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Phenotypic characterization of oral mucosa: what is normal?

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BACKGROUND: Knowledge of the phenotypic pattern of oral squamous epithelium is important in the histopathologic evaluation of lesions including cancer. The literature on normal epithelium is controversial as the phenotype has not been evaluated in samples from completely healthy tissue donors without a history of tobacco and alcohol exposure.
METHODS: In this study, we evaluated normal upper lip fornix and gingival mucosa from carefully selected young healthy donors without a history of smoking and alcohol exposure, and keratin types 8, 10, 14, and 17, filaggrin, and Ki67 were investigated in these donors. The results were compared with profile of epithelium from leukoplakia.
RESULTS: The results demonstrated that the phenotypic patterns of gingiva and upper lip fornix mucosa were different. Surprisingly, a high proportion of gingival samples exhibited keratin 8 and a suprabasal signal for keratin 14. These patterns were compared with that of human oral leukoplakia, and some phenotypic similarities were noted.
CONCLUSIONS: These results demonstrated oral epithelium phenotypic plasticity based on functional requirements of the microenvironment, which can be used in diagnosis.

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Keywords: gingiva; keratin; leukoplakia; merkel cell; microenvironment

Introduction

The oral cavity is a very special environment that is under permanent mechanical, chemical, and microbiological stress. Oral mucosa is covered by squamous epithelium that must fulfill all requirements to maintain homeostasis in this part of the upper aerodigestive tract. Squamous epithelium is a multilayered sheet of cells that is morphologically, functionally, and phenotypically stratified. The proliferating cells (including cells with epithelial stem cell properties) adhere to the basement membrane. However, proliferating cells can also be observed suprabasally during the course of inflammation or dysplasia. Cells in the basal epithelial layer usually express keratins 14 or 19. On the other hand, suprabasal mitotically inactive cells express keratin 10. Keratinized epithelium also expresses filaggrin molecules suprabasally (1–3). Knowledge of the molecular topology of the multilayered sheath is important as the detection of differentiation-specific proteins can be used during histopathologic inspection of pathological lesions in the oral cavity to refine the diagnosis and direct personalized medicine (4). The detection of keratin 8 seems to be particularly useful. This intermediate filament is not expressed postnatally in squamous epithelium in contrast to monolayer epithelia such as intestinal epithelium. The presence of keratin 8 in oral lesions is usually accepted as a marker of malignancy (5).

When the expression of usual differentiation-dependent markers is evaluated, it is difficult to define what normal expression is. The number of studies describing the phenotype of oral epithelium from normal donors is very limited. Usually, tissue harvested some distance from the tumor or tooth decay is considered 'normal' (6). The influence of smoking and alcohol abuse on oral epithelium has also not been evaluated. In addition, it is known that distant inflammation or tumor can significantly influence the microenvironment (for example by dysregulation of cytokine production) and thus, the phenotype of the epithelium can be affected.

This study evaluated the phenotypic pattern of markers used to describe squamous epithelium in tissue samples obtained from carefully selected, young healthy donors without a history of smoking and alcohol abuse. We evaluated samples of gingiva as mechanically and microbiologically exposed tissue and mucosa from the upper lip

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fornix which is not mechanically stressed. The obtained data were compared with the differentiation pattern observed in leukoplakia.

Materials and methods

Patient characteristics

All tissue samples were obtained from healthy young male and female donors up to 20 years of age without a history of smoking and alcohol abuse. These donors did not have signs of inflammation including periodontal disease. The gingiva samples (number of donors was $n = 24$) were harvested during extraction of the 3rd molar with no tooth decay for orthodontic purposes. Mucosa from the upper fornix (number of donors is $n = 10$) was obtained before surgical prolongation of the maxilla. The size of sample was approximately 3 mm^3. We also evaluated samples of human oral leukoplakia obtained from 10 patients. The characteristic of patients with leukoplakia is summarized in Table 1. All samples were obtained with informed consent from the tissue donors after agreement from the Local Ethical Committee according to the Declaration of Helsinki.

Tissue sample processing and immuno- and lectin cytochemistry

The tissues were immersed in Tissue-Tek (Sakkura, Zoeterwoude, the Netherlands) a cryoprotective agent at 4°C, frozen in liquid nitrogen, and then stored in containers with pieces of ice to prevent drying of the specimens at −80°C until further processing. Tissue sections 7 μm thick were prepared using Cryocut (Reichert-Jung, Vienna, Austria). Tissue-Tek was washed from the sections with phosphate-buffered saline (PBS, pH 7.2). They were then fixed with paraformaldehyde for 5 min and subsequently washed three times with PBS for 5 min each time. After treatment with Triton X-100 (Sigma–Aldrich, Prague, Czech Republic)

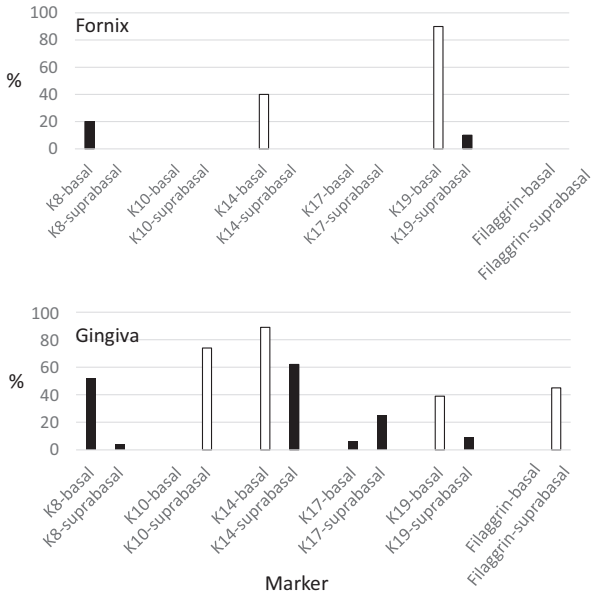

Figure 1 Expression of selected keratins and filaggrin in epithelium covering the upper lip fornix and gingiva. Unexpected expression is shown in black.

Table 1 Characteristic of patients with leukoplakia

No.	*Sex*	*Age*	*Location*	*Dysplasia*	*Keratin 8*	*Keratin 14*	*Keratin 17*	*Ki67 (suprabasal)*
1	F	42	Buccal mucosa, right	No	–	+	–	–
2	M	62	Buccal mucosa, right	No	+	–	–	–
3	M	69	Angle of mouth, left	No	–	+	+	+
4	F	52	Tongue	No	+	+	+	+
5	M	46	Tongue	No	–	+	–	+
6	M	67	Buccal mucosa, left	No	+	+	–	+
7	M	55	Tongue	No	–	–	–	
8	M	43	Tongue	No	–	+	+	+
9	M	55	Buccal mucosa, left	No	+	–	–	–
10	M	21	Gingiva, left	No	+	+	+	+

M, male; F, female.

Table 2 Antibodies used for immunocytochemical analysis

Primary antibody	*Producer*	*Secondary antibody/Fluorochrome*	*Producer*
Wide spectrum cytokeratin/P	Abcam, Cambridge, UK	Swine anti-rabbit/FITC	DAKO Cytomation, Glostrup, Denmark
Cytokeratin 8/P	Sigma-Aldrich, Prague, Czech Republic		
Cytokeratin 17/P			
Filaggrin			
High molecular weight cytokeratin/M	DakoCytomation, Glostrup, Denmark	Goat anti-mouse/TRITC	Sigma-Aldrich, Prague, Czech Republic
Cytokeratin 10/M			
Cytokeratin 19/M			
Ki-67/M			
Cytokeratin 14/M	Sigma-Aldrich, Prague, Czech Republic		

P, rabbit polyclonal; M, mouse monoclonal.

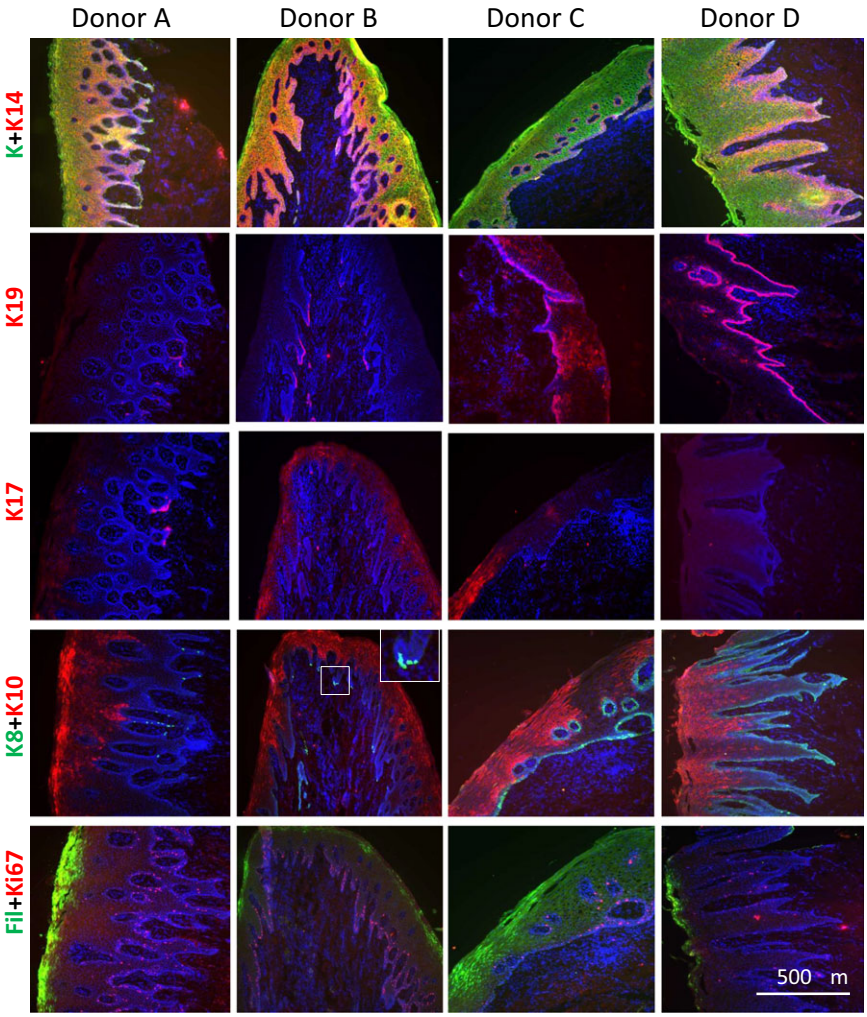

Figure 2 Expression of keratin 8, 10, 14, 17, and 19, filaggrin, and Ki67 in gingival samples collected from four donors demonstrates an interindividual variability in gingival phenotype including non-typical expression. Bar is 500 μm.

(0.1% for 4 min) and washing, the sections were treated with the 1st step antibodies summarized in Table 1 diluted according to the supplier's recommendations. The labeled antibodies diluted as recommended were used as the 2nd step reagents (Table 2). The specificity of the reaction was tested by the isotype non-relevant antibody in the case of monoclonal antibodies and by omission of the 1st step antibody in the case of polyclonal antibodies. Nuclei were counterstained with 4′,6-diamidin-2-phenylindol (DAPI, Sigma–Aldrich). The specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and evaluated using a Nikon-Eclipse 90i microscope equipped with the computer-assisted image analysis system LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic) and a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Osnabrück, Germany).

Results

Upper lip fornix

As expected, all epithelial cells were positive for keratin visualized by antibodies against a panel of keratins. Epithelium from 20% of the samples was positive for keratin 8 with the signal located only in cells of the basal layer. Keratin 14, an obvious marker of basal cells, was observed basally in a number of samples somewhat lower than is 50%. Samples from 90% of donors exhibited keratin 19 positivity in the basal layer, and one sample was even positive suprabasally. Positive signals for keratins 10 and 17 were not detected, and no signal for filaggrin, the marker of keratinization, was observed which was expected. Basal cells in almost 3/4 of samples exhibited Ki67, a marker of proliferation. Suprabasal expression of Ki67 was very rare. Other studied markers were negative. Only one sample contained Merkel cells double positive for both keratins 8 and 19 in the basal cell layer. These data are summarized in Figs 1 and 3.

Gingiva

More than 1/2 of the samples exhibited a distinct signal for keratin 8 different from Merkel cells in the basal layer of epithelium, and the same positivity was rarely detected suprabasally. Suprabasal cells in 3/4 of the studied samples exhibited suprabasal positivity for keratin 10. A signal for

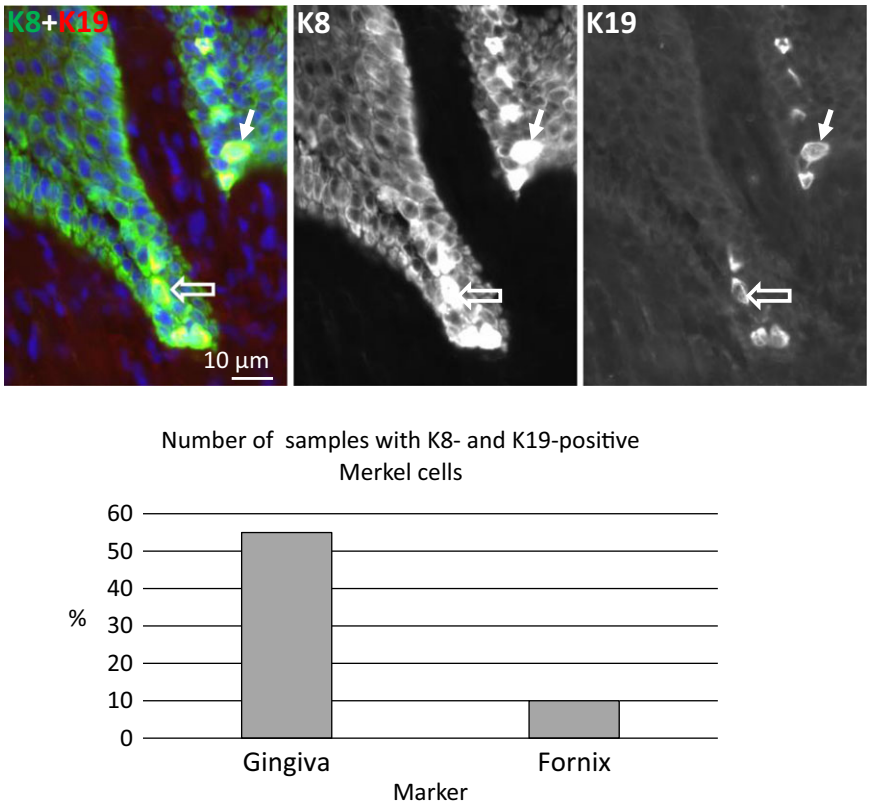

Figure 3 Occurrence of Merkel cells double positive for keratin 8 and 19 in gingival epithelium including quantitative evaluation of the upper lip fornix and gingiva. Bar is 10 µm.

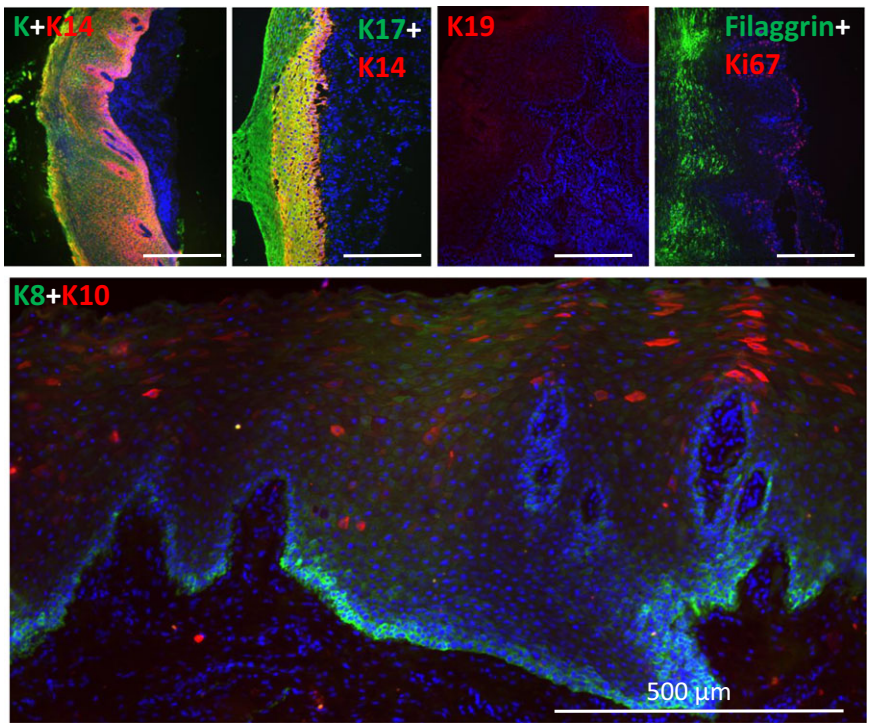

Figure 4 Representative samples of leukoplakia with detection of keratin (K) 8, 10, 14, 17, 19, filaggrin, and Ki67. Bar is 500 µm.

the basal presence of keratin 14 was detected in almost all samples of epithelium and was present suprabasally in cells from 2/3 of donor samples. Keratin 17 was also positive basally as well as suprabasally, but in a minority of samples. Basal positivity for keratin 19 was observed in 40% of samples, and the signal for keratin 19 was rarely detected suprabasally. Almost 50% of samples was keratinized and exhibited a suprabasal signal for filaggrin (Figs 1 and 2). Ki-67 was expressed basally similar to that in fornix epithelium (Fig. 2). More than 50% of samples contained Merkel cells positive for both keratins 8 and 19 in the basal layer of the epidermis (Fig. 3).

Leukoplakia

The patient characteristics are summarized in Table 1. The most prominent sign of leukoplakia epithelium was strong suprabasal expression of keratin 14 (70% of samples). This aberrant epithelium also frequently expressed keratin 17 (40% of samples) and keratin 8 basally (50%) in cells different from Merkel cells. In contrast, keratin 19 was presented in non-Merkel basal cells in sample of one donor. Suprabasal expression of this keratin was also observed in epithelium from one patient. Other samples exhibited no signal. The presence of keratin 10, a typical marker of suprabasal, terminally differentiated cells was very rare similar to the occurrence of filaggrin, a marker of keratinization. Proliferating cells marked by presence of Ki67 were located basally and suprabasally (Table 1; Fig. 4).

Discussion

Samples of mucosa from the upper lip fornix and gingiva exhibited different phenotypic patterns. While the differentiation pattern of upper lip epithelium was as expected, the pattern of epithelium covering the gingiva was quite surprising in relation to the expression of keratins 8 and 14. These differences between the fornix and mechanically exposed gingiva may be due to mechanical forces, as the mechanoreceptor Merkel cells positive for keratins 8 and 19 (7–9) are frequently found in gingival epithelium and are absent in the fornix. These findings reflect the action of mechanical forces associated with mastication (10). These data correspond with the detection of filaggrin in gingiva also observed by other researchers, which was related to keratinization (11) and stimulated by mechanical forces (12). The forces associated with the process of chewing must be reflected in oral cavity tissue close to the teeth (13). Mechanical factors such as tension seem to be an important factor in squamous epithelium function, as these forces stimulate gene expression and epithelial to mesenchymal transition (14, 15). Gingival epithelium frequently exhibits keratins 8 and 17 and suprabasally keratin 14, which can be interesting from a histopathologic point of view. Keratin 8 is usually absent in squamous epithelium postnatally, and its detection is an important tool in histopathologic diagnosis (6, 16). However, previous observations summarized by Presland and Dale (17) support our findings and indicate that keratin 8 may be present in normal gingiva, especially in the junctional part of epithelium. Similarly, detection of keratins 17–19 can have some diagnostic relevance in study of oral epithelium dysplasia (18–20). However, it should be noted that basal expression of keratin 19 is also physiological in oral epithelium (21). Data presented in this study demonstrated some similarity between gingival epithelium and leukoplakia namely in basal expression of keratin 8 and suprabasal expression of keratins 14 and 17. Postnatal keratin 8 expression seems to be associated with dysplasia or cancer formation as was observed by numerous authors and employed in diagnostics (6, 22–24). The suprabasal presence of keratin 14 observed in normal gingiva is also considered a marker of abnormal epithelium including basal/squamous cancer (21, 25–27). The surface of teeth represents an ideal surface for the formation of bacterial biofilms (31). Stimulation of a pro-inflammatory micromilieu in the dental vicinity may also result in the unique properties of the gingiva and is consistent with previous observations on the influence of the pro-inflammatory microenvironment on the expression of keratin 8 by normal keratinocytes (28, 29) and reflects their differentiation plasticity (30).

In conclusion, this study performed in carefully selected normal samples of human gingiva and mucosa of the upper lip fornix obtained from healthy normal donors without a history of alcohol and tobacco abuse demonstrated the difference between the mucosal phenotype of the gingiva and upper lip fornix. The specificity of biomechanics and immune conditions in both locations may be associated with this difference. The presence of some phenotypic markers in the gingiva is similar to abnormal epithelium as demonstrated in leukoplakia samples. These data provide evidence of epithelial plasticity and may be diagnostically relevant, because differentiation pattern typical for abnormal epithelium can be also exhibited in normal oral epithelium.

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Conflict of interests

Authors declare no conflict of interest.