Desmoglein 3 and keratin 10 expressions are reduced by chronic exposure to cigarette smoke in human keratinised oral mucosa explants

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Objective: Oral mucosa is a physiological barrier against several exogenous stimuli, among which cigarette smoke represents a source of reactive oxidizing compounds. No morphological evidences exist on the smoke effects induced in the human oral epithelium. In this study we performed a preliminary light and transmission electron microscopy morphological evaluation focussing in particular on keratinocyte intercellular adhesion and terminal differentiation in chronic smokers.

Design: Human biopsies were obtained from healthy young chronic smoker women (n = 5) compared with a parallel group of non-smoker healthy volunteers (n = 5), as the smoking habit among women is ever more spreading. Samples were processed for light and transmission electron microscopy. On paraffin sections Masson’s and Dane and Herman’s histochemical staining were performed. Biomarker expressions of intercellular adhesion (desmoglein 3, Dsg3), terminal differentiation (keratin 10, K10 and keratin 14, K14), and basal membrane preservation (laminin) were investigated by immunofluorescence.

Results: In both groups the epithelial structural integrity, homeostasis, and the basal membrane were comparable. Dsg3 and K10 expressions were affected in smokers with the former significantly reduced (p < 0.05). Ultrastructural analysis showed hypertrophic keratinocytes in the upper spinous layer and morphologically preserved desmosomes throughout the epithelial compartment.

Conclusions: The reduction of Dsg3 and K10 expressions indicates that the overall process of keratinocyte terminal differentiation was altered. These preliminary results strongly suggest that Dsg3 and K10 can represent valuable immunomarkers to evaluate the tissue attempt to respond to an exogenous stress such as chronic cigarette smoke, but further samples need to be analysed.

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1. Introduction

In the oral mucosa, a cornified stratified squamous epithelium is supported and fed by the underlying connectival lamina propria. The interposed basal membrane, made up of type IV collagen, proteoglicans, and glycoproteins, allows the preservation of the epithelial–mesenchymal cross talking. The mechanical and structural integrity of the epithelium is
guaranteed by the presence of cell–cell junctions and the continuous renewal process of maturation and differentiation of the keratinocytes, known as terminal differentiation (TD). During TD, keratinocytes migrate from the deepest proliferative layer (basal layer) to the upper compartments, i.e., spinous and granular layers, finally reaching the horny layer. This process is characterised by profound molecular and morphological modifications both in the cytoskeletal arrangement and in the junctional complex. The main keratins of the stratified cornified epithelia are keratins 5 and 14 (K5/K14) in the basal layer and K1/K10, K4/K13 in the superficial layers.

Epithelial cell–cell junctions encompass tight junctions, adherens junctions, and desmosomes. These latter are the most abundant in the stratified epithelia and intercellular cohesion is mediated by desmosomal cadherins, desmocollins (Dscs) and desmogleins (Dsgs). These are glycoproteins that anchor adjacent epithelial cells by calcium-dependent interactions between their extracellular protein domains. In addition, they have a transmembrane domain and a cytoplasmic tail providing a binding platform for the armadillo family members: plakoglobin and plakophilin. Up to now three isoforms for desmocollins (Dsc1–3) and six isoforms of desmogleins (Dsg1–3, Dsg1-β, Dsg1-γ and Dsg4) have been characterised. Dsg1 and Dsg3 are the specific isoforms of oral keratinised epithelium, with Dsg3 more expressed than Dsg1.

Oral mucosa is one of the most important physiological barriers against environmental stresses, chemical damages, mechanical agents, and bacterial infections thanks to the presence of closely adherent cells joined by abundant intercellular junctions. The continuous differentiation pattern allows the epithelial renewal, playing a key role in maintaining the barrier effectiveness.

The strategies adopted by the oral mucosa in toto and by single keratinocytes to protect the underlying tissues are different according to the stress they must face. Thus, the knowledge of the oral epithelial homeostasis in basal conditions and after exposure to exogenous stimuli represents a crucial issue of oral biology. Among the different environmental stresses that human oral mucosa faces, cigarette smoke plays a pivotal role and is responsible for the production of highly reactive oxidant species such as aromatic polycyclic compounds, aldehydes, and free oxygen radicals. Several studies investigated the effects exerted in respiratory airways by different smoke compounds. From a clinical point of view, oral modifications occur in the epithelial lining, as melanosis in the masticatory mucosa, ikerkeratosis, and acanthosis.

The aim of this study was a preliminary structural and ultrastructural evaluation of human oral mucosa cytoarchitecture in smokers. In particular, expression of TD epithelial biomarkers as parameters of intercellular adhesion (Dsg3), terminal differentiation (K10 and K14), and basal membrane preservation (laminin) were investigated by immunofluorescence.

2. Materials and methods

All chemicals were from Sigma Aldrich (Milan, Italy) unless stated otherwise.

Biopatic fragments were obtained from the gingival mucosa of the upper and lower dental arches during the extraction of the wisdom teeth of smoking (n = 5) and non-smoking (n = 5) healthy volunteer women aged 20–40 years, who understood and signed the informed consent accordingly with Helsinki Declaration. The investigation was approved by the Institutional Ethical Committee.

Two groups of individuals were recruited: phenotypically normal non-smokers and phenotypically normal chronic smokers. The latter group was chosen among subjects reporting an addiction between 5 and 10 cigarettes/day from 5 (n = 3), 10 (n = 1) or 15 (n = 1) years, without history of current respiratory tract infection, chronic bronchitis, or lung cancer. Women were excluded if reported a history of alcohol consumption, or recent use of non-steroidal anti-inflammatory drugs or other anti-inflammatory medications. In smokers, biopsies were taken from apparently non-lesioned areas, namely without oral inflammation.

Immediately after excision, each biopsy was fragmented using a sterile scalpel into two different specimens processed in parallel for light (LM) and transmission electron (EM) microscopy. Histochemical and immunofluorescence analysis were performed using a Nikon Eclipse 80i microscope equipped with a Nikon digital camera DS-5Mc (Nikon, Tokyo, Japan), ultra-structural analysis with a Jeol CX100 transmission electron microscope (Jeol, Tokyo, Japan).

2.1. Histochemical analysis

For LM analysis, samples (3 mm × 3 mm) were immersion fixed in 4% formalin in phosphate buffer saline (PBS) 0.1 M pH 7.4 for 5 h at room temperature, routinely dehydrated with ascending ethanol, and paraffin embedded. 4 μm serial sections were cut using a manual rotary microtome (RM2155, Leica, Wien, Austria) and collected on 3-amino-propytriethoxysilane-coated glass slides. Paraffin sections were de-waxed, rehydrated, and stained with Masson’s trichrome staining for the evaluation of the cytoarchitecture and three-dimensional structure of the oral epithelium. Nuclei were stained with Weigert’s iron haematoxylin, and slides were immersed in ponceau 2R/ fuchsin solution, orange G diluted in phosphomolybdic acid, and fast green FCF, with washing in 1% acetic acid solution between each colouration step. Adjacent sections were stained following the tetrachronic method of Dane and Herman to demonstrate the presence of keratins and prekeratins. The reaction is based on the use of Mayer’s emallume, fuchsin B, alcian blue, and orange G.
2.2. Immunofluorescence analysis

Intercellular adhesion, terminal differentiation and basal membrane preservation were investigated by immunofluorescence on 4 \mu m paraffin sections. In negative technical controls the primary antibody was always omitted and replaced by Bovine Serum Albumin (BSA) 1% in PBS 0.1 M pH 7.4. In all slides nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) diluted 1:50000 in distilled water.

To evaluate intercellular adhesion, a prediluted monoclonal mouse anti-human Dsg3 antibody (Progen Biotechnik, Heidelberg, Germany) was used as previously described. For each subject at least three sections were placed on one slide, two slides per experiment were considered, and at least three experiments were then carried out (18 sections examined). On each slide, one section represented the internal negative technical control.

Dsg3 expression was quantitatively assessed on the whole epithelial area as already described modifying a protocol previously proposed and extensively used. Digital microphotographs of the whole epithelial area with the same exposure time for positive and negative sections were acquired. The mean optical density (OD) of the whole epithelium in each section was measured thanks to Image Pro Plus software (version 4.5.0.19; Media Cybernetics, Bethesda, MD, USA). The mean OD of negative controls, i.e. the non-specific autofluorescence, was later subtracted to the mean OD of the correspondent positive sections. Results were expressed as mean OD + standard deviation (SD) for each subject in the non-smoking and smoking groups and were then compared through the non-parametric Mann–Whitney’s test (significance set at p < 0.05).

To verify the basal membrane continuity, sections treatment with pepsin 0.1% in HCl 2N pH 1.5 for 25 min at 37 °C was necessary before primary antibody incubation. A polyclonal rabbit anti-laminin antibody was used (Monosan, Uden, The Netherlands) diluted 1:100 in PBS/BSA 1% for 1 h at room temperature. Repeated washing with PBS/BSA 1% solution were done before incubation for 1 h at room temperature with a FITC-conjugated goat anti-rabbit immunoglobulin (Molecular Probes–Invi-trogen, Carlsbad, California) diluted 1:50 in PBS/BSA 1%. Laminin immunofluorescence also allowed to detect blood vessels in the underlying connective tissue.

The expression of terminal differentiation biomarker K14 was revealed using a monoclonal mouse anti-human K14 antibody (LL002; Santa Cruz Biotechnology Santa Cruz, California, USA). Unmasking with pepsin 0.05% for 15 min at room temperature was followed by the incubation first with the anti-K14 antibody (1:200 in PBS/BSA 1%) overnight at 4 °C and then with the same FITC-conjugated goat anti-mouse immunoglobulin described for Dsg3.

To localise K10, samples were immersed in sodium citrate buffer 0.01 M pH 6 and put in autoclave at 95 °C for 5 min. Sections were incubated with PBS/BSA 3% for 20 min at room temperature and then with mouse anti-K10 antibody (Progen Biotechnik, Heidelberg, Germany) diluted 1:10 in PBS/BSA 1% overnight at 4 °C. As secondary antibody the already mentioned FITC-conjugated goat anti-mouse immunoglobulin was used.

2.3. Ultrastructural analysis

Gingival fragments (2 mm × 2 mm) were fixed in 3% glutaraldehyde in Sorensen buffer 0.1 M pH 7.4 overnight at 4 °C and Araldite embedded. 2 μm semithin sections were stained with toluidine blue and analysed with the already mentioned light microscope. Ultrathin sections (60 nm) were cut using a diamond knife, with a Reichert Ultracut R ultramicrotome (Leica, Wien, Austria) and stained with lead citrate and uranyl acetate.

On digital electron microphotographs taken at an original magnification lower than 5000×, intercellular spaces were measured with the above cited image analysis system. At least five microphotographs per sample were analysed and at least 15 measurements were performed on each microphotograph. Results were expressed as mean length (μm) + SD for non-smoking and smoking groups. Data of non-smokers and smokers were then compared through the non-parametric Mann–Whitney’s test (significance set at p < 0.05).

3. Results

3.1. Histochemical analysis

In both non-smokers and smokers the physiological arrangement of keratinocytes in a stratified squamous epithelium anchored to the basal membrane and supported by the underlying connective tissue was preserved without morphological differences between the two experimental groups, as showed by Masson’s trichrome stained paraffin sections (Fig. 1A and B). Toluidine blue stained araldite semithin sections (Fig. 1C and D) confirmed at a microscopic level the grossly normal appearance of oral mucosa in the smoking group, as previously detected by our clinicians. Nuclear counterstaining with Weigert’s iron haematoxylin demonstrated that cell density was not modified throughout all epithelium in smokers. In the epithelial compartment, columnar basal cells were noted and spherical and flattened keratinocytes were present, respectively, in the spinous and granular layers.

In all samples of the non-smokers’ group a homogeneous staining specific for keratins and prekeratins was evident in the living epithelium after Dane and Herman’s staining; the stratum corneum showed an intense orange shade (Fig. 1E). Abundant brown granules were present in the cytoplasm of granular keratinocytes (arrowheads in Fig. 1E). In the oral epithelium of smoking subjects the colour shade decreased proceeding from the basal compartment towards the uppermost layers (Fig. 1F) with an abrupt reduction of colour intensity between medium and upper spinous layer. Moreover, this staining revealed scattered hypertrophic cells in the spinous layer with a pale cytoplasm. In the suprabasal layers of non-smoking subjects
and alterations in its localisation in smokers were evident (Fig. 2B). In the non-smoker group Dsg3 expression gradually increased proceeding from the basal to the spinous layer, softened in correspondence of desmosomes of granular keratinocytes, and disappeared in the stratum corneum. In the oral epithelium of smokers Dsg3 expression was restrained to the spinous compartment, while basal/granular cells and corneocytes showed a weak or no specific labelling of their cell membrane (arrows in Fig. 2B).

When all the epithelium was globally considered, a significant reduction of Dsg3 expression measured as OD was found in the smokers' group (0.039 ± 0.009 in non-smokers vs. 0.023 ± 0.006 in smokers, \( p < 0.05 \), Mann–Whitney’s test) (Fig. 3).

### 3.2.2. Basal membrane

In both experimental groups laminin immunoreactivity showed the preservation of the continuous protein network underneath the basal layer following the profile of the epithelial–mesenchymal interface (Fig. 2C and D). Laminin immunofluorescence also revealed a normal pattern of blood vessel distribution in the papillary connective tissue.

### 3.2.3. Terminal differentiation

In non-smokers the specific cytoplasmic labelling confirmed the expression of K14 in basal keratinocytes and only slightly in the suprabasal cells (Fig. 2E), with a similar distribution if compared to smokers' specimens (Fig. 2F).

In non-smokers K10 immunofluorescence was detected starting from the lower spinous up to the horny layer, but not in the basal keratinocytes (Fig. 4A). In all smokers an aberrant distribution of this protein was evident (Fig. 4B–F). Interestingly, every specimen showed its own pattern of K10 localisation, presenting scattered keratinocytes with a very weak or absent immunolabelling (arrows, Fig. 4B, D, and F). K10 expression in other samples was either non-homogeneous (Fig. 4C) or even absent (Fig. 4E) in the uppermost epithelial layers.

### 3.3. Ultrastructural analysis

Transmission electron microscopy allowed us to verify that in both groups basal cells were similarly arranged in a cylindrical monolayer, had numerous mitochondria and voluminous nuclei oriented along the cell axis with a regular chromatin electrondensity (Fig. 5A and C). At higher magnification, morphologically preserved hemidesmosomes and unaltered desmosomes were evident in all samples, even though intercellular spaces were increased (Fig. 5C) between the basal keratinocytes of smoking subjects compared to non-smokers. The quantitative analysis demonstrated that this difference was statistically significant (0.604 ± 0.018 vs.
0.352 ± 0.068; p < 0.05, Mann–Whitney’s test). In smokers abundant infiltrated leukocytes were found throughout the epithelium (Fig. 5D) and, above all, in the lower spinous layer (Fig. 5E) where membrane limited cellular remnants were often found adjacent to leukocytes (arrowheads in Fig. 5E). Furthermore, Langerhans cells were abundant in the spinous layer (arrow in Fig. 5F). Even the connective tissue appeared rich in infiltrated leukocytes, especially near the epithelial–mesenchymal interface where we could recognise macrophages and mast cells (Fig. 5G). The control samples presented, as usual, only scattered mast cells and macrophages in the connective tissue, whilst no Langerhans’ cells were detected between oral keratinocytes in considered samples. In both groups fibroblasts showed normal morphological features.

While spinous keratinocytes of non-smokers were always characterised by abundant electrondense keratin tonofilaments and numerous desmosomal junctions (Fig. 5B), in all samples from smoking subjects the upper spinous layer hosted scattered hypertrophic cells in which keratin bundles were not so evident and seemed disassembled (Fig. 5H). However, desmosomes were well preserved, intercellular spaces were narrow, and nuclei showed homogeneously distributed chromatin without any morphological sign of apoptosis. In both groups the granular layer was characterised by flattened keratinocytes with a centralised nucleus and a clearly visible nucleolus; corneocytes were always present.

4. Discussion

The relationship between cancer and cigarette smoke is clinically well documented, but most studies focussed on the changes induced in airway epithelial cells and in lungs. The present work represents a preliminary but important brick in basic oral biology as it reports the morphological effects induced in the first target organ of the chronic smoke exposure, i.e. human keratinised oral mucosa, with a particular attention to keratinocytes, which are the earliest cytotype facing such exogenous stress.
In non-smokers and smokers the structural integrity of the oral mucosa was comparable, no evidences of apoptosis were found, and K14 immunostaining showed that the basal layer maintained unaltered its homeostasis. In previous studies, cultured oral mucosa showed profound alterations of the normal cytoarchitecture, stratification, and keratinisation after 15 days of exposure to nicotine. A long-term systemic nicotine treatment induced morphological modifications in the cheek mucosa of rats, with a consequent damage of the cellular and tissue integrity. The mismatch between our observations and the above cited studies can be related to the different experimental settings, the administration routes, the exposure times, and the different exogenous stresses, i.e. nicotine versus cigarette smoke.

In the present study, the epithelial–mesenchymal cross talking needed for keratinocyte maturation and differentiation was preserved, as shown by laminin immunoreactivity. Leukocyte infiltration was evident throughout the oral mucosa, both in the connective tissue and in the epithelial compartment of smokers. Even though smokers are more susceptible to developing periodontal diseases, smoking masks overt inflammation signs as a consequence of the inhibited production of cytokines. Thus, grossly normal-appearing oral mucosa without overt gingival inflammation reported by our clinicians in these subjects is in accordance with the previous report. However, up to now, no morphological evidence was obtained on this issue and the presence of mononuclear leukocytes may be related with a change in their gene expression as suggested by an in vitro study on mononuclear blood cells exposed to tobacco smoke. A recent work revealed by immunohistochemistry an increased number of Langerhans cells in the oral mucosa of smokers, reflecting a smoking-related change in mucosal immune function. These Authors also suggested that, smoking cessation will result in normalisation of the number of Langerhans cells in the oral mucosa and improved immune function. Leukocyte infiltration was present in all biptic samples of the smokers’ group, but an inter-individual variability was observed and didn’t seem to be correlated with the smoke exposure.

In the epithelium, the cytoskeletal apparatus and the desmosomal molecular composition resulted profoundly affected by cigarette smoke starting from the suprabasal compartment, indicating that keratinocyte TD was altered. Ultrastructural analysis, Dane and Herman’s histochemical staining, and K10 expression strongly supported the hypothesis that a dysplastic transformation occurred in the upper epithelial layers of chronic smokers probably as a consequence of K10 loss which is considered as one of the first marker during malignant conversion. The quantitative analysis for K10 expression was not performed as its aberrant expression was absolutely evident, but the inter-individual differences seem not to be correlated with the individual smoking habits. The reported ultrastructural cytoskeletal disassembly can be explained considering that keratins are the most susceptible proteins to oxidative stress. In particular, keratin intermediate filaments are privileged targets of carbonylation, an irreversible non-enzymatic...
oxidative reaction due to cigarette smoke. Recent studies indicate that keratins might be more than just structural reinforcement and that changes in the specific composition of the keratin intermediate filament cytoskeleton can have dramatic consequences on the biological behaviour of epithelial cells. On the basis of these premises, the aberrant K10 expression can be interpreted as an adaptive response to a chronic injury aimed at preventing the barrier disruption in environmentally challenged oral mucosa.

In the preservation of the physiological barrier of the oral mucosa, a pivotal role is played by desmosomal cadherins. In oral mucosa Dsg1 and Dsg3 show a reciprocal expression throughout the epithelial compartment. To our knowledge, this is the first study addressing the smoke outcome on the molecular composition of the most important epithelial intercellular junctions, i.e. desmosomes. Up to now no evidences are available on the direct relationship between the expression of the mucosal type desmosomal cadherin Dsg3 and cigarette smoke. The effect on tight junctions was taken into account and a reduced expression of E-cadherin and zona occludens-1 (ZO-1) was reported in co-cultures of smokeless tobacco pretreated fibroblasts and immortalised keratinocytes. Additionally, ZO-1 disassembly was observed in bronchial cultured epithelial cells following cigarette smoke exposure.

In the present study Dsg1 labelling was similar in non-smokers’ and smokers’ groups (data not shown), whilst Dsg3 expression was detected in correspondence of the upper spinous layer, i.e. in the compartment where hypertrophic keratinocytes were found. This observation suggests that in these cells the cytoskeletal disassembly was not accompanied by alterations either of the molecular composition or of the ultrastructural features of the desmosomal complex. However, in all smokers, Dsg3 immunoreactivity significantly decreased in the innermost and outermost epithelial layers. Differently from Dsg1, the expression of Dsg3 was not clearly correlated with a worse prognosis in tobacco-induced head and neck cancer, and contradictory observations were
reported regarding its role in head and neck carcinogenesis.\(^{40,41}\) In the latter study this desmosomal cadherin was overexpressed in a subtype of head and neck squamous carcinoma cells, but not in other non-squamous malignancies, leaving its role still controversial. In transgenic mice, an altered distribution of desmoglein isoforms affected the epithelial permeability\(^{42}\) and, thus, the reported Dsg3 decrease leaving its role still controversial. In transgenic mice, overexpression of carcinoma cells, but not in other non-squamous malignancies,\(^{43}\) increased nasal epithelial ciliary beat frequency associated with lifestyle tobacco smoke exposure.\(^{44}\)

The present observations together with previous studies\(^{21}\) suggest that, in the oral epithelium, Dsg3 can be a valuable immunomarker of acute and chronic response to exogenous stimuli, targeting more the molecular composition than the morphological structure of desmosomes. However, further studies on a larger smoking population are needed to strengthen this preliminary analysis. Ongoing studies in our laboratory are aimed at elucidating parallel cellular effects induced by cigarette smoke on intercellular filament carbonation, qualitative/quantitative analysis of the leukocyte infiltration, cell proliferation, and tight junction protein expression. A particular attention will be devoted to the early events induced in organ explants of human oral mucosa after acute exposure to a single cigarette smoke, considering both keratinised and non-keratinised regions of the buccal cavity. Another issue will be the comparison between male and female smokers to further elucidate the gender-dependent effects of cigarette smoke.

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