +	9/11 basal - Suprabasal + 2/11 basal ++ suprabasal -
K19.1	25	19	Basal + Suprabasal - All layers -	10/11 basal +/++ Suprabasal - 1/11 basal - Suprabasal -
K18.174	26	18	All layers -	All layers -
K8.17.2	27	8	all layers -	6/11 all layers - 5/11 basal - Suprabasal +

-=no staining; +=weak staining; ++=moderate to intensive staining.

dent observers. The specific immunocytochemical staining was scored for the presence or absence of positive basal and/or suprabasal reactions in epithelia.

Results

Histopathology

Epithelial hyperplasia and basal cell hyperplasia were constant findings in the specimens from the snuff-affected oral mucosa. Hyperkeratinization of the epithelium was noted in all the specimens (10 parakeratosis and 1 orthokeratosis). Vacuolization of the cells in the superficial epithelial layers was noted in six specimens. In two samples vacuolization was extensive. A very mild mononuclear inflammatory cell infiltrate was recorded in the connective tissue immediately beneath the epithelium in every specimen. An increased mitotic rate was present in some specimens but none of the lesions fulfilled the histological criteria of epithelial dysplasia (Fig. 1A, B). No correlation could be identified between the duration of snuff use and the histological features described.

Immunocytochemistry

The results are shown collectively in Table 1.

Stratification-related cytokeratins

Immunohistochemical staining with MAb KA1 (CK 4, 5 and 6) revealed a weak fluorescent reaction both in the basal and the suprabasal cell layers of the normal and affected epithelium.

The control epithelium was negative with MAb KA5 (CK 1, 9, 10, 11). Weak immunofluorescence was observed in all suprabasal epithelial cell layers of the smokeless tobacco lesions (Fig. 2). In some of these specimens the reaction products were concentrated in patches scattered throughout the epithelium.

A strong staining reaction with MAb 6B10 (CK 4) was seen to be distributed in the suprabasal cell layers both in the normal controls and in ten specimens of the snuff user's mucosa, whereas the basal cells in both groups were negatively stained. One specimen of the snuff-affected mucosa was completely negative.

The staining pattern with anti-CK 14 (CK 14) showed weak reactivity in the basal cells. The suprabasal cell layers were negative in the control specimens. The staining in eight of the snuff user's specimens was like that of the controls. All layers in three of the snuff user's specimens were negative.

Simple epithelial cytokeratins

A strong staining reaction with MAb 4F5 (CK 7 and 19) was confined to the basal cell layer in the normal controls, with faint staining in the suprabasal cell layers. Nine specimens from the group of snuff users showed weak staining in the basal and the suprabasal cells. In 2/11 specimens no staining reaction could be detected with MAb 4F5 and all epithelial cell layers were negative.

A strong reaction with MAb 2A4 (CK 8, 18, and 19) was observed in the basal cell layer of the control tissue. In ten specimens from the snuff user's epithelium, a weak reaction was seen in the basal cell layer, with no reaction to the antibody in the suprabasal region. In one case neither basal nor suprabasal cell layers were stained.

MAb anti-CK 7 (CK 7) was distributed weakly in the suprabasal cell layers

Fig. 3. Suprabasal staining with some positive reaction in the basal cells of snuff user's mucosa. Anti-CK7 specific for CK 7. Reaction in connective tissue is due to unspecific staining. (×250)

and in some occasional basal cells (Fig. 3). In some specimens of the snuff user's group, the suprabasal staining seemed to be patchy, with heterogeneous CK 7-immunoreactivity.

MAb K4.62 (CK 19) showed clear staining in the basal cell layer of the normal sulcus mucosa and a very weak reaction suprabasally (Fig. 4A). In nine of the snuff user's specimens, weak immunoreactivity was detected throughout the suprabasal layers of the epithelium but not in the basal cells (Fig. 4B). A strong basal staining reaction, without suprabasal staining, could be observed in two specimens of snuff user's keratosis (Fig. 4C).

K19.1 (CK 19) showed a marked reaction in the basal cells in one of the control specimens, whereas all layers in the other control were negative. Ten specimens from the snuff user's epithelium showed strong (6/11) or weak (4/11) immunoreactive staining in the basal cells. In four of the specimens, the staining reaction was observed to be discontinuous. The staining was negative suprabasally (Fig. 4D). One of the specimens was completely negative.

MAb K18.174 (CK 18) failed to stain the oral vestibular sulcus epithelium.

MAb K8.17.2 (CK 8) showed no reaction in the control specimens. Six of the snuff user's specimens were negative and five showed a patchy suprabasal staining reaction (Fig. 5).

Discussion

The CK expression seen in the normal oral vestibular sulcus epithelium was in accordance with previous reports by other authors (5, 28, 29). The histopathology of the snuff user's lesions was

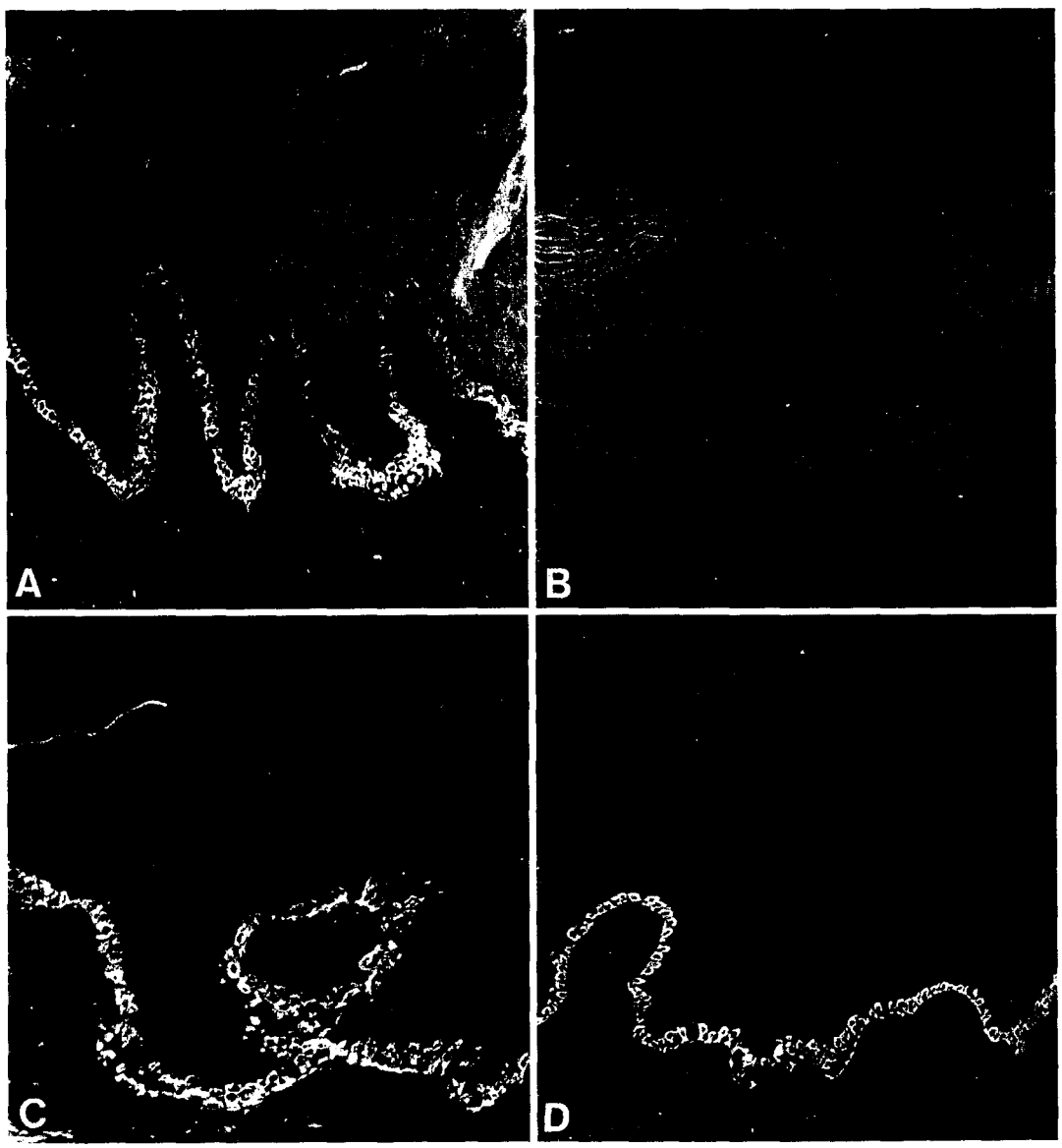

Fig. 4. A) Antibody K4.62 specific to CK 19 showing strong staining in basal cells and weaker staining in suprabasal cells of normal vestibular epithelium (×250). B) Antibody K4.62 in snuff user's vestibular epithelium. Negative reaction for CK 19 in the basal cells, with weak suprabasal staining. The membrane staining seen here is due to an antigenic interrelationship between desmoplakins (24). (×250) C) A strong reaction with antibody K4.62 is shown in the basal cells of snuff-affected epithelium, with a negative suprabasal reaction. The strong connective tissue staining is due to unspecific binding of the MAb (×250). D) Antibody K19.1 specific to CK 19 showing strong staining in basal cells of snuff user's epithelium (×250).

Fig. 5. A suprabasal mosaic-like staining pattern is seen with antibody K8.17.2 specific to CK 8 in snuff affected epithelium (×250).

also consistent with that of similar material described earlier (11). None of the histological features detected could be associated with a long history of snuff use. Neither did the duration of the snuff use seem to correlate with the changes seen in the CK profile. The immunohistochemical stainings with the MAbs proved to be useful tools for demonstrating the alterations in CK expression that could not have been ascertained by using routine light microscopy alone. According to our results, immunostaining was seen to be confined to the epithelial cells. The control stains for the immunoreactions showed negative results. Therefore it seems obvious that the positive fluorescence shows the presence of the antigen in question. However, a possible masking of antigens cannot be completely excluded in cases showing no reaction (30, 31), and a genuine cross-reaction can never be ruled out in positive cases.

A weak reaction was seen in all epithelial cell layers with MAb KA1 recognizing stratification-related CK 4, 5 and 6. Of these polypeptides, CK 5 resides in the basal cells together with CK 14 (32). Type II CK 4 is usually found as the pair to acidic type I CK 13. CK 6 normally forms a pair with CK 16. Together, CK 6 and 16 have been considered as "hyperproliferative" and they have been connected with fast cellular turnover in the epithelium (1, 32). Monospecific antibody to CK 4 was also included in the study. The suprabasal immunoreactivity with MAb KA1 could be due to CK 4, which is usually detected in non-cornified epithelia.

Reaction to MAb KA5 for the demonstration of CK 1, 9, 10 and 11 was negative in all layers of the normal epithelium, whereas a reaction could be observed throughout the suprabasal layers of the snuff user's epithelium. The reaction with this MAb suggests that the use of snuff could contribute to an alteration in the formation of these CKs and change the CK staining pattern of the vestibular mucosa to resemble more that of a cornifying type of epithelium (33). Similar changes have been found in oral leukoplakias (28). Unfortunately, MAb KA5 (like MAbs 4F5, 2A4 and KA1) detects several CK proteins, which is a disadvantage compared with the monospecific antibodies for a single CK that gives more specific information about tissue formation.

Non-cornified stratified epithelia typically express a pair of CKs, 5 and 14, in the basal cells (5, 34). Our result of a staining reaction with anti-CK 14 in the basal cells is in line with that. The negative reaction to CK 14 in some specimens of the snuff user's group could be due to masking of the antigen or a cellular disturbance giving rise to embryonic-like cells in the basal cell area (6).

MAbs 4F5 and 2A4 showed a strong staining reaction confined to the basal cell layer of the normal specimens, whereas the specimens from snuff user's mucosa showed in several cases a weaker CK expression, which with 4F5 extended throughout the epithelium. MAb 4F5 reveals CK 7 and 19, and MAb 2A4 reveals CK 8, 18 and 19, respectively (22). The CKs shown by these antibodies are mainly found in simple epithelia but may gradually become more conspicuous in oral epithelium with dysplastic changes (34, 35). Moreover, simple epithelia demonstrate CK 19, which is also found in stratified epithelia. The staining reaction confined to the basal cells with antibodies 4F5 and 2A4 was apparently due to CK 19, which was variably revealed, however, with the monospecific MAbs K4.62 and K19.1.

CK 7 has been detected in diverse simple epithelia (5, 36) and should not be detectable in stratified or in non-keratinizing epithelia. However, in our series CK 7 seemed to be invariably present suprabasally in all the specimens including those from the normal mucosa. The earlier findings (29) of CK 7 immunoreactivity being present in the normal buccal epithelium were in concert with our results for the vestibular sulcus epithelium but were in contrast to the findings in some other studies (5, 36). It seems that CK 7 should not be unambiguously regarded as a prognostic marker of malignancy as has been previously suggested (5, 36, 37). The suprabasal staining reaction with MAb 4F5 was in accordance with the results for anti-CK 7.

CK 19 has been suggested to be extensively expressed in human carcinomas (38). Furthermore, the presence of CK 19 in the suprabasal cells has been proposed to correlate with the premalignant state of the tissue and to be of important diagnostic value (39, 40). Interestingly, in our study MAb K4.62 gave a suprabasal reaction in some of the specimens, whereas another MAb for the same CK, K19.1, did not reveal any immunopositivity suprabasally. The staining pattern of CK 19 seemed to vary in the snuff user's group between the different MAbs. A variable suprabasal staining reaction for CK 19 with MAb K4.62 in normal buccal mucosa has also been shown in some other studies (29, 41). There may be several possibilities for differential expression of CK 19. The variation in CK 19 expression in the control specimens might be due to the presence or absence of inflammation (37). Also, CK 19 expression can be speculated to reflect the degree of tissue cornification (36, 42) or it could merely suggest enhanced cell proliferation in epithelia. In addition, the differential staining result with the two MAbs used against CK 19 may be a consequence of masking of epitopes.

Simple epithelial CK 8 has been shown to be expressed in oral dysplasias

by immunohistochemistry (5) and not to be a normal constituent of stratified squamous epithelia (37). The expression of this CK has been suggested to emerge gradually in dysplasia, which may link the expression of CK 8 with malignancy (37). In our material, CK 8 was present in the epithelium of several specimens of the snuff user's keratosis. The presence of CK 8 could not, however, be correlated with any of the histological features of our material. No dysplastic changes could be diagnosed histologically in any of the specimens. Bosch *et al.* (37) have shown that early changes in CK expression are more extended at the mRNA than at the protein level, suggesting that histological alterations may become detectable later. Accordingly, it can be speculated that changes in CK expression may appear earlier than the histological alterations.

CK expression has been related to the degree of tumor differentiation (43, 44). Multiple changes have been reported to occur in CK expression as a lesion progresses to premalignancy and malignancy (37). Smoking is one of the predisposing factors for squamous cell cancer involving the oral mucosa (45). Previous studies have shown that no differences are found in CK staining pattern in normal buccal mucosa in smokers and nonsmokers, whereas in mucosa showing tobacco-induced lesions changes are evident (28, 29). In the present study on snuff user's keratosis, alterations seemed to occur in the CK expression pattern of the vestibular sulcus epithelium at the site of the placement of the snuff. Some samples showed induction of cornifying types of CKs, whereas in other samples expression of simple epithelial type of CKs was noticed. Whether the alterations observed here are indicative of a premalignant change is, however, uncertain. Further studies of the subject are needed.

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