Proliferation and differentation markers in snuff-induced oral mucosal lesions

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Abstract

Background: Regular use of snuff is known to cause whitish oral mucosal lesions of variable severity at the usual quid placement site. The main aim of this study was to elucidate cellular mechanisms involved in snuff-induced epithelial changes.

Methods: Expression patterns for markers of cell proliferation (PCNA, Ki-67), cell cycle regulation (p53, p21), keratin changes (pankeratin, CK18, CK19), cell stress (HSP 70) and collagen type IV in 14 snuff-induced oral mucosal lesions and 12 control samples were analyzed by immunohistochemistry (IHC).

Results: On light microscopy, all snuff-induced lesions were characterized by a hyperkeratinized and thickened epithelium. Some vacuolized cells, markers of cell degeneration, were frequently seen (in 9/14 of the samples) in the superficial layers in epithelia. Expression of PCNA and Ki-67 was found in a statistically significantly fewer cells in snuff-induced lesions ($P < 0.001$) than in the controls. This indicates that epithelia in snuff-induced lesions are not thickened as a result of increased cellular proliferation, but by protracted turnover of differentiating cells. Of cell cycle markers, p21 was found be up-regulated in 4/14 snuff-induced lesions, probably by p53-independent pathways. Only two snuff-induced lesions showed p53 positivity. However, the number of stained cells with p53 and p21 was not statistically different from that in controls. Expression of CK18, but not any alterations in CK19 expression, was seen in 5 of 14 snuff-induced lesions. Snuff also seems to stimulate the expression of collagen type IV, possibly by basal cells, as indicated by the thickened staining of the basal lamina.

Conclusions: The findings of this study showing suppressed cellular proliferation and infrequent p53 dysfunction in snuff lesions may partly explain why dysplastic changes are seldom seen in mucosal lesions induced by the Scandinavian type of snuff.

Keywords: Ki-67; oral mucosa; PCNA; smokeless tobacco; snuff

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The most characteristic oral mucosal change due to snuff use is a hyperkeratinized, white patch developing at the snuff application site after a few months of use. Typically, the lesions are reversible

after discontinuation of use (1–3). Other changes such as discoloration (3), abrasion (4), cervical erosion (5), deterioration of periodontal health (5), and focal gingival recession (6) of the teeth may also develop as a result of snuff use.

The severity of oral lesions in snuff users has been shown to depend on the form (loose vs. portion packed) of the snuff used, placement of the quid (one vs. several sites), hours and amount of daily use as well as on the total years of snuff use and the user's age (7). On the basis of their clinical appearance, including wrinkling, thickening, and colour changes, the lesions can be classified into four grades of severity (8).

The major concern with snuff-induced lesions is their suggested potential to develop into pre-cancerous lesions and oral malignancy. Previous retrospective studies have shown a strong correlation between snuff use and the incidence of oral cancer (9). However, the most recent epidemiologic studies from Scandinavia on snuff-related carcinogenesis do not support these findings (10,11). Importantly, histopathologic studies on snuff-associated oral lesions have infrequently found dysplastic changes (12–15).

The variable results of epidemiologic studies on snuff-related oral carcinogenesis are probably due to differences in the chemical composition of the snuff products used in different geographical areas. Snuff contains a variety of chemicals; the most important and most abundant carcinogenic agents being tobacco-specific N-nitrosamines (16,17). Scandinavian snuff is a non-fermented, almost sterile product, in which tobacco-specific nitrosamines are generated less than in corresponding products manufactured by fermentation (18,19).

Oral carcinogenesis is a complex multi-step process, resulting from a series of genetic alterations leading to aberrations in the regulation of cellular proliferation and differentiation. Because little is known of the molecular level of the biologic events underlying the histologic changes due to snuff use, a variety of interplaying factors was investigated. The present study was undertaken to assess the expression of proliferation markers PCNA and Ki-67, tumor-suppressor gene p53, and its downstream target p21, as well as some cytokeratins, heat shock protein (HSP) 70, and collagen type IV in 14 biopsy specimens from snuff-induced lesions.

Materials and methods

Study subjects and samples

Biopsy specimens from 14 men with mucosal snuff dippers' lesions were obtained (between 1990 and 1998) from the Student Health Service and Institute of Dentistry, University of Turku, Finland. Table 1 shows the characteristics of snuff users. All snuff-induced lesions were located in the upper labial sulcus of the anterior region. The mean age of the subjects was 24 years (SD ± 3.2, range 18–32 years). The average time of snuff use was 5.9 years (SD ± 4.1, range 2–15 years) with a mean frequency of 6.8 (SD ± 2.6, range 2–10) times per day. All study subjects were using loose snuff and 3 were also using portion-packed snuff. The snuff used was exclusively of the non-fermented, Scandinavian type of moist snuff. Five of the 14 subjects were also current smokers with a more occasional than regular smoking habit. No data on their alcohol use were available.

The samples of the study group were complemented with 12 control biopsy specimens from normal buccal mucosa from the upper first molar region. These control samples were obtained from subjects included in another study on oral mucosal changes in coeliac disease (study approved by the Ethical Committee of the University of Turku, 1996). A healthy oral mucosa was the inclusion criterion. They had no past or present history of snuff use or any other tobacco habits. The majority (8/12, 67%) of the controls were men.

Table 1. Characteristics of snuff users

Sample	Age (years)	Clinical grade	Snuff type	Dosage/day	Usage time (years)	Smoking
1	18	2	n.k	n.k	n.k	n.k
2	26	2	loose	7–8	13	yes (occasionally)
3	32	1	loose/portion	10	15	no
4	24	2	loose	6–7	7	no
5	21	1	loose	3–4	3	no
6	25	2	loose	10	4	no
7	25	2	loose	5–10	3	no
8	25	1	loose	10	$3^1/_2$	yes
9	23	3	loose	10	10	yes (occasionally)
10	23	2	loose/portion	4	2	yes
11	27	1	loose	6	3	no
12	21	n.k	loose	2–3	2	yes (occasionally)
13	25	1	loose/portion	3–4	4	no
14	26	2–3	loose	5–10	7	no

n.k = not known

Table 2. Antibodies used in the immunohistochemical analyses

Antibody	Manufacturer	Clone	Dilution	Pretreatment	Positive control samples
PCNA	Dako, Glostrup, Denmark	PC 10	1:50	Microwave + prot.K	Skin
Ki-67	Immunotech, Marseille, France	MIB-1	1:50	No pretreatment	Skin
p53	Dako, Glostrup, Denmark	DO 7	1:1*	Microwave	Breast cancer
p21	Nova Castra, Newcastle, UK	4D 10	1:10	Microwave	Verruca
Pankeratin (5, 6, 8, 17, 19)	Dako, Glostrup, Denmark	MNF 116	1:50	Microwave + prot.K	Skin
Cytokeratin 18	Dako, Glostrup, Denmark	DC 10	1:20	Microwave + prot.K	Prostrate
Cytokeratin 19	Dako, Glostrup, Denmark	RCK 108	1:100	Microwave + prot.K	Breast
HSP 70	Sigma, Saint Louis, MI, USA	BRM-22	1:250	No pretreatment	Minor salivary gland
Collagen type IV	Dako, Glostrup, Denmark	CIV 22	1:50	Microwave + prot.K	Fibrous hyperplasia

* Ready-to-use reagent

The mean age of the controls was 46 years (SD ± 15, range 25–72), i.e. higher than that of the study subjects.

Histology

Five-μm-thick sections from formalin-fixed (10% neutral buffered formalin), paraffin-embedded samples were cut on organosilan-pretreated glass slides for hematoxylin-eosin (HE) staining and immunohistochemistry. On light microscopic evaluation, special attention was paid to the degree of keratinization, epithelial vacuolization, basal cell proliferation, inflammatory changes, and dysplastic changes of the squamous epithelium.

Immunohistochemistry (IHC)

All samples from study and control subjects were analyzed using IHC for evaluation of the expression of proteins of cell proliferation (Ki-67, PCNA), cell cycle regulation (p53, p21) and keratins (pankeratin, cytokeratins 18 and 19), cell stress (HSP 70), and collagen type IV. Table 2 summarizes the pretreatment methods used for antigen retrieval, dilutions of the individual monoclonal antibodies, and the positive controls for IHC. The sections were stained using the avidin-biotin complex technique of the Dako ChemMate Detection Kit (peroxidase/DAB-rabbit/mouse; Dako, Glostrup, Denmark) and an automatic device for IHC analysis (DAKO ChemMate TM 500, BioTek Solutions, USA), according to the manufacturers' instructions. Sections of samples used as negative controls were treated without the primary antibody. Mayer's hematoxylin was used for counterstaining.

Quantitation of IHC staining

The immunoreactivity of the samples was graded on the basis of the quantity of positively stained cells and staining intensity. When these parameters were summarized, the staining of the samples could be categorized into four groups: negative (–), weak (+), moderate (+ +) and strong (+ + +) staining. The localization of staining in the epithelium was also recorded.

PCNA, Ki-67, p53, and p21 positivity was additionally determined by counting stained cells in 3 randomly selected fields at a magnification of 400×, except for PCNA at a magnification of 250×. The results of cell counts are given as means of percentages of positive cells of all cells counted in a defined field. Statistical differences between the controls and the study group were investigated using the *t*-test for equality of means (2-tailed).

Results

Histology

Snuff-induced lesions

All snuff-induced lesions were characterized by a thick hyperkeratinized surface layer. Six of the samples showed parakeratinization, 6 showed orthokeratinization and 2 samples both. The keratinized surface showed a chevron pattern in 5 samples. Some dyskeratotic cells were also present in 1 sample in the upper parts of the epithelium.

In 9 of 14 samples, small areas of vacuolated cells with a pyknotic nucleus were seen in the superficial layers of the stratum granulosum. In 2 of these samples, vacuolisation was more prominent. Keratohyaline granules were seen in 5 of the 14 snuff users' samples.

These snuff users' lesions gave an overall impression of an epithelium with thickened and elongated rete ridges. No changes compatible with dysplasia were seen in any of the specimens.

Mild chronic inflammation in lamina propria, localized to the juxta-epithelial part, was seen in 50% of the samples, with lymphocyte predominance, but plasma cells were also present. Some lymphocyte traffic into the basal parts of the epithelium was also seen. No evidence for fungal hyphae was seen in any of the samples.

Control samples

The control samples consisted of thick, stratified epithelia with normal structure and a non-keratinized surface layer. No vacuolization was present in the surface layers, but loose architecture of cells in the intermediate layers typical of normal buccal mucosa was found.

Immunohistochemistry

Tables 3 and 4 summarize the results of IHC staining. The main finding of the immunohistochemical analysis is that PCNA and Ki-67 expression was clearly lower in snuff lesions than in controls. With p53 and p21 no difference between study samples and controls were found.

Table 3. Results of the immunohistochemical analysis of PCNA, Ki-67, p53, p21, HSP 70, pankeratin, CK18, and CK19 in snuff user lesions and control samples

Sample	PCNA	Ki-67	p53	p21	HSP70	pan-CK	CK19	CK18
S1	+++	++	+	+++	++	+++	+	++
S2	++	++	–	–	++	+++	+	+
S3	+	+	–	–	++	+++	+	–
S4	+++	++	–	–	+	++	+	–
S5	+++	++	–	–	++	++	+	–
S6	+++	++	–	–	+++	++	+	–
S7	++	++	–	+	+	++	+	–
S8	+	+	–	–	+	++	+	–
S9	++	++	–	+	+++	+++	+	–
S10	++	++	–	–	++	++	+	–
S11	+++	++	–	+	++	++	+	–
S12	+	+++	–	+++	+	+++	–	++
S13	++	+++	–	+++	+	+++	+	+++
S14	+++	++	+	+++	+	+++	+	
C1	+++	++	–	+	++	++	+	–
C2	+++	++	–	+	+	++	+	–
C3	+++	++	–	+	+	++	–	–
C4	+++	++	–	+	+++	++	+	–
C5	+++	++	–	+	+	++	–	–
C6	+++	++	–	+	++	++	+	–
C7	+++	++	–	+	++	++	+	–
C8	+++	++	–	n.a	++	++	+	–
C9	+++	++	–	+	++	++	–	–
C10	+++	+	–	+	+	++	+	–
C11	+++	++	–	+	+	++	–	–
C12	+++	++	–	+	+	++	+	–

S = snuff users lesion, C = control, n.a = not assessed

Table 4. Percentage of positively stained cells by IHC

	Mean ± SD		
	Controls	Snuff users lesions	Level of significance
p53	2.0 (±1.7)	3.1 (±4.6)	0.464
p21	36.9 (±7.6)	28.6 (±31.3)	0.398
PCNA*	100 (±0)	34.4 (±16.15)	0.000
Ki-67	71.5 (±6.6)	24.6 (±9.7)	0.000

* Counted field in the lower third of the epithelium

Proliferating nuclear antigen (PCNA) and Ki-67

In biopsy specimens from snuff users' lesions, PCNA (Fig. 1a) and Ki-67 (Fig. 1b) nuclear staining was present in the basal layer and in some scattered cells of the parabasal layers. Three snuff users' samples showed weak staining with PCNA. In the controls, strong nuclear staining with PCNA was seen a in all basal cells and with a lower intensity in the cells of the lower third of the epithelium. Ki-67 staining occurred in fewer cells than PCNA staining. Overall, the quantity of PCNA and Ki-67 stained cells was greater in controls than in snuff users. Although the number of stained cells was clearly higher in the controls the staining intensity was lower than that of snuff user's lesions. The number of positively stained nuclei was statistically significantly lower in snuff lesions than in controls both by PCNA and Ki-67 staining (Table 4).

P53

The quantity of p53 stained cells in snuff users did not differ from that in controls statistically (Table 4). In snuff users, only 2 samples showed positivity with the p53 antibody (10% cut-off value). Nuclear positivity was found in the basal layer (Fig. 1c). These non-smoking individuals had a lesion clinically graded as category 2. Nuclear positivity was also seen in single scattered basal cells in the other snuff users' samples and in the control samples in each section.

P21

p21 expression was found in 7/14 of the snuff-induced lesions (Fig. 1d). Staining intensity was weak in 3 and strong in 4 snuff-induced lesions. Nuclear staining was seen in parabasal layers extending to the granular layer, with slightly decreasing intensity towards the epithelial surface. The 2 samples showing basal p53 positivity also showed strong suprabasal staining with p21. In comparison, all controls showed weak p21 staining confined to cells predominantly in the parabasal layers. However, the difference in the quantity of stained cells between controls and study subjects was not statistically significant.

Pankeratin

With pankeratin (detecting cytokeratins 5, 6, 8, 17 and 19), staining was seen in all epithelial cell layers, but with enhanced strong

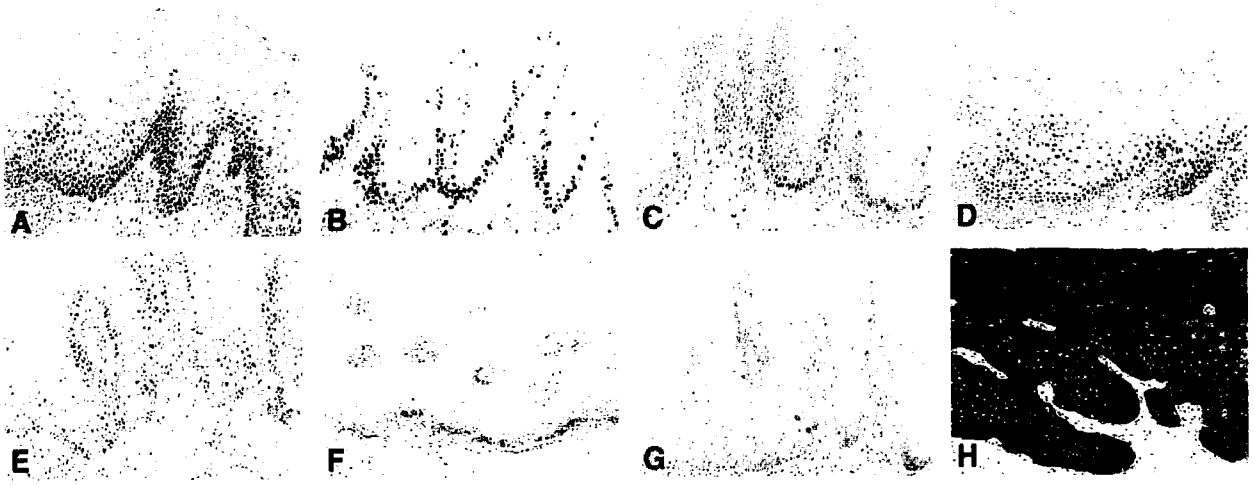

Fig. 1. Immunohistochemical staining of snuff users' lesions. (A) PCNA positive cells in the basal layers. (B) Ki-67 positive staining in the basal layers. (C) p53 positive cells in the basal layer. (D) p21 positive suprabasal cells. (E) nuclear HSP expression in basal and parabasal cells. (F) CK 19 staining in basal cells. (G) CK 18 staining in basal and suprabasal layers. (H) strong pankeratin expression.

staining confined to the basal and parabasal layers (Fig. 1h). In snuff users' lesions, 7 of 14 followed the same staining pattern as the control samples. In the other 50% of snuff users' samples, higher staining intensity was seen in the whole thickness of the epithelium.

CK 18

CK 18 immunoreactivity was seen in 5 samples of the 14 snuff user lesions (Fig. 1g). In 2 of these, staining was faint and mainly confined to the basal cell layer. In 2 samples, moderate staining was mainly localized to the basal cells, but weak suprabasal staining was also seen, and one sample showed staining in the whole thickness of the epithelia. Of snuff users' samples 4 of 5 with CK18 positivity also showed staining with p21. A few strongly stained cells showing cytoplasmic staining, identified as non-dendritic Merkel cells, known to express CK18, were also seen in all snuff users' samples.

CK 19

With the monoclonal antibody to CK 19, a 40-kDa cytokeratin intermediate filament protein, granular cytoplasmic staining in the basal cells in a bandlike manner or as patches of a few cells was seen in all samples (Fig. 1f) from the study subjects and most controls. In the controls, 4 samples were totally negative for CK19.

Heat Shock Protein (HSP) 70

HSP 70 could be detected in every biopsy sample obtained from snuff users (Fig. 1e) and controls. The staining pattern was variable in the groups, and no marked differences were found between controls and study subjects. In snuff-induced lesions, strong nuclear expression was localized to basal cells but also seen in parabasal cells, whereas the controls showed strong staining with HSP in the nucleus, usually confined to the basal layer. Cytoplasmic staining was seen in the upper part of the epithelium, with variable intensity in both groups.

Collagen type IV

The staining intensity and thickness of the stained threadlike layer of collagen type IV were clearly increased in 10 of 14 snuff dipper's lesions compared to controls.

Discussion

The histology of the biopsy specimens from snuff-induced lesions in the present study was characterized by ortho- or parakeratinized epithelia with epithelial thickening, which is in agreement with the findings of several previous studies (2, 8, 14). This epithelial thickening has been thought to be a consequence of hyperproliferation.

According to the present results, the thickening may rather be related to the increased turn over time of cells in the epithelium. Keratinization and thickening of the epithelium have been regarded as a protective response helping to impede the delivery of tobacco constituents to the cells and the nucleus. The hyperkeratinization becomes quickly distinguishable, even 7 days after exposure of the mucosa to snuff (20). No correlation between the duration of snuff use or daily dose to the degree of keratinization was observed in this study. The high pH (about pH8–9) of Scandinavian snuff (21) and the calcium salts present in snuff (22) may be responsible for this excessive keratinization. Mild cellular vacuolization was a frequent finding in the superficial layers of the snuff-induced lesions but not in control samples. This change has been regarded as a degenerative response of the epithelia to high alkalinity of the snuff (8). Alkalinity is used to enhance nicotine delivery, but it also maximizes the toxic effects of the snuff product (23).

None of the 14 biopsy specimens from snuff-induced lesions showed epithelial dysplasia, supporting the view that premalignant or malignant changes are rare in snuff-induced lesions. Jungell and Malmström investigated 21 snuff lesions in Finnish army recruits and found 1 case of mild dysplasia interpretable as a reactive change towards subepithelial inflammation (14). In an extensive study of 15,000 snuff users, Smith and co-workers found no dysplasia in 157 biopsied lesions (15). Similarly, Axell and co-workers recorded no dysplastic changes in 114 analyzed snuff dipper's lesions (8). In a study by Larsson and co-workers, oral lesions with changes representing some parameters used to define dysplasia were found in 29 subjects of 252 snuff or chewing tobacco users (2). However, after discontinuation of the habit, the histologic changes were reversible.

Most snuff lesions show at least some inflammatory changes (8). The present study agrees with this finding, although the inflammatory reactions seen were mainly very mild. However, we were not able to find any eosinophils, which have been a common finding in snuff-induced lesions in several previous studies (2, 8).

Immunohistochemistry

In snuff users' lesions, PCNA and Ki-67 immunopositivity was found in a significantly lower quantity of cells than in the control samples. This indicates that snuff does not cause epithelial thickening by inducing hyperproliferation of epithelial cells, but rather by extending the life-span of differentiated cells before desquamation. Some *in vitro* studies have shown that tobacco extracts and some tobacco constituents such as nicotine may suppress cell proliferation (24, 25). However, the results are somewhat contradictory as there are also studies showing that smokeless tobacco extracts and purified tobacco-specific nitrosamines increase the proliferation of labial mucosal cells *in vitro* (26). In smokers, increased immunopositivity with Ki-67 has also been reported (27).

In the present study, the number of Ki-67 -positive epithelial cells was lower than that of PCNA, even if the patterns of expression generally parallelled. This feature has been observed previously (28). This finding may relate to the fact that these antigens do not mark the same phases of the cell cycle. Ki-67 reaches a maximum during G2, S and M phases but is not found in G0 (29), whereas PCNA is present throughout the cell cycle and furthermore also involved in DNA repair. Furthermore, PCNA is characterized by a longer half-life and is thus detectable in many cells that have already passed the cell cycle. The result indicates thus that the majority of the cells are in the resting stage.

Our study suggests that p53 immunopositivity is infrequent in snuff users' lesions, as only two samples showed a very modest increase in p53 staining compared with the controls. On the basis of immunohistochemical staining alone, it is impossible to determine whether the positive staining is due to p53 mutation or wild type expression. A similar result of a low frequency of enhanced p53 expression was reported in a study by Ibrahim *et al.* in Swedish snuff users' lesions, where 2 of 15 lesions expressed p53 confined to the nuclei of the epithelial basal layer (30). By contrast, Wedenberg and co-workers described snuff lesions without any dysplastic features, but with a high frequency of p53 over-expression by IHC, using an antibody that recognises both mutated and wild type of the protein (31). Highly discordant results were also obtained by Wood and co-workers, who found significantly elevated expression of p53 in snuff-induced lesions, as compared to normal mucosa obtained from the same individuals (32). The comparison of immunohistochemical stainings in studies with p53 may be affected by methodological differences such as variations in fixation, type of antibodies and section pretreatment. In all above p53 studies, except in the study of Wood *et al.*, the p53 antibody DO-7 microwave pretreatment was used for antigen unmasking. The results of Wood were interpreted using the CM^{-1} and p53–1801 clone of p53, of which the later has been reported to be less sensitive than DO-7 (33). Reliance upon the demonstration and quantitation of p53 depends of the reproducibility of the method, which in this study was achieved using a standardized protocol and an automatic appliance.

Interestingly, a few scattered basal cells showed p53 positivity in nearly all snuff users' samples and in controls. Similar results were recently reported in another study, suggesting that p53 is expressed at low levels in tissues in the complete absence of gene mutations, reflecting the activation of normal p53 (34). However, the up-regulation of wild type p53 may be a consequence of ongoing DNA damage (35).

In 4 snuff users' lesions, markedly increased immunoreactivity for p21 was found compared to controls. p21 positivity was never seen in basal cells but in the overlying cells committed to differentiation. In normal mucosa, p21 is weakly expressed by suprabasal keratinocytes, but is consistently negative in basal cells (36). p21 induction has been shown to play a central role in cell-cycle control during the terminal differentiation of keratinocytes (37), probably induced by p53-independent pathways (38). p21 blocks the replication of DNA and the progression of the cell cycle into the S-phase by binding to cyclin and cyclin-dependent-kinases (CDK) and by interaction with PCNA.

The lining mucosa of a normal sulcular epithelium is stratified and non-keratinized and does not generally express CKs 7, 8, 18 and 19 characteristic of simple or transitional epithelia. In the present study, snuff-induced lesions showed more intense pankeratin expression than control samples. Furthermore, CK18 was also detectable in 5 snuff samples. CK 8, which is co-expressed with CK 18, has been shown to be expressed aberrantly in tobacco chewers' leukoplakia (39). Expression of CK 18 similar to CK 19 has been implicated in premalignant and malignant lesions (40). In the present study, almost all samples showed weak immunopositivity for CK19 without any association to CK 18 expression. Consistent with our results, one study showed no up-regulation of CK19 expression in Swedish snuff-induced lesions (41), whereas another study showed contradictory results with CK 19 expression in basal and also suprabasal cells (42).

Heat shock proteins (HSP) have an important role in protecting and promoting the recovery of cells from physiologic and pathologic stress. A wide range of stimuli (e.g. elevated temperatures) have been found to modify their expression. An important finding in association with snuff is that nicotine induces HSP expression (43). In addition, HSP 70 has been shown to be up-regulated by smoking in lung adenocarcinomas (44) and in oral squamous cell carcinomas (45). HSPs are also expressed in normal tissue. A shift from cytoplasmic to nuclear localization is usually associated with cell stress. In the present study, a greater proportion of positive nuclei was detected in snuff users' samples than in control samples, which may be due to cellular stress caused by snuff. However, further studies are needed to assess the significance of this finding.

In addition to changes in the epithelia, snuff use may induce changes in the underlying lamina propria such as amorphous deposits in the dermal papillae, as a result of altered collagen structure (8). Increased expression of tenascin has also been reported (46). The present study showed that snuff stimulates the synthesis of collagen type IV probably by basal cells, which may also explain the amorphous deposits at the transition between the lamina propria and the submucosa found by other researchers.

To conclude, this study shows that snuff does not induce epithelial cell proliferation but possibly increases the life span and differentiation of epithelial cells leading to hyperkeratosis and epithelial thickening. This finding may partly explain why dysplastic and cancerous changes are only seldom encountered in mucosal lesions caused by the use of the Scandinavian type of snuff.

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