Expression of keratins 8, 18, and 19 in epithelia of atrophic oral lichen planus

Keratins form intermediate filaments of the cytoskeleton in keratinocytes and have roles in cell structure, signaling, intracellular transport, and cell death. Oral lichen planus (OLP) is an oral inflammatory disease with derangements in basal keratinocytes and disruption of the basal membrane. Here, we focused on epithelial expression of keratins 8, 18, and 19 because these proteins are known to modulate cell death. Biopsies were taken from buccal oral mucosa of persons with normal oral mucosa (n = 10) or atrophic OLP (n = 10). Cultured normal oral keratinocytes (n = 4) showed expression of mRNA and protein for keratins 8, 18, and 19. Immunohistochemistry showed consistent staining for keratins 8 and 18 in basal keratinocytes of normal oral mucosa. In OLP, staining for keratin (K)8 was mostly negative and staining for K18 was weak. Keratin 19 was expressed irregularly in most biopsies of normal oral mucosa and not at all in OLP. Several mononuclear leukocytes in the cellular infiltrate showed membrane staining for K8 and K18. Positive staining for K16 confirmed partial collapse of the basal cell layer in OLP. The basal cell niche in OLP therefore appeared to be partly populated with keratinocytes demonstrating a higher degree of differentiation (K8 K18 K19 K16); consequently, such areas may be more susceptible to the action of cell death factors released from the cell infiltrate as a result of lacking the protective, normal keratin present in the basal epithelial cell layer of normal oral mucosa.

Oral lichen planus (OLP) is a common chronic inflammatory disease that leads to derangements in basal keratinocytes and disruption of the basal cell membrane (1). The keratinocyte cytoskeleton consists of proteins that build actin filaments, microtubules, and intermediate filaments. Monomeric intermediate filament proteins are composed of a central α-helical rod domain flanked by amino (N) and carboxyl (C) terminals with globular structures (2). Lacking a signaling sequence, the translation is executed by free cytoplasmic ribosomes. Keratins self-assemble by dimerization of a sequence homology type I (acid) keratin with a sequence homology type II (basic) keratin, as coiled coils. Similar heterodimers assemble in an antiparallel and staggered manner and form the soluble tetramer unit of keratin that self-elongates into intermediate filaments (3). During mitosis and wound-healing processes, intermediate filaments are easily disassembled and stocked into tetramers that can be recycled and used to rebuild new filaments. While some keratins, such as keratin 8 (K8), can form dimers with several partners [keratin 18 (K18), keratin 19 (K19), and keratin 20 (K20)], others, such as K18, do not have alternative partners (4). Heteropolymerization stabilizes the keratin filament structure, and non-polymerized keratins are rapidly degraded; thus, loss of a filament partner can lead to keratin destabilization (5). Type I keratins (e.g., K18 and K19) are caspase substrates, while the type II keratins (e.g., K8) are not (6).

Keratins not only provide mechanical cell stability and integrity, they also participate in various regulatory functions, including cell signaling, the cell cycle, and cell death (reviewed in (4)). Altered expression of keratins has an impact on polymerization and has been associated with inflammation (7), cellular stress, metastasis, epithelial barrier dysfunction (4), and higher sensitivity to tumor necrosis factor (TNF)-induced cell death (8). Evidence for crucial cytoprotective functions of keratins is found in many diseases that are associated with genetic keratin variants (9). Notably, deficiencies in expression of K8, K18, and K19 have been found to alter intracellular cell-death signaling, and defective variants of these keratins are associated with diseases in which abnormal cell death is seen (e.g., in the liver, where hepatocellular ballooning and Mallory–Denk body formation can be found) (10). Such cell-death regulation can take place at
different levels, for example, by caspase binding and activation of the death effector domain containing DNA binding domain (DEDD), ensuring degradation of substrates in an ordered manner (11); sequestration of intracellular scaffolding proteins that participate in death receptor signaling (12, 13); modulation of TNF-induced nuclear factor of kappa-light-chain-enhancer of activated B cells (NF-κB) activation (8); targeting of Fas receptors to the cell surface (13); and protection of mitochondrial function (14). Phosphorylation of K8/K18 can also act as a phosphate ‘sponge’, keeping phosphorylation away from, and prohibiting activation of, pro-apoptotic proteins (2). In the absence of K18, K8 can dimerize with K19: the functions of the K8/K19 complex are probably similar to those of the K8/K18 complex, but this has not been explored in detail (4). Additionally, loss of K8 phosphorylation at pS73 and pS431 in an oral squamous cell carcinoma (OSCC)-derived cell line increases cell migration and enhances tumor growth (15). Finally, loss of K8/K18 in the same cell line reduced tumorigenic potential, altered cell shape, and decreased the expression of integrin αβ4 and activity of its downstream signaling pathways, even though the expression of K5/keratin 14 (K14) was scarcely affected (16).

Previous investigations have shown that epithelial expression of keratins is altered in OLP compared with normal oral mucosa and this can therefore affect the functions mentioned above: keratin 1 (K1)/keratin 10 (K10) (17–19), K14 (19, 20), keratin 6 (K6)/keratin 16 (K16), and K19 (19) are reported to be upregulated in OLP, whereas keratin 4 (K4) (21), K4/keratin 13 (K13) (18, 19), keratin 5 (K5)/keratin 15 (K15) (19), and K19 (22, 23) are reported to be downregulated. Data on K8 and K18 expression in oral keratinocytes of normal oral mucosa are inconsistent, varying from reports of absence to sporadic presence (24, 25). While K19 has been found to be downregulated in OSCC (23), the pairwise expression of K8 and K18 in OLP has not been systematically studied. In OSCC, consistent expression of K8 and K18 has been reported (4, 15). As OLP has malignant potential, we hypothesized that expression of K8, K18, and K19 could be altered in this disease. The aim of this study was therefore to evaluate the expression of K8 and K18 and some of their phosphorylated forms in normal oral mucosa and in atrophic OLP. Expression of K19 was also examined as it can be coexpressed with K8. Positivity for K16 staining was used as a marker to distinguish between basal and suprabasal epithelial cell layers (4). As cytokines can regulate expression of several keratins (26), we also tested the hypotheses that inflammatory cytokines from OLP can be modulators for K8, K18, and K19 expression.

Material and methods

Biopsy material

Volunteers were recruited at the Dental Faculty of the University of Oslo and in private dental offices in the region of Oslo, Norway, on the basis of clinical diagnosis and subsequent histopathological assessment by oral pathologists at the Dental Faculty of the University of Oslo and at the Norwegian National Hospital. Buccal oral mucosa biopsies were taken from healthy persons with clinically normal oral mucosa and from patients with OLP, and were either formalin-fixed and paraffin-embedded (FFPE) or snap-frozen in isopentane on dry ice. Normal oral mucosa biopsies were taken from clinically healthy buccal mucosa. The OLP biopsies were taken from an erythematos part of buccal OLP lesions. Clinical and histopathological diagnoses were made according to established criteria (27). None of the patients with OLP were undergoing treatment for their disease. Ten FFPE and 10 frozen biopsies were randomly drawn from a larger group of patients with OLP. Biopsies from patients with normal oral mucosa were similarly drawn from a larger group, with attempts made to match these as much as possible to the OLP biopsies with respect to patient age. Four oral mucosal biopsies for use in cell culture were obtained from healthy volunteers during third molar extractions. These samples were taken from the distal part of the releasing incision in the buccal mucosa. The demographic data of the volunteers included in the study are shown in Table 1. All persons had a Caucasian background.

The study was carried out according to the Helsinki Declaration’s principles for biomedical research and was approved by the Regional Ethics Committee (REK Sør), Oslo, Norway. Written and oral informed consent was obtained from all donors.

Immunostaining

For all tissue-staining techniques, FFPE sections were used, except for detection of M30 and K18 pS33, for which cryosections were required. Four-micron-thick FFPE sections were deparaffinized prior to heat-induced epitope retrieval in 0.05% citraconic anhydrid (Sigma-Aldrich, St Louis, MO, USA) for 15 min at 100°C using a decloaking chamber (Biocare Medical, Pacheco, CA, USA). Immunostaining for keratins was done using the avidin–biotin complex method as previously described (18). Slides were incubated with primary antibodies against keratin 4 (K4) (21), conjugated secondary antibodies, and DAB substrate. The sections were mounted with Aquamount (Pall, New York, NY, USA). Sections were also stained with hematoxylin.

Table 1

Demographics of the volunteers of the study

| Variable                        | Persons with normal oral mucosa | Patients with oral lichen planus |
|--------------------------------|---------------------------------|--------------------------------|
| n                              | 10                              | 10                             |
| Age (yr)                       | 53 (33–73)                      | 52 (30–62)                     |
| Gender (male/female)           | 5/5                             | 4/6                            |
| Smoker/non-smoker              | 2/8                             | 0/10                           |
| Cryosection groupa              | 10                              | 10                             |
| Age (yr)                       | 40 (26–74)                      | 59 (49–70)                     |
| Gender (male/female)           | 5/5                             | 4/6                            |
| Smoker/non-smoker              | 2/8                             | 0/10                           |
| Culture groupb                 | 4                              | NA                             |
| Age (yr)                       | 28 (23–38)                      | NA                             |
| Gender (male/female)           | 1/3                             | NA                             |
| Smoker/non-smoker              | 0/4                             | NA                             |

Values are given as n, n/n or median (range). NA, not applicable.

aBiopsies taken from this group were processed as formalin-fixed and paraffin embedded (FFPE).

bBiopsies taken from this group were processed as cryosections.
USA) and cooled on ice before staining. Cryosections (4 µm) were fixed with acetone at −20°C for 10 min before staining. Staining was preceded by a blocking step of 30 min with 5% serum matching the species of the secondary antibody, after which the sections were incubated overnight at 4°C with the primary antibody. Primary antibodies for single staining were directed against K8, phosphorilated K8 (pS432), phosphorylated K18 (pS33), and K19 (clones EPI628Y, 21H19L3, IB4, and A53-B/A2.26, respectively; all from Thermo Fisher Scientific, Frederick, MD, USA), K18 (clone SB38b; Southern Biotechnology, Birmingham, AL, USA), and cleaved K18 (clone M30; Roche Diagnostics, Basel, Switzerland). Bound antibodies were amplified by incubation with mixed secondary antibodies (biotinylated horse anti-mouse or goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA) for 1 h at 21°C, and finally with peroxidase conjugated ABC reagent (Vector Laboratories). The antibody label was visualized using 3,3′-diaminobenzidine, and nuclei were counterstained with hematoxillin. Negative-control stains consisted of isotype-matched staining of normal oral mucosa with rabbit Ig (Dako, Agilent Technologies Denmark, Glostrup, Denmark), mouse IgG2a and IgG2b (Sigma-Aldrich) at the same concentrations as the primary antibodies they matched with, and are shown in Figure S1, along with positive-control stains for K8, K18, and K19, carried out on minor salivary gland tissue.

For double-immunofluorescence staining, primary antibody pairs of different subclasses, or raised in different species, were incubated simultaneously. The following unlabeled primary antibodies were used: K8 and K18 as above; K16 (clone LL25; Thermo Fisher Scientific), CD4 (clone SP35; Cell Marque, Rocklin, CA, USA), CD8 (clone SP16; Epitomics, Burlingame, CA, USA), CD3, and CD11c (clones LN10 and 5D11 respectively; all from Novocastra, Leica Biosystems, Nussloch, Germany); CD3, CD45, CD45R0, CD68, and HLA-DR (rabbit polyclonal, mouse clones 2B11 + P7D2, UCHL1, KP1, and CR3/43, respectively; all from Dako); and CD45RA (clone 2H4; Coulter, Miami, FL, USA). The secondary antibodies used were biotinylated goat anti-rabbit IgG (Vector Laborato ries), biotinylated goat anti-mouse IgG2b, Alexa 488-labeled goat anti-mouse IgG1 and IgG2a (Southern Biotechnology), cyanine (Cy3)-labeled donkey anti-rabbit IgG, and Cy2-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Europe, Ely, UK), and the biotinylated antibodies were visualized with Cy2- or Cy3-labeled streptavidin (GE Life Sciences, Uppsala, Sweden). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA) before coverslips were mounted with polyvinyl alcohol mounting medium containing DABCO (Sigma-Aldrich).

Cell culture

Primary oral keratinocyte cultures were prepared from biopsies obtained after surgical third-molar removal. After transport in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) with 25 µg ml⁻¹ of bovine pituitary extract (GIBCO), 1 ng ml⁻¹ of epidermal growth factor (GIBCO), and 1x Antibiotic-Antimycotic solution in a humidified atmosphere of 5% CO₂ in air at 37°C. In the experiments, cells from passages 4–6 were used.

RT-qPCR

Cells were seeded at a density of 500,000 cells per well in 6-well plates. After 4 h, medium was replaced with medium lacking bovine pituitary extract and epidermal growth factor. The next day, cells were stimulated with 50 ng ml⁻¹ of interleukin-6 (IL-6), 10 ng ml⁻¹ of interferon-gamma (IFN-γ), 50 ng ml⁻¹ of TNF-α or a combination of IFN-γ plus TNF-α (all from Peprotech, London, UK) for 24 h. Cells were washed twice with cold PBS before lysis in RLT buffer (Qiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). RNA was purified by the use of an RNeasy mini kit, run on a QiaCube (Qiagen). The quantity and purity of the RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Total RNA (600 ng) was transcribed into cDNA using a mixture of reverse transcriptase and random nonamers (Eurogentec, Seraing, Belgium). Each cDNA synthesis reaction was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), in a total volume of 50 µl, as follows: 10 min at 25°C; 30 min at 48°C; and 5 min at 95°C (termination). The following primers were designed using NIH primer blast: KRT8: forward, ACCGAATTGGTCCTCATCAAG and reverse, CCGGATCTCCTCTCATATAG; KRT18: forward, GGAAGTAAAGGCGCTTAAACAG and reverse, GTACTTTGTCTAGCTCTTCTC; KRT19: forward, GAG GAGAAAATCAGTACGCT and reverse, CCATGACCT CATATTGGCTT. Detection of mRNA was performed using a SYBR Green qPCR Master Mix (Bimake, Munich, Germany). Each real-time PCR reaction consisted of 2.5 µl of RNase-free water, 12.5 µl of Master Mix, 1 µl each of forward and reverse primers, and 8 µl of template. Reactions were carried out on a Stratagene MX3005p qPCR system (Agilent Technologies, Santa Clara, CA, USA) for 40 cycles (95°C for 10 s, 51°C for 15 s, 72°C for 20 s) after an initial 10 min incubation at 95°C. All reactions were performed in duplicate. Ribosomal protein S26 was used as a reference gene and to calculate ΔCt values.

Western blotting

Keratinocytes were grown, without stimulation, to 80% confluence in T25 flasks, washed twice in PBS and then scraped off in cold PBS, collected in reaction tubes, and centrifuged at 4°C for 5 min at 450 g. The pellet was lysed in CellLytic M Cell Lysis Reagent (Sigma-Aldrich) with HaltTM Protease Inhibitor Cocktail and EDTA solution (1:100; Pierce, Rockford, IL, USA) on ice for 10 min before centrifugation at 4°C for 10 min at 16,000 g. The pellet was washed with 1 ml of high-salt buffer (10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% Triton X-100) with protease inhibitor for 1 h at 4°C under rotation. After centrifugation (4°C, 10 min, 16,000 g), the pellet was resuspended in 200 µl of 2x Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) supplemented with 2.5% β-mercaptoethanol, heated to 95°C for 10 min, and then 5 µl was loaded into each well.
of 15-well 10% SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to 0.45-μm pore-size nitrocellulose membranes using a semi-dry transfer system (Bio-Rad). The membranes were blocked with 5% bovine serum albumin (BSA) (Jackson ImmunoResearch Europe) in wash buffer (50 mM Tris–HCl, 154 mM NaCl, 0.1% Tween-20) for 1 h at 21°C. The blots were then incubated with primary antibody solution overnight at 4°C (clones EP1628Y and SB38b, 1:1,000; clone A53-B/A2.26, 1:200), washed three times in wash buffer for 10 min, then incubated with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG (1:6,000; Jackson ImmunoResearch Europe) for 2 h at 21°C. The blots were washed in wash buffer before visualization with a substrate solution containing 5-bromo-4-chloro-3-indolyolphosphate and nitro-blue tetrazolium (Sigma-Aldrich).

**Immunocytofluorescence**

Keratinocytes were seeded on glass coverslips at a density of 250,000 cells per well in a 24-well culture plate without stimulation. After 24 h, the coverslips were washed with cold PBS and the cells fixed with a prechilled mixture of methanol-acetone (7:3, v/v) for 5 min at −20°C. The coverslips were washed with PBS, then blocked with 1% BSA before overnight incubation with primary antibody solution (clones EP1628Y, A53-B/A2.26, and SB38b 1/200). After washing, the slides were incubated with Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Europe) or biotinylated horse anti-mouse IgG (Vector Laboratories) followed by Cy2-labeled streptavidin (GE Healthcare, Amersham, UK). Nuclei were stained with DAPI (Thermo Fisher Scientific), before the coverslips were washed and mounted with polyvinyl alcohol mounting medium containing DABCO (Sigma-Aldrich). Photographs were taken using a Nikon E90i microscope equipped with a DS-Ri1 camera using the NIS-elements software (Nikon Instruments Europe, Amstelveen, the Netherlands). The final photographs were composed using Adobe Photoshop CS5 (San Jose, CA, USA).

**Statistical evaluation**

To evaluate the effect of the addition of cytokines on the transcription of keratins, univariate \( t \)-tests were carried out on \( \Delta A C_t \) values, using IBM SPSS V25 (IBM, Armonk, NY, USA). Two-sided values of \( P < 0.05 \) were considered to indicate statistical significance.

**Results**

**Keratin expression in cell culture**

RT-qPCR was carried out to detect \( KRT8, KRT18, \) and \( KRT19 \) mRNAs in four primary oral keratinocyte cultures. \( KRT8, KRT18, \) and \( KRT19 \) mRNAs were readily detected (\( \Delta A C_t = 4.2, 1.5, \) and 4.5, respectively) and transcription was significantly increased (\( P < 0.05 \)) after addition of IFN-\( \gamma \) (\( KRT78 \) and \( KRT19 \)), TNF-\( \alpha \) (\( KRT19 \)), or IFN-\( \gamma \) + TNF-\( \alpha \) (\( KRT8, KRT18, \) and \( KRT19 \)) (Fig. 1A). Western blotting using extracts from primary oral keratinocyte cultures derived from healthy persons showed expression of K8, K18, and K19 proteins, as indicated by bands of the correct calculated molecular weights (Fig. 1B). Immunocytostaining of cells also confirmed expression of the same molecules in cultured oral keratinocytes (Fig. 1C).

**Keratin expression in tissues**

The results of tissue staining are summarized in Table 2. In all biopsies from normal oral mucosa, K8 showed strong cytoplasmic staining throughout the basal and parabasal cell layers (Fig. 2A). Most other epithelial layers showed weak cytoplasmic staining. Occasionally, cells in the basal and lower spinous layers showed intense cytoplasmic staining. In OLP, the transition area from histologically non-affected to affected mucosa of OLP showed K8 staining that was reduced in intensity as well as in the number of K8-positive layers (not shown). In atrophic areas, only scattered, weak staining was observed in the basal cell layer, and to a lesser extent in the parabasal cell layer, of nine OLP biopsies; cells in the other epithelial layers were not stained (Fig. 2D). In the remaining OLP biopsy, no staining was seen.

In the cellular infiltrate in OLP, some K8-positive cells were detected in the inflamed connective tissue (Fig. 3A). These cells did not express CD3, CD45RA, CD45R0 (data not shown), or CD68 in double-immunofluorescence staining reactions. Some cells co-expressed K8 and CD11c, and within the epithelium, sporadic K8⁺ cells co-stained for HLA-DR.

**K8 pS432**

In all 10 biopsies of normal oral mucosa, several K8 pS432-positive cells were found scattered within the stratum basale (Fig. 2B) and weak cytoplasmic staining was found in six. In the 10 biopsies taken from patients with OLP, K8 pS432-positive single cells in the basal or parabasal layers and/or faint staining throughout the epithelium were seen in some. In three biopsies, there was no staining (Fig. 2E).

**K18**

In normal oral mucosa, basal cells showed clear, positive staining for K18 (\( n = 10 \)), and in some cases the more superficial epithelial layers stained weakly (\( n = 6 \)) (Fig. 2C). Both cytoplasmic and nuclear staining was observed. In OLP, there was no staining in nine biopsies (Fig. 2F). In one biopsy, weak cytoplasmic staining was observed without clear basal staining.

Besides some K18-positive endothelial cells, some single, stained cells were found in the subepithelial connective tissue in seven of the 10 biopsies from normal oral mucosa and in six of the 10 OLP biopsies (Fig. 3B). These cells were negative for CD4 (data not shown) but positive for CD8, CD11c, CD45, CD68, or HLA-DR.

**K18 pS33**

In normal oral mucosa, weak K18 pS33 staining was seen throughout all epithelial layers (Fig. 2G).
Cytoplasmic staining appeared as small dots in two biopsy samples, while nuclear staining appeared as larger dots in nine. Two of the 10 OLP biopsies showed cytoplasmic staining, four showed nuclear dots, and the remaining four were stain-negative (Fig. 2J). The cellular infiltrate in OLP biopsies was also stain-negative for K18 pS33.

Cleaved K18 (M30)

Cleaved K18, as detected with the M30 antibody, was observed in one single epithelial cell in two of 10 biopsies from normal oral mucosa, and the remaining eight were stain-negative for cleaved K18 (Fig. 2H). Staining for cleaved K18 was seen in colloid body structures in nine out of 10 biopsies from OLP and in a few scattered cells, situated around the basal layer, in five biopsies (Fig. 2K). One biopsy was stain-negative for cleaved K18.

K19

Staining of K19 in normal oral mucosa was highly variable: in four biopsies, most basal cells were positive for K19; in three, some minor areas of coherent basal cells were positive for K19; and in three, a few single basal cells were positive for K19 (Fig. 2I). Nine OLP biopsies were stain-negative for K19; in the remaining OLP biopsy, some areas of basal cells showed weak staining for K19 (Fig. 2L).

Identification of the basal epithelial cell layer

Staining for K16 was applied to identify basal and parabasal cell layers as K16-negative and DAPI-positive (Fig. 4). In normal oral mucosa, epithelial K16 staining was absent in basal and parabasal cell layers and first seen in more superficial epithelial cell layers (Fig. 4A). By contrast, in OLP biopsies the epithelial
layers displaying K16-negative staining were much thinner or totally absent in some areas (Fig. 4B).

Discussion

The K8/K18 and K8/K19 protein complexes are foremost expressed in single-layer epithelia, such as in the intestine, but they can also be expressed in more complex epithelia (4). The latter was found to be the case for oral buccal mucosa as we detected K8, K18, and K19 protein in cells from primary oral keratinocyte cultures, from normal oral mucosa, by western blotting and immunocytofluorescence, and KRT8, KRT18, and KRT19 mRNAs by RT-qPCR. Transcription of K8, K18, and K19 in the epithelium of normal oral mucosa was previously also found in a microarray study (21). Our immunohistochemical tissue-staining results showed strong expression of K8 and K18 in basal and parabasal keratinocytes of normal, oral non-keratinized stratified squamous epithelium. Some staining for K8 was also seen in the spinous cell layer of normal oral mucosa. While the strongest staining for K8 in healthy squamous epithelia is mostly confined to the basal layer, some K8 protein and KRT8 mRNA can be found in more superficial epithelial layers, as has been shown in esophagus and vagina (28). In our hands, K19, the second K8-binding partner, showed more variable expression in normal oral mucosa than K18, a finding similar to that reported in a previous study (23). However, our immunohistochemical K8/K18 staining results contrast the findings of other investigations; MAEDA et al. (29), MATTHIAS et al. (25), VAI DyA et al. (30), and VAN DER VELDEN et al. (31) did not detect notable amounts of K8 and/or K18 in adult or fetal normal oral mucosa. The differences in staining for K8 and K18 between our study and these previous immunohistological studies are likely to be caused by the different tissue-processing methods and antibodies used. We have indeed tested the affinity of several poly- and monoclonal antibodies towards different keratin domains; varying results were obtained (data not shown). Not all antibodies stained stratified keratinocytes similarly on FFPE and cryosections, probably because of differences in epitope availability. From the antibodies tested, we selected the rabbit antibody (clone EP1628Y) for detection of K8, and the mouse antibody (clone SB38b) for detection of K18 because they gave similar and consistent staining on both FFPE-HIER and cryo-prepared tissue sections, which was comparable with the signals achieved with most of the antibodies tested. Expression of K8 has been reported to be stronger in head and neck malignancies than in normal oral mucosa (25); however, we think that the expression of K8 was underestimated in normal oral mucosa because, in the present study using the antibodies tested, we selected the rabbit antibody recognizing K7 and K18. VAN DER VELDEN et al. (31) did not detect expression of K8 or K18 in basal cells of lichen planus rubor, with the exception of Merkel cells, and the expression of K19 was reduced. MATILLA et al. (32) found that K19 was expressed in

Table 2

| Target keratin | Normal oral mucosa (n = 10) | OLP (n = 10) |
|----------------|-----------------------------|-------------|
| K8             | Strong staining in basal and parabasal layers, positively stained single cells distributed throughout the stratum spinosum, and weak cytoplasmic staining in most other epithelial layers (n = 10) | Weak, intermittent staining of basal cell layer and to a lesser extent the parabasal cell layer (n = 9). Stain-negative (n = 1) |
| K8 pS432       | Scattered staining in basal cells (n = 10). Additional faint staining throughout the epithelium (n = 6) | A few positive single cells in the basal or parabasal layers only (n = 4). Faint staining throughout the epithelium (n = 2). Both of the above (n = 1). Stain-negative (n = 3) |
| K18            | Staining of nearly all basal cells (both cytoplasmic and nuclear) (n = 10). Weak parabasal staining (n = 6) | Stain-negative (n = 9). Weak cytoplasmic staining throughout the epithelium without clear basal staining (n = 1) |
| K18 pS33       | Cytoplasmic dots throughout the epithelium (n = 2). Nuclear dots (n = 9). Stain-negative (n = 1) | Cytoplasmic staining throughout the epithelium (n = 2). Nuclear dots (n = 4). Stain-negative (n = 4) |
| Cleaved K18 (M30) | Staining of one single cell (n = 2). Stain-negative (n = 8) | Staining in colloid bodies (n = 9). Staining of few scattered cells (n = 5). Stain-negative (n = 1) |
| K19            | Staining of most basal cells (n = 4), some minor areas of coherent basal cells (n = 3), or single basal cells (n = 3) | Stain-negative (n = 9). Staining of basal cells in minor areas (n = 1) |
nearly 30% of all biopsy samples from patients with OLP, while Shimada et al. (23) found significantly reduced expression of K19 in 150 OLP biopsies. As the disease activity of OLP fluctuates between periods of remission, quiescence, activity with pronounced inflammation, and acute ulceration, the expression of the investigated keratins might vary between these phases.

To limit variability, in the present study biopsies were taken from erythematous areas of OLP, and samples were processed using controlled fixation and treatment procedures.

In healthy tissue, the keratin intermediate filaments K8/K18 and K5/K14 of basal keratinocytes maintain the mechanical strength and shape of cells through
Fig. 3. Double-immunofluorescence (IF) staining of inflammatory cells expressing keratin (K)8 (A) or K18 (B) (red), and cellular surface markers for immune cells (green). The middle images in each panel show the overlay of red- and green-stained cells (i.e., double-IF stain). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Original magnification: 40 x.

Fig. 4. Double-immunofluorescence staining of normal buccal mucosa (A) and oral lichen planus (B). Formalin-fixed and paraffin-embedded sections were stained for keratin (K)16 (green) and for K18 (red), while nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Inserts show the same picture, omitting the red or green signal. Original magnification: 20 x.
their connection with desmosomes and hemidesmosomes (4). The latter is particularly important for retaining firm adhesion between the epithelium and the underlying connective tissue. Unbalanced processing, stress, and genetic variants of the keratin partners K8/K18 have been shown to affect the keratin cytoskeleton negatively in liver diseases, resulting in hepatocellular ballooning and Mallory–Denk body formation (10). In OLP, basal keratinocytes show signs of hydropic degeneration, whereby cells become vacuolated, separated, and disorganized. These morphological changes can be related to the collapse of keratin intermediate filaments, as seen in the present study. Although basal keratinocytes in both normal oral mucosa and OLP express K5/K14 (19, 20), which also binds to hemidesmosomes, loss of K8/K18 can alter cell shape and reduce expression of the hemidesmosomal integrin α6β4 and its downstream signaling pathways, even with unchanged expression of K5/K14 (16). Reduction in integrin α6β4 leads to degeneration of hemidesmosomes and loss of adhesion to the basal lamina (36). Therefore, loss of K8, K18 and K19 (as seen here in OLP) can contribute to weaknesses in the epithelial tissue–connective tissue interface.

Considering the cause of the changes in expression of K8, K18, and K19 in OLP compared with normal oral mucosa, one explanation could be that release of inflammatory mediators, such as IFN-γ, TNF-α, or IL-6, in the lesional area (33), can downregulate expression of K8, K18, and K19. Our experiments in which primary oral keratinocyte cultures were incubated with those cytokines, did not, however, point in this direction, as expression of KRT8, KRT18, and KRT19 mRNAs was increased by treatment with IFN-γ/TNF-α. Such increases in expression of keratin mRNAs are in accordance with similar in vitro experiments in which increased synthesis of the psoriasis-associated K17 is observed after treatment of keratinocytes with IFN-γ (34). Another explanation for reduced or absent expression of K8, K18, and K19 could be that the basal cells are destroyed and are thus no longer present. This notion is supported by the results of the current study and previous observations that in some areas of OLP biopsies, antibodies reactive with K16 – a marker for most epithelial cell layers except for the basal and parabasal cell layers (4) – showed that the basal and parabasal layers of K16-negative keratinocytes, which are usually three- to five cells thick in normal oral mucosa, were markedly thinner in OLP. In OLP, we even observed staining of K16 in cells that were lying in the basal cell position. This suggests that the basal cell compartment in some areas of OLP is lost and is populated by more differentiated keratinocytes. As parabasal cells do not express K18 (Fig. 2F), this could account for the lack of expression of K18 in the basal cell layer in OLP.

The DEDD is constitutively bound to K18 and can also associate with the cysteine protease caspase-3 and caspase-9. Upon induction of apoptosis, conformational changes in DEDD recruit and concentrate procaspase-3 and procaspase-9 at the K8/K18 filaments. This is followed by activation of caspases, cleavage of K18, and finally release of activated DEDD and the activated caspases. This process ensures degradation of the substrates in an ordered manner (11). In the early stage of apoptosis in keratinocytes, active caspase-3 cleaves K18 at the 393DALD-S sequence (37), creating a neo-epitope that specifically is recognized by the monoclonal antibody M30, and this reactivity is lost when, or if, the epithelial cells become necrotic (38). In the present study, hardly any M30-positive cells were observed in normal oral mucosa, whereas several M30-positive cells were observed in OLP. This finding is in agreement with previous studies indicating limited apoptotic cell death of keratinocytes in OLP (39).

Loss of normal expression of K8, K18, or K19 will have different consequences for epithelial cells. Well-known tasks, as for other keratins, are related to mechanical stability and integrity of the epithelial cells, but K8, K18, and K19 are also involved in regulating intracellular cell-death signaling circuits. For example, the K8/K18 complex has the ability to sequester and withhold crucial intracellular scaffolding proteins that participate in death receptor signaling, mediating apoptosis. Examples of this include the tumor necrosis factor receptor type 1 (TNFR1)-associated death domain (TRADD) that binds K8/K18 directly, and Fas-associated protein with death domain (FADD) that binds K8/K18 through DEDD via its death domain. The scaffolding TRADD and FADD proteins participate in death signaling from TNFR1, Fas receptor, death receptor (DR)3, DR4, and DR5 by being part of the death-inducing signaling complex (DISC). Binding of TRADD and FADD to K8/K18 reduces apoptotic signaling by hindering participation of TRADD (12) and FADD (13) in DISC formation. In addition, K8 and K18 both bind the cytoplasmic domain of TNFR2 and can thereby moderate TNF-induced NF-kB activation (8). The severe reduction of K8 and K18 in OLP might partially explain the strong upregulation of NF-kB p50 and FADD in OLP that we observed earlier (39). The K8/K18 complex also contributes to targeting of Fas receptors to the cell surface. Binding of K8/K18 to microfilaments by plectin can modulate lipid raft dynamics and thereby clustering of, and signaling by, Fas (13). Likewise, K8/K18 filaments interact with the DR5 receptor, thereby hindering its translocation to the cell surface and binding to the TNF-related apoptosis-inducing ligand (TRAIL) (40). Recently, it was also shown that K8/K18 has a protective effect on mitochondrial anatomy and function (14). Mitochondria in epithelial cells in OLP can have abnormal anatomy (41) and this can therefore also be related to deficient expression of K8/K18. Thus, loss of K8 and K18 may render epithelial cells more susceptible to cell death, an outcome that is highlighted in K8 knockout mice and cell lines showing increased sensitivity to the actions of apoptosis-inducing ligands such as TNF-α and Fas (8, 12, 13). As such, the deficient expression of K8 and K18 in OLP epithelium may leave basal layer keratinocytes more susceptible to death-induced signaling. Incidentally, the reduced expression of K8/K18 in OLP is in contrast to that seen in OSCC, where K8
expression is upregulated (25). The upregulation of K8 in OSCC may be implicated in the resistance of affected keratinocytes to cell death.

Both K8 and K18 have multiple sites that are suitable for post-translational modification (reviewed in (2)). Phosphorylation of K8 and K18 proteins has been observed during cellular events that regulate their filament organization, solubility, and distribution, as well as in processes concerning cell migration, mitosis, and protection from ubiquitination and apoptosis. Keratin phosphorylation may also act as a phosphate ‘sponge’, thus preventing phosphorylation of, and accordingly prohibiting the activation of, pro-apoptotic proteins (2). During epithelial cell mitosis and apoptosis, K8 and K18 filaments are disassembled into soluble tetramers while still being confined to a dimer structure. This process is facilitated by phosphorylation of serine and threonine residues in the head and tail regions. The functional significance of most phosphorylation sites in K8 and K18 is still elusive. Keratin 8 S432 is phosphorylated by extracellular signal-regulated kinase 1/2 (ERK1/2) in response to epidermal growth factor (42) during mitosis and early during apoptosis (37). This leads to decreased migration of OSCC cells (2). In normal oral mucosa, some basal and suprabasal cells expressed K8 pS432, which might be related to mitosis. This was not observed in OLP, which might point to reduced epithelial restitution. Phosphorylation of K18 S33 regulates keratin filament solubility, organization, distribution, and association with binding proteins (43). The phosphorylation of K18 throughout most of the epithelium of normal oral mucosa indicates that this post-translational modification plays a part in the normal keratinocyte-differentiation process. Hence, the deviating expression of K18 pS33 observed in OLP epithelium further indicates disturbed keratinocyte differentiation. How lack of K18 and its phosphorylated form can cause this disturbance opens an interesting line for further investigations.

A number of cells in the cellular infiltrate of OLP showed membranous staining for K8 and K18. Double-immunofluorescence staining revealed that the K8-positive cells in the cell infiltrate did not express CD3, CD45RA, CD45R0, or CD68 epitopes, but some expressed CD11c. This is an intriguing observation. The information on the expression or presence of keratins in leukocytes is scarce. Keratins can be expressed by large-cell anaplastic lymphomas (K8 and K18) (44), myelomas (pan-keratin) (45), and B-1 B-cell progenitors (K5) (46). Here, we showed that certain leukocytes, possibly dendritic cells as they expressed CD11c or HLA-DR, display membranous staining with K8 and K18 antibodies. This staining can be caused by production of the keratins by the leukocytes themselves, as shown in some of the above listed cell types. Alternatively, the keratins may have been released by damaged keratinocytes and bind to the cell membrane of the leukocytes. Fragmentation of K18 occurs also during apoptosis (6, 38, 47), and fragmented K18 can reorganize into keratin aggregates that are shed into cell-culture medium (37).

Taken together, expression of K8, K18, and K19 are disturbed in OLP compared with normal oral mucosa. This might be caused by total or partial collapse of the basal cell layer in atrophic OLP and population of this layer by more differentiated, K8* K18* K19* cells. As K8, K18, and K19 and their complexes have different important functions, including maintenance of cell structure and regulation of the cell cycle and cell death, the changes may aggravate the epithelial pathology in OLP.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Control stainings.