European Journal of Oral Sciences

Eur J Oral Sci 2020; 128: 369–378
DOI: 10.1111/eos.12732

The Authors. Eur J Oral Sci published by John Wiley & Sons Ltd

European Journal of Oral Sciences

Composition of hemidesmosomes in basal keratinocytes of normal buccal mucosa and oral lichen planus

Olav Schreurs, Maria G. Balta, Andreas Karatsaidis, Karl Schenck
Institute of Oral Biology, University of Oslo, Oslo, Norway

Schreurs O, Balta M, Karatsaidis A, Schenck K. Composition of hemidesmosomes in basal keratinocytes of normal buccal mucosa and oral lichen planus. Eur J Oral Sci 2020; 128: 369–378. © 2020 The Authors. Eur J Oral Sci published by John Wiley & Sons Ltd

Oral lichen planus (OLP) is a chronic inflammatory disease displaying ultrastructural disturbances in epithelial hemidesmosomes. The expression of several key hemidesmosomal components in OLP as well as in normal buccal mucosa is, however, unknown. The aim of the study was therefore to examine intracellular and extracellular components involved in hemidesmosomal attachment, in OLP (n = 20) and in normal buccal mucosa (n = 10), by immunofluorescence. In normal buccal mucosa, laminin-$\alpha3\gamma2$, integrin-$\alpha6\beta4$, CD151, collagen α-1(XVII) chain, and dystonin showed linear expression along the basal membrane, indicating the presence of type I hemidesmosomes. Plectin stained most epithelial cell membranes and remained unphosphorylated at S4642. In OLP, most hemidesmosomal molecules examined showed disturbed expression consisting of discontinuous increases, apicolateral location, and/or intracellular accumulation. Plectin showed S4642-phosphorylation at the basement membrane, and deposits of laminin-$\alpha3$ and laminin-$\gamma2$ were found within the connective tissue. The disturbed expression of hemidesmosomal proteins in OLP indicates deficient attachment of the basal cell layer, which can contribute to detachment and cell death of basal keratinocytes seen in the disease.

Olav Schreurs, Institute of Oral Biology, University of Oslo, PB 1052 Blindern, N-0316 Oslo, Norway

E-mail: schreurs@odont.uio.no

Key words: CD151; collagen α-1(XVII) chain; dystonin; integrin-$\alpha6$; integrin-$\beta4$; laminin-$\alpha3$; laminin-$\gamma2$; plectin

Accepted for publication July 2020

Oral lichen planus (OLP) is a commonly occurring chronic inflammatory disease of the oral mucosa. The overall estimated pooled prevalence of OLP is 0.89% among the general population and 0.98% among clinical patients (1). Clinically, OLP occurs in quiescent and active forms that can vary with time (2–4). The reticular and atrophic variants are the most frequent disease forms and can be localized on the buccal mucosa, tongue, gingiva, lips, palate, and floor of the mouth (2). Patients with oral lichen planus can also exhibit cutaneous, genital, scalp, nail, esophageal, or ocular lichen planus (5). Histologically, OLP is characterized by a dense inflammatory infiltrate in the lamina propria, primarily consisting of T-lymphocytes and epithelial changes, such as local destruction of basal keratinocytes (liquefaction degeneration), epithelial atrophy, acanthosis, presence of Civatte bodies, and hyperkeratosis (2,6).

Hemidesmosomes are multimolecular complexes that ensure stable adhesion of keratinocytes to the underlying basal membrane. In the stratified epithelium of the skin, the hemidesmosomal protein complex called type I hemidesmosome consists of integrin $\alpha6\beta4$, plectin 1a, CD151 (tetraspanin-24), dystonin (230 kDa bullous pemphigoid antigen), and collagen α-1(XVII) chain (180 kDa bullous pemphigoid antigen) (Figure S1) (reviewed in 7). Simple epithelia, such as the intestinal epithelium, lack type I hemidesmosomes but display complexes consisting of integrin $\alpha6\beta4$, CD151, and plectin only, which are called type II hemidesmosomes. The composition of the hemidesmosomes in oral mucosa is not yet fully known. Integrin $\alpha6\beta4$ connects the basal keratinocytes to a component of the basal membrane, laminin-332, a trimolecular complex secreted by basal keratinocytes and consisting of $\alpha3$, $\beta3$, and $\gamma2$ chains. On the intracellular side of the hemidesmosomes, plectin and dystonin connect to the cells' intermediate filaments, which in keratinocytes are keratins. The keratins form filaments that extend from the nucleus to hemidesmosomes and desmosomes in the internal leaflet of the plasma membrane. Under healthy conditions, basal keratinocytes of oral, non-keratinizing stratified epithelia express the keratin dimers K5/K14 and K5/K15 that can bind to dystonin and plectin 1a, and K8/K18 and K8/K19 that can bind to plectin (8–12).

In OLP, ultrastructural studies have revealed disruption of the basal membrane and destruction of hemidesmosomes (13–15). At the molecular level, disturbances in hemidesmosomes and their attachment have also been described. Immunostaining has shown that the integrin $\alpha6$ and $\beta4$ chains are upregulated, can appear circumferentially around basal cells, and, to some extent,

are also found in the suprabasal epithelial layers (16–18). This is in contrast to normal oral mucosa, where they are expressed as a continuous band (18,19). Immunostaining for laminin-332, laminin-111, and collagen type IV in OLP has been described as a absent, discontinuous, intensified, or thickened bands with finger-like projections into the connective tissue, while it was seen as a continuous band along the basement membrane in normal oral mucosa (18–20). Intracellularly, K8, K18, and K19 are absent or strongly reduced in OLP, as compared with normal oral mucosa (10).

A premise for the existence of hemidesmosomes is the presence of integrin-$\alpha6\beta4$ and its substrate laminin-332, the linker protein plectin, and keratin filaments. Hemidesmosomes of the steady-state epithelium of oral mucosa may well have the same composition as those of the skin, but the expression of CD151, collagen α-1 (XVII) chain, dystonin, and plectin in normal basal oral mucosal keratinocytes is unknown. Furthermore, as plectin has a crucial role in the assembly of hemidesmosomes (21), it is important to know its expression in OLP, in view of the hemidesmosomal damage that has been previously described. Therefore, the aim of this study was to examine the key intracellular and extracellular components that are involved in hemidesmosomal attachment, in healthy buccal mucosa and in OLP.

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, Oslo, Norway. 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, or snap-frozen in isopentane on dry ice. OLP biopsies were taken from buccal mucosa of patients who were diagnosed for OLP at an earlier visit, and were either displaying a pure white reticular pattern (RET-OLP), or RET-OLP in combination with erythematous areas, the atrophic type of lichen planus (ATR-OLP). In the latter cases, biopsies were taken from the red areas. Clinical and histopathological diagnosis was made according to established criteria (22). None of the patients with OLP were under treatment for their disease. Biopsies were randomly drawn from a larger group of OLP patients. Biopsies from patients with normal oral mucosa were similarly drawn from a larger group but attempted to match as much as possible in age to the OLP biopsies. Four buccal mucosal biopsies for use in cell culture were obtained from healthy volunteers during third molar extractions. Those samples were taken from the end 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 Ethical Committee (REK Sør), Oslo, Norway. Written and oral informed consent was obtained from all donors.

Table 1

Demographics of the volunteers of the study

Variable	Persons with normal oral mucosa (NOM)*	Patients with reticular oral lichen planus (RET-OLP)†	Patients with atrophic oral lichen planus (ATR-OLP)‡
		FFPE group§	
n	10	10	10
Age (yr, range)	52 (33-73)	60 (42-76)	58 (49-78)
Gender (male/female)	5/5	3/7	3/7
Smoker/non-smoker	2/8	1/9	0/10
		Cryosection group¶	
n	10	10	10
Age (yr, range)	51 (28-64)	60 (42-76)	58 (49-77)
Gender (male/female)	4/6	3/7	3/7
Smoker/non-smoker	2/8	0/10	0/10

Values are given as *n*, *n*/*n*, or median (range).
*0/10 persons were identical in the FFPE and cryosection group
†8/10 patients participated in both FFPE and cryosection group.
‡6/10 patients had a corresponding biopsy in the cryosection group.
§Biopsies taken from this group were processed as formalin-fixed and paraffin embedded (FFPE).
¶Biopsies taken from this group were processed as cryosections.

Immunofluorescence staining

For all stainings, formalin-fixed and paraffin-embedded (FFPE) sections were used, with an exception for the detection of dystonin, which required cryosections. Four-micron thick formalin-fixed and paraffin-embedded sections were deparaffinized prior to heat-induced epitope retrieval in 0.05% citraconic anhydride (Sigma-Aldrich, St Louis, MO, USA) for 15 min at 100°C using a decloaking chamber (Biocare Medical, Pacheco, CA, USA), and they were cooled on ice prior to staining. Cryosections (4 μm) were fixed with acetone at −20°C for 10 min prior to staining. Staining was preceded by a blocking step of 30 min with 5% serum matching the species of the secondary antibody, and incubation overnight at 4°C with the primary antibody. For double immunofluorescence staining, primary antibody pairs raised in different species were incubated simultaneously.

Unlabeled primary antibodies were rabbit anti-CD151, rabbit anti-collagen α-1(XVII) chain, and mouse anti-laminin-$\alpha3$ (Atlas Antibodies, Bromma, Sweden), rat anti-integrin-$\beta4$ (clone 439-9B; Abcam, Cambridge, UK), goat anti-integrin-$\alpha6$ and mouse anti-laminin-$\gamma2$ (E-6) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and guinea

pig anti-plectin (reactive with all alternatively spliced isoforms of plectin expressed in the epidermis, i.e. isoforms 1, 1a, and 1c; Progen Biotechnik, Heidelberg, Germany). Plectin pS4642 was detected with an antibody originally generated to detect an identical epitope in desmoplakin around pS2849 (a kind gift from Dr. Kathleen J. Green, Northwestern University, Chicago, IL, USA). For detection of dystonin, mouse anti-dystonin was used (clone 279; Cosmo Bio, Tokyo, Japan). Dystonin has three main isoforms, where only the epithelial isoform 3 (BPAG1e) is involved in hemidesmosome formation (23). Native protein of cornea was used to generate the mouse anti-dystonin presently used. The linear staining along the basal membrane of normal oral mucosa obtained with this antibody was not observed when another antibody towards dystonin was applied (rabbit polyclonal; Atlas Antibodies), as its immunizing sequence is not present in the much shorter isoform 3 (data not shown). Collagen α-1(XVII) chain is a transmembranous protein that is cleaved within the juxtamembranous non-collagenous 16A domain with successive shedding of the ectodomain 120 kDa linear IgA disease antigen-1. The antibody used here (Atlas Antibodies) detects epitopes within the intracellular amino acids 10-106, just outside of the dystonin binding region. This domain is identical in both known isoforms. Additional details on the primary antibodies are shown in Table S1.

The secondary antibodies used were biotinylated horse anti-mouse and rabbit anti-rat IgG (Vectorlabs, Burlingame, CA, USA), biotinylated donkey anti-guinea pig IgG, Cyanine (Cy)2-labeled donkey anti-rabbit, and donkey anti-goat IgG (Jackson ImmunoResearch Europe, Cambridgeshire, UK). The biotinylated antibodies were visualized with Cy3-labeled streptavidin (GE LifeSciences, 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).

Monochrome photographs were taken from each protein stained with a Nikon E90i microscope equipped with fluorescent filter blocks for DAPI, Cy2, Cy3, and Cy5, and 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). For visualizing all four channels in one single picture, the DAPI signal was converted to grey, while signals from either Cy3 or Cy5 were converted to blue. The staining of the images was assessed by OS and KS.

Results

A summary of the protein expression in the examined tissues is given in Table 2.

Laminins

Laminin-α3 was expressed along the basement membrane of all patients. In normal oral mucosa (Fig. 1A), this was seen as a thin, nearly continuous band. Circumferential membranous laminin-α3 staining of basal and suprabasal cells was observed in one patient. No clear cytoplasmic staining was observed. In both forms of OLP, the expression along the basement membrane was interrupted with small breaks. Cytoplasmic staining was stronger than in normal oral mucosa. In RET-OLP (Fig. 1B), the basal membrane of seven patients showed thickening, doubling, or streaks into the connective tissue, or combinations of these irregularities. In ATR-OLP (Fig. 1C), the expression along the basal membrane was very weak or only local in four patients, while other irregularities were not seen.

Laminin-γ2 also showed a band-like expression along the basement membrane of all patients (Fig. 1D–F). In normal oral mucosa, one patient showed circumferential membranous staining of basal and suprabasal cells and some streaks into the connective tissue. Both forms of OLP (Fig. 1E,F) showed interrupted expression along the basement membrane with irregularities in four patients in each group. The irregularities included cytoplasmic expression and minor breaks, doubling, thickening, and streaks of the basal membrane (Fig. 1G). Cytoplasmic expression was seen in many basal cells of nearly all OLP patients, with the strongest expression in samples of RET-OLP. Circumferential membranous staining of basal and suprabasal cells was observed in three RET-OLP biopsies only.

Integrins

Expression of integrin-α6 appeared as small dots along the basal membrane of all samples of normal oral mucosa (Fig. 2A; red color), while three biopsies showed additional cytoplasmic staining in some basal and suprabasal cells. Increased expression with breaks was observed in all OLP biopsies (Fig. 2B,C; red color) except for two patients with ATR-OLP that did not show staining along the basal membrane. The cytoplasm of basal and suprabasal cells stained in half of the samples from ATR-OLP but none in RET-OLP, while circumferential membranous staining of basal and suprabasal cells was observed in five ATR-OLP and three RET-OLP samples.

Integrin-β4 expression (Fig. 2A–C; green color) was seen in the area of the basal membrane in nearly all samples. In normal oral mucosa, the cytoplasm of some basal and suprabasal cells stained weakly in six samples, while one sample did not show staining. In OLP, staining along the basal membrane was enhanced. Two patients with RET-OLP and one with ATR-OLP showed staining of the apicolateral membranes or the cytoplasm of the basal cells. The cytoplasm of basal and suprabasal cells stained in four patients with ATR-OLP, while three other samples showed circumferential membranous staining of basal cells.

CD151

The staining for CD151 showed a similar location for all groups, although the signal was clearly enhanced in OLP (Fig. 2A–C; blue color): most biopsies showed partial staining along the basal membrane and some did not show any staining, while cytoplasmic staining was observed in about half of the cases. Small intense

Table 2

Summary of immunohistological stainings for hemidesmosomal proteins in clinically normal oral mucosa (NOM) and reticular (RET-OLP) or atrophic (ATR-OLP) forms for oral lichen planus

Protein	Staining observed	NOM (n = 10)	RET-OLP (n = 10)	ATR-OLP (n = 10)
Laminin-α3	Along basal membrane	10	10	8
	- thickened, doubled, and/or with streaks	0	7	0
	- partial or very weak	0	0	4
	Cytoplasm of basal cells	0	9	10
	Membranes of basal and suprabasal cells	1	0	0
Laminin-γ2	Along basal membrane	10	10	10
	- with thickening, doubling, and/or streaks	1	4	4
	Cytoplasm of basal cells	3	8	10
	Membranes of basal and suprabasal cells	1	3	0
Integrin-α6	Along basal membrane	10	10	8
	- partial	0	0	7
	Cytoplasm of basal and suprabasal cells	3	0	5
	Membranes of basal and suprabasal cells	0	3	5
Integrin-β4	Along basal membrane	9	8	9
	- partial	9	0	9
	Cytoplasm of basal and suprabasal cells	6	0	4
	Membranes of basal cells	0	0	3
	Only cytoplasm or apicolateral membranes of basal cells	0	2	1
	No staining	1	0	0
CD151	Partial along basal membrane	7	7	7
	No staining of basal membrane	3	3	3
	Cytoplasm of basal and suprabasal cells	6	5	5
	Dots through entire epithelium	3	5	5
Plectin 1a	Along basal membrane	10	10	10
	Cytoplasm of entire epithelium	7	10	8
Plectin pS4642	Along basal membrane	3	10	10
	- partial	3	0	0
	Cytoplasm and membranes of epithelium	10	10	10
Col17A1	Basal membrane and basal cells	10	10	10
	Cytoplasm of basal cells	1	10	7
	Dotted in suprabasal layers	0	5	0
	Strong stained single basal cells	0	0	5
Dystonin	Basal membrane, with	10	9	10
	- breaks	0	9	4
	- thickening	0	4	2
	Cytoplasm of basal and suprabasal cells	1	9	10

dots were seen in the cytoplasm of the entire epithelium in approximately half of the patients.

Plectin

In all patients, plectin was expressed at most epithelial cell membranes, including the basal membrane, and with cytoplasmic staining of variable strength in the proximity of the membrane (Fig. 2D–F; red color). Intra-epithelial leukocytes, many of them displaying dendrites, stained strongly for plectin. In normal oral mucosa, three patients showed weak phosphorylation of plectin at the basal membrane of a few cells, while the remaining seven did not express pS4642 along the basal membrane (Fig. 2D; green color). In RET-OLP (Fig. 2E), the basal and suprabasal cells expressed cytoplasmic and membranous plectin more intensely than in ATR-OLP (Fig. 2F) and normal oral mucosa (Fig. 2D). The basal membrane of all OLP samples showed minor breaks in the plectin staining, and all showed plectin phosphorylation. Staining for plectin pS4642 was also seen between all epithelial cells, due to its reaction with desmosomes (see Material and methods). It was, however, absent in intraepithelial leukocytes.

Collagen α-1(XVII) chain

Staining for the collagen α-1(XVII) chain was found along the basal membrane in all samples (Fig. 2G–I; blue color). In normal oral mucosa, one sample showed staining of the cytoplasm of basal cells, while staining of apicolateral membranes of basal cells was observed in FFPE sections (data not shown) but was less obvious in cryostat sections. All samples of RET-OLP (Fig. 2H) showed cytoplasmic as well as circumferential membranous staining of basal cells, and half of them showed small dots in the suprabasal layers. Also, in ATR-OLP (Fig. 2I), all samples displayed circumferential membranous staining of basal cells, while five of them showed enhanced staining of single basal cells.

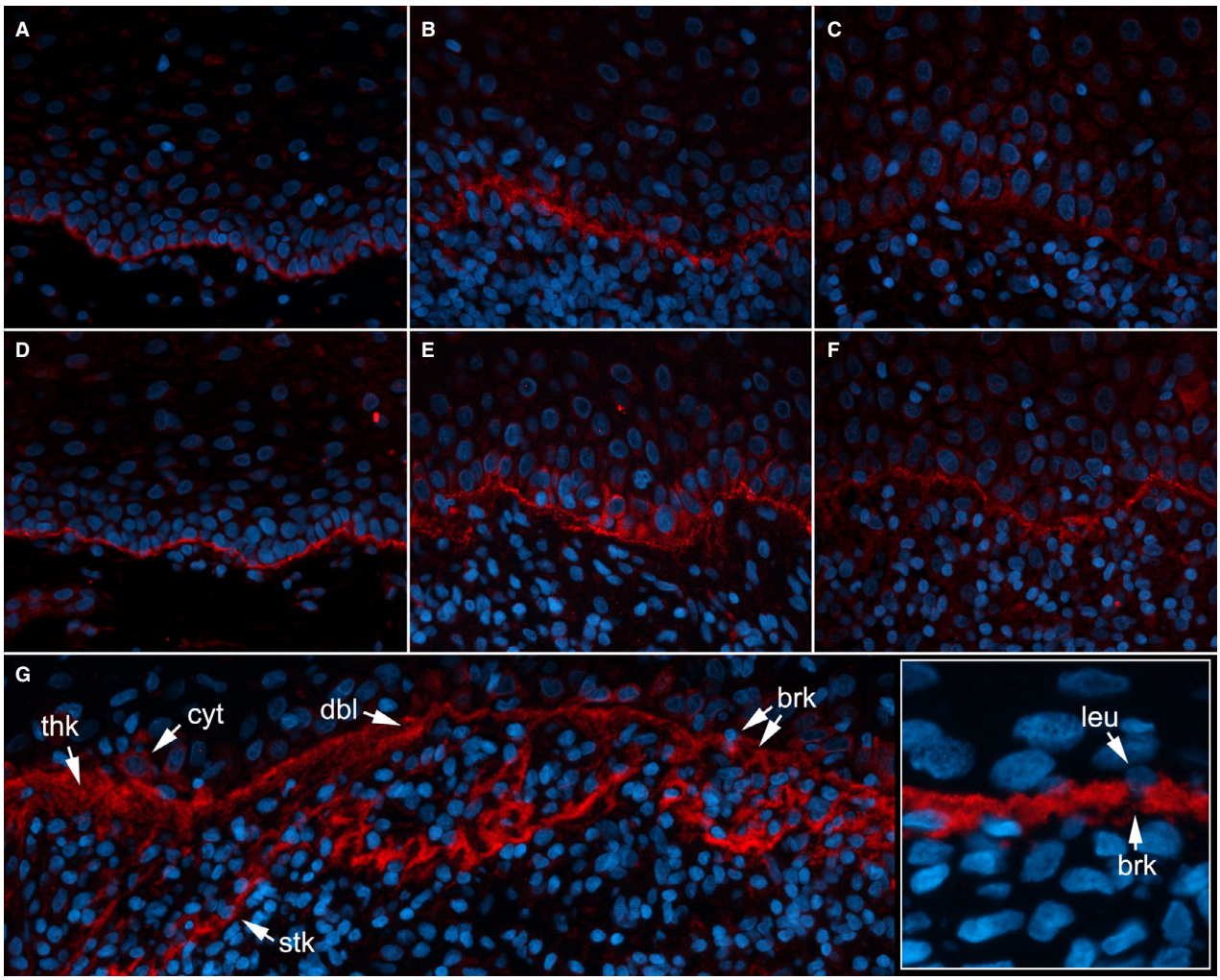

Fig. 1. Immunofluorescent staining of normal buccal mucosa (A, D) and buccal mucosa from reticular (B, E, G) and atrophic (C, F) oral lichen planus. (A–C) Staining for laminin-α3 (red color). (D–G) Staining for laminin-γ2 (red color). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) shown in blue (A-G). Original magnification main pictures 40x. Abbreviations: cytoplasmic expression (cyt), minor breaks (brk), doubling (dbl), thickening (thk), and streaks (stk) of the basal membrane, minor break with a passing leukocyte (leu). [Colour figure can be viewed at wileyonlinelibrary.com]

Cytoplasmic staining was observed in seven samples of ATR-OLP.

Dystonin

Nearly all samples showed expression of dystonin in their basal cells, but this was restricted to the basal membranes in normal buccal mucosa only (Fig. 2G; red color). Cytoplasmic expression of dystonin was found in one sample of normal buccal mucosa. In one patient with RET-OLP, dystonin expression was hard to find, while the remaining samples showed staining of the cytoplasm of basal and suprabasal cells as well as the basal interrupted staining of the membrane (Fig. 2H). Four samples showed a thickened band of staining. All samples of ATR-OLP (Fig. 2I) displayed cytoplasmic staining of basal and suprabasal cells, and staining of the basal membrane showing breaks (four samples) or with thickening (two samples).

Discussion

Hemidesmosomes connect keratinocytes to the extracellular matrix and are the major attachment sites for intermediate filaments. In steady-state buccal mucosa, our stainings showed that all hemidesmosomal proteins described for epidermal tissues also are expressed and are in similar localizations as described for skin (24). The co-localization of dystonin and collagen α-1(XVII) chain with plectin, integrin $\alpha 6\beta 4$, and CD151 at the basal membrane of the basal epithelial cell layer indicates the presence of type I hemidesmosomes. It is not possible to tell whether type II hemidesmosomes also are formed, as those complexes merely contain fewer components than hemidesmosomes type I and do not carry specific markers. Hemidesmosomes type II are, however, likely to be present as they are probably precursors for the larger hemidesmosome type I complexes (25). The anti-plectin antibody currently used is not

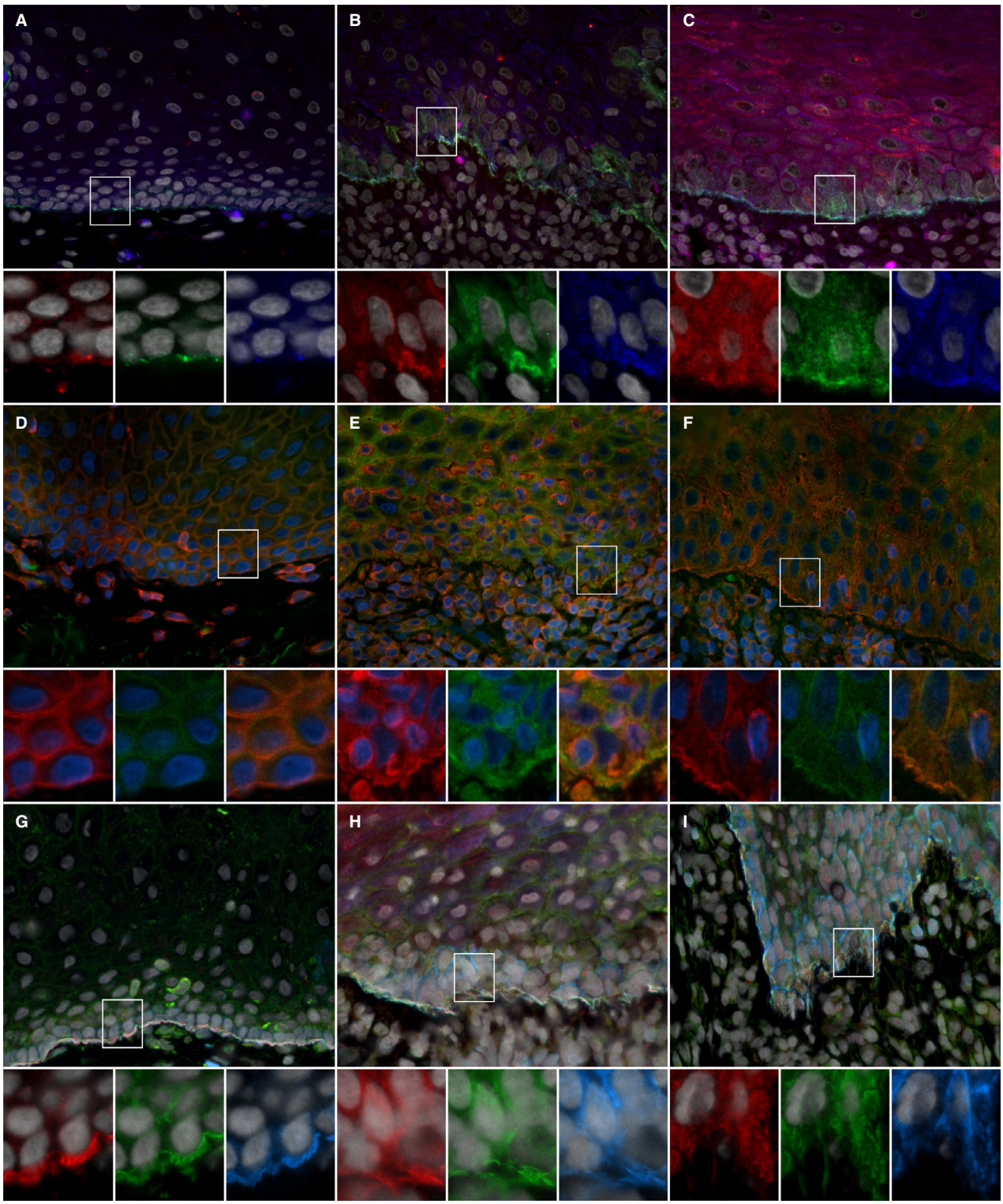

Fig. 2. Immunofluorescent staining of normal buccal mucosa (A, D, G) and buccal mucosa from reticular (B, E, H) and atrophic (C, F, I) oral lichen planus. (A-C) Triple staining for integrin-α6 (red), integrin-β4 (green), and CD151 (blue). Inserts show magnified single stainings. (D-F) Double staining for plectin (red) and plectin pS4642 (green). Inserts show magnified single stainings (left and center) and double stainings (right). (G–I) Triple staining for dystonin (red), plectin (green), and collagen α-1(XVII) chain (blue). Inserts show magnified single stainings. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) shown in blue (D-F) or digitally converted into grey (A-C and G-I). Original magnification main pictures 40x. [Colour figure can be viewed at wileyonlinelibrary.com]

isotype-specific, but as plectin 1a is the predominant isoform found in keratinocytes and the only isoform found in hemidesmosomes (26), the staining observed is therefore most likely due to reaction with this isoform. Plectin binds to the integrin β4 chain, which was also detected at the basal membrane of the basal epithelial cell layer, alongside integrin α6 and laminin-γ2. Plectins link intermediate filaments with microtubules and actin filaments and anchor intermediate filaments to hemidesmosomes and desmosomes. During terminal epithelial differentiation, plectin disconnects from integrin β4 and the intermediate filaments, and relocates to the cytoplasm in a calcium-depending fashion, before it is degraded (27). The cytoplasmic staining we observed, therefore, probably reflects the normal epithelial differentiation process. Plectin pS4642 stained only weakly along parts of the basal membrane of some normal oral mucosa, indicating that most plectin remained unphosphorylated at S4642, and therefore was bound to intermediate filaments. Incidentally, the plectin pS4642 antibody presently used also binds to the desmoplakin pS2849 epitope in desmosomes (see Material and methods). Therefore, staining was also found around the entire cell membrane of most cells throughout the epithelium. Plectin, as seen in the hemidesmosomes of normal oral mucosa, creates binding sites for both dystonin and collagen α-1(XVII) chain, which were expressed linearly along the basement membrane. Transmembrane collagen α-1(XVII) chain was also found on apicolateral membranes of basal keratinocytes. The ectodomain of collagen α-1(XVII) chain is constitutively shed from the cell surface and embedded into the extracellular matrix, a process that is upregulated upon wound healing (28,29). The presently used anti-collagen α-1(XVII) chain antibody detects an intracellular domain of hemidesmosome-bound collagen α-1(XVII) chain and not the earlier produced, cleaved and embedded ectodomain of the molecule (Figure S1). The collagen α-1(XVII) chain staining of apicolateral plasma membranes is, therefore, probably due to the presence of non-hemidesmosomal collagen α-1(XVII) chain that might have other roles, like involvement in adherence junctions (30).

In OLP, all hemidesmosomal proteins that we examined could be detected but they were in part abnormally positioned or interrupted, as compared with normal buccal mucosa. The interprotein dependency for the stabilization of the hemidesmosome complex can be a key to explaining the aberrant expression of these proteins in OLP, since the absence or incorrect processing of only a single protein can affect the assembly, location, and adherent properties of the complex, as reviewed by Litjens and co-workers (21) and summarized in Figure S1, and as detailed below.

Basal membrane staining of laminin-α3 and γ2 staining was irregular but more staining was seen within the cytoplasm of basal cells in OLP as compared to normal oral mucosa. Correct secretion and deposition of laminin-332 into the extracellular matrix and the successive cleavage of the α3 and γ2 chains are prerequisites for positioning integrin $\alpha6\beta4$ at the basement membrane (7,31). The thickening, doubling, and remnants of the α3 and γ2 chains of laminin in the connective tissue are probably due to remodeling in response to the epithelial damage seen in OLP (Fig. 1G). Frequent trafficking of immune cells that pass the basal membrane in OLP can also contribute to disruptions in laminin-332 deposition (Fig. 1G). Simultaneously, keratinocytes that repopulate the basal cell layer need to produce and secrete new laminin-332, and this may explain the increased cytoplasmic laminin-332 we observed. It is indeed known that disconnection from and injury to the basement membrane induces laminin-332 secretion (32).

Hemidesmosomes only avidly bind to processed laminin-332. Cytokines, like transforming growth factor-β and tumor necrosis factor, which are both released in OLP lesions (33,34), may influence the expression of the individual laminin-332 chains differently (35–37). The cytoplasmic accumulation of laminin-γ2 in OLP could be due to unbalanced production of laminin single chains, as the secretion of laminin-332 is dependent on the newly synthesized α3-chain that joins the preformed $\beta3\gamma2$ heterodimer (Figure S1) (38). Therefore, we also examined the presence of the α3-chain, but as we observed cytoplasmic accumulation, this did not point to such misbalance. Defects in laminin-332 are described in other diseases, including junctional epidermolysis bullosa and mucous membrane pemphigoid, and are due to mutations or to autoantibodies (39). Mutations in LAMA3 can lead to protein misfolding that affects trimerization of the α3, β3, and γ2 chains, which reduces its secretion (32). This is less likely to be the case in OLP, as this would lead to a significant lack of deposition of laminin-332 and blister formation, which we did not observe.

Integrin $\alpha6\beta4$ showed interruptions along the basal membrane and circumferential membranous staining of basal and suprabasal cells. Cytoplasmic staining of basal and suprabasal cells was visible in ATR-OLP but absent in RET-OLP. The cytoplasmic presence might be triggered by phosphorylation of integrin β4, which releases plectin from the hemidesmosomal complex, followed by endocytosis, and recycling of integrins (40). This can be induced by growth factors, tumor necrosis factor, or calcium signaling (reviewed in 7). Another explanation for a possible increase in phosphorylation of integrin β4 can be loss of K8/18 expression in basal keratinocytes in OLP epithelium (10), as keratins can prohibit phosphorylation by acting as a phosphate 'sponge' (41). The reduced expression of K8/18 and K5 in basal keratinocytes in OLP epithelium (10,42) can also explain the cytoplasmic localization of integrin-$\alpha6\beta4$ in ATR-OLP because keratins stabilize hemidesmosomes through regulation of integrin-β4 turnover and keep integrin-$\alpha6\beta4$ localized to the cell membrane (40). In RET-OLP, the more quiescent form of OLP, K8/18 expression is less affected (O. Schreurs, A. Karatsaidis, K. Schenck, unpublished data), and this might keep integrin-$\alpha6\beta4$ membrane-located as presently observed.

Unlike observations in normal oral mucosa, plectin expression in OLP frequently displayed small disruptions at the basal membrane and was clearly S4642-

phosphorylated in all OLP samples. Plectin can only localize at hemidesmosomes when allied with keratins. Plectin has a broad intermediate filament binding affinity and can bind keratins K5, K6, K8, K14, K15, and K18 (43). This association leads to a conformational change that enables plectin to bind integrin-β4 (44). Phosphorylation of plectin pS4642 decreases plectin's ability to associate with intermediate filaments (9). The increased staining of plectin pS4642 along the basal membrane in OLP may therefore point towards a disabled binding of keratins. This coincides with the failing expression of K18 that we previously observed (10), since K18 is able to bind to plectin only (11). Release of plectin from keratin also initiates the release of plectin from integrin-β4. This is followed by loss of the binding sites for collagen α-1(XVII) chain and dystonin, and this will finally resolve the hemidesmosome, resulting in lower binding strength of the epithelium to the underlying connective tissue.

Positioning of collagen α-1(XVII) chain in hemidesmosomes is dependent on an intracellular binding site that is created by the association of integrin-β4 and plectin (43). Both forms of OLP displayed increased staining for collagen α-1(XVII) chain in the cytoplasm, which coincides with the increase in phosphorylation of plectin S4642. This observation also strengthens our concept that hemidesmosomes in OLP are destabilized.

Dystonin showed both thickening and breaks of the basal lamina and this was more prominent in RET-OLP than in ATR-OLP. Both OLP forms showed an increased cytoplasmic dystonin localization in basal and parabasal cells. While the amino-terminus of dystonin interacts with a binding site that is created by the association of integrin-β4 and collagen α-1(XVII) chain, the carboxy-terminus binds K5/14 and K6/15 but not K8/18 (12,43). Therefore, we interpret this cytoplasmic localization as failing recruitment of dystonin to hemidesmosomes, probably due to changes in the association between integrin-β4 with plectin. Loss of dystonin's involvement will drive hemidesmosomes towards type II, with a weakened binding between keratin filaments and the basal cell membrane. It is therefore possible that the hemidesmosomal plaques in OLP contain a larger proportion of type II hemidesmosomes than in normal oral mucosa.

Keratins are also important for formation and stability of desmosomes in two ways: they mediate localization of hemidesmosomes through binding to plectin (44), and they are able to absorb intracellular phosphatase activity. Keratins associate with hemidesmosomes by binding to the universal intermediate filament binding sites on plectin, or the more distinguishing binding sites of dystonin-3 that bind K5/14 and K6/15 but not K8/18 (12). In basal keratinocytes of OLP, the K8/18 complex has collapsed (10) and K5 is reduced (42) and this will have consequences for the phosphorylation of both integrin-β4 and plectin, as well as for the localization of plectin. In addition, hyperphosphorylation of keratins with subsequent depolymerization of filaments into soluble tetramers most likely leads to the release of plectin from hemidesmosomes.

The present findings are in keeping with the damage seen in the basal zone area in erosive (45) and sclerotic (46) lichen planus of the vulva, including defective expression of integrin $\alpha 6\beta 4$, dystonin and collagen α-1 (XVII) chain. In the skin, the expression in lichen planopilaris of laminin-5 and type IV collagen are disrupted and not linear along the basal membrane zone, with finger-like projections of the staining protruding into the dermis (47). Lichen planus in the skin also shows uneven or absent immunostaining for laminin-5, laminin-1, and collagen type IV (48).

Collectively, in OLP, the currently observed disrupted laminin-332 deposition, disrupted integrin-α6 expression, and increased plectin phosphorylation along the basal membrane, concomitant with cytoplasmic accumulation of both dystonin and collagen α-1(XVII) chain, indicate deficient hemidesmosomal attachment to the basal membrane and a reduced connection between the basal membrane and the cells' structural stabilizers, the intermediate filaments. The expression of the latter structures is also disturbed in OLP as the basal cells in OLP do not show normal presence of K8/18 (10). In addition, flattening of the epithelial rete ridges reduces the contact surface of the epithelium with the connective tissue, further weakening the attachment of the epithelium to the connective tissue. Impairment of hemidesmosomal proteins often leads to blistering diseases, like pemphigoid or different forms for epidermolysis bullosa, and is commonly due to gene mutations or autoantibodies towards these proteins. Blister formation is not part of the pathology in OLP, apart from in the relatively rare bullous form. Still, it is conceivable that damage to the epithelial-connective tissue interface is an important pathogenic factor in OLP that leads to tissue damage accompanied by cell death. Basal keratinocytes that, due to lack of mechanical strength in hemidesmosomes, loose contact with the basal membrane will indeed enter anoikis, a programmed cell death program, because normal signals sensed through integrins are lost (49). Under pathological circumstances, significant cell death may ensue (50), in extreme conditions evidenced by liquefaction degeneration. Remnants of the α3 and γ2 chains of laminin may be left behind, and these can be the doublings and streaks we observed.

Several hemidesmosomal proteins may serve as autoantigens. Collagen α-1(XVII) chain-reactive T cells have been found in dermal lichen planus, and autoantibodies towards collagen α-1(XVII) chain antibodies have been described in oral lichen planus (51–54). Autoantibodies have been found to be reactive with integrin-α6 in oral pemphigoid, as well as with the collagen α-1(XVII) chain and dystonin in mucous membrane pemphigoid (55), bullous pemphigoid and in elderly patients with chronic non-bullous pruritic disorders (56). As hemidesmosomes are damaged in OLP, they may release native or modified antigens that can play a pathogenic role. Yet, if OLP is an autoimmune

reaction to a hemidesmosomal protein, it has a pathogenic phenotype that does not induce blister formation.

In sum, we have shown that type I hemidesmosomes occur in healthy buccal mucosa, as is the case for skin. The hemidesmosomal plaques are, however, strongly altered in OLP. The causes for this disruption might lie on the level of each of the components of the hemidesmosomes, as correct hemidesmosome assembly and stability depends on correct expression and/or post-translational modification of every component. The changes in protein expression and location seen in OLP indicate complications in the establishment of type I hemidesmosomes, leading to a poor attachment of the epithelium to the underlying connective tissue and a more vulnerable epithelial barrier. Based on the present study and other available data, it is not possible to tell whether these changes in hemidesmosomes are at the root of the pathology of OLP, perhaps via the generation of native or modified hemidesmosomal autoantigens. Alternatively, the altered distribution of hemidesmosomal proteins can be caused by apoptotic disruption of keratinocytes induced by immune cells.

Acknowledgements – The authors thank Drs. Else K. Breivik Hals (TannSpes and Lovisenberg Diaconal Hospital, Oslo, Norway) and Bjarte Grung (Drammen oral kirurgi, Drammen, Norway) for providing the biopsies.

Conflicts of interest – The authors declare no conflicts of interest.

References

1. Li C, Tang X, Zheng X, Ge S, Wen H, Lin X, Chen Z, Lu L. Global prevalence and incidence estimates of oral lichen planus: a systematic review and meta-analysis. *JAMA Dermatol* 2020; **156**: 172–181.
2. Andreasen JO. Oral lichen planus. 1. A clinical evaluation of 115 cases. *Oral Surg Oral Med Oral Pathol* 1968; **25**: 31–42.
3. Silverman S Jr, Gorsky M, Lozada-Nur F. A prospective follow-up study of 570 patients with oral lichen planus: persistence, remission, and malignant association. *Oral Surg Oral Med Oral Pathol* 1985; **60**: 30–34.
4. Thorn JJ, Holmstrup P, Rindum J, Pindborg JJ. Course of various clinical forms of oral lichen planus. A prospective follow-up study of 611 patients. *J Oral Path Med* 1988; **17**: 213–218.
5. Eisen D. The evaluation of cutaneous, genital, scalp, nail, esophageal, and ocular involvement in patients with oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1999; **88**: 431–436.
6. Hedberg N, Ng A, Hunter N. A semi-quantitative assessment of the histopathology of oral lichen planus. *J Oral Pathol* 1986; **15**: 268–272.
7. Walko G, Castañón MJ, Wiche G. Molecular architecture and function of the hemidesmosome. *Cell Tissue Res* 2015; **360**: 529–544.
8. Moll R, Divo M, Langbein L. The human keratins: biology and pathology. *Histochem Cell Biol* 2008; **129**: 705–733.
9. Bouameur JE, Schneider Y, Begré N, Hobbs RP, Lingasamy P, Fontao L, Green KJ, Favre B, Borradori L. Phosphorylation of serine 4642 in the C-terminus of plectin by MNK2 and PKA modulates its interaction with intermediate filaments. *J Cell Sci* 2013; **126**: 4195–4207.
10. Schreurs O, Karatsaidis A, Balta MG, Grung B, Hals EKB, Schenck K. Expression of keratins 8, 18, and 19 in epithelia of atrophic oral lichen planus. *Eur J Oral Sci* 2020; **128**: 7–17.
11. Chavez JD, Schweppe DK, Eng JK, Zheng C, Taipale A, Zhang Y, Takara K, Bruce JE. Quantitative interactome analysis reveals a chemoresistant edgotype. *Nature Commun* 2015; **6**: 7928.
12. Fontao L, Favre B, Riou S, Geerts D, Jaunin F, Saurat JH, Green KJ, Sonnenberg A, Borradori L. Interaction of the Bullous Pemphigoid Antigen 1 (BP230) and Desmoplakin with Intermediate Filaments Is Mediated by Distinct Sequences within Their COOH Terminus. *Mol Biol Cell* 2003; **14**: 1978–1992.
13. Johnson FR, Fry L. Ultrastructural observations on lichen planus. *Arch Dermatol* 1967; **95**: 596–607.
14. Jungell P. Immunoelectron microscopic study of the basement membrane in oral lichen planus. *J Cutan Pathol* 1990; **17**: 72–76.
15. Paul M, Shetty DC. Analysis of the changes in the basal cell region of oral lichen planus: an ultrastructural study. *J Oral Maxillofac Pathol* 2013; **17**: 10–16.
16. Becker J, Schuppan D. Altered expression of extracellular matrix proteins and integrins in oral lichen planus (OLP). *J Oral Path Med* 1995; **24**: 159–164.
17. Köse O, Lalli A, Kutulola AO, Odell EW, Waseem A. Changes in the expression of stem cell markers in oral lichen planus and hyperkeratotic lesions. *J Oral Sci* 2007; **49**: 133–139.
18. Ramirez-Amador V, Dekker NP, Lozada-Nur F, Mirowski GW, MacPhail LA, Regezi JA. Altered interface adhesion molecules in oral lichen planus. *Oral Dis* 1996; **2**: 188–192.
19. Haapalainen T, Oksala O, Kallioinen M, Oikarinen A, Larjava H, Salo T. Destruction of the epithelial anchoring system in lichen planus. *J Invest Dermatol* 1995; **105**: 100–103.
20. Neppelberg E, Loro LL, Øijordsbakken G, Johannessen AC. Altered CD40 and E-cadherin expression – putative role in oral lichen planus. *J Oral Path Med* 2007; **36**: 153–160.
21. Litjens SHM, de Pereda JM, Sonnenberg A. Current insights into the formation and breakdown of hemidesmosomes. *Trends Cell Biol* 2006; **16**: 376–383.
22. Al-Hashimi I, Schifter M, Lockhart PB, Wray D, Brennan M, Migliorati CA, Axéll T, Bruce AJ, Carpenter W, Eisenberg E, Epstein JB, Holmstrup P, Jontell M, Lozada-Nur F, Nair R, Silverman B, Thongprasom K, Thornhill M, Warnakulasuriya S, van der Waal I. Oral lichen planus and oral lichenoid lesions: diagnostic and therapeutic considerations. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007; **103**(Suppl): S25.e1–S25.e12.
23. Künzli K, Favre B, Chofflon M, Borradori L. One gene but different proteins and diseases: the complexity of dystonin and bullous pemphigoid antigen 1. *Exp Dermatol* 2016; **25**: 10–16.
24. Green KJ, Jones JC. Desmosomes and hemidesmosomes: structure and function of molecular components. *FASEB J* 1996; **10**: 871–881.
25. Sterk LM, Geuijen CA, Oomen LC, Calafat J, Janssen H, Sonnenberg A. The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin α6β4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol* 2000; **149**: 969–982.
26. Andrä K, Kornacker I, Jörgl A, Zörer M, Spazierer D, Fuchs P, Fischer I, Wiche G. Plectin-isoform-specific rescue of hemidesmosomal defects in plectin (–/–) keratinocytes. *J Invest Dermatol* 2003; **120**: 189–197.
27. Kostan J, Gregor M, Walko G, Wiche G. Plectin isoform-dependent regulation of keratin-integrin α6β4 anchorage via Ca^{2+}/calmodulin. *J Biol Chem* 2009; **284**: 18525–18536.
28. Jacków J, Löffek S, Nyström A, Bruckner-Tuderman L, Franzke CW. Collagen XVII shedding suppresses re-epithelialization by directing keratinocyte migration and dampening mTOR signaling. *J Invest Dermatol* 2016; **136**: 1031–1041.
29. Nishimura M, Nishie W, Shirafuji Y, Shinkuma S, Natsuga K, Nakamura H, Sawamura D, Iwatsuki K, Shimizu H. Extracellular cleavage of collagen XVII is essential for correct cutaneous basement membrane formation. *Hum Mol Genet* 2016; **25**: 328–339.

30. Natsuga K, Watanabe M, Nishie W, Shimizu H. Life before and beyond blistering: the role of collagen XVII in epidermal physiology. *Exp Dermatol* 2019; **28**: 1135–1141.
31. Rousselle P, Beck K. Laminin 332 processing impacts cellular behavior. *Cell Adhes Migr* 2013; **7**: 122–134.
32. Schneider H, Mühle C, Pacho F. Biological function of laminin-5 and pathogenic impact of its deficiency. *Eur J Cell Biol* 2007; **86**: 701–717.
33. Khan A, Farah CS, Savage NW, Walsh LJ, Harbrow DJ, Sugerman PB. Th1 cytokines in oral lichen planus. *J Oral Path Med* 2003; **32**: 77–83.
34. Weber B, Schlapbach C, Stuck M, Simon HU, Borradori L, Beltraminelli H, Simon D. Distinct interferon-gamma and interleukin-9 expression in cutaneous and oral lichen planus. *J Eur Acad Dermatol* 2017; **31**: 880–886.
35. Amano S, Akutsu N, Ogura Y, Nishiyama T. Increase of laminin 5 synthesis in human keratinocytes by acute wound fluid, inflammatory cytokines and growth factors, and lysophospholipids. *Br J Dermatol* 2004; **151**: 961–970.
36. Korang KC, Angela M, Uitto J, Mauviel A. Differential cytokine modulation of the genes LAMA3, LAMB3, and LAMC2, encoding the constitutive polypeptides, α3, β3, and γ2, of human laminin 5 in epidermal keratinocytes. *FEBS Lett* 1995; **368**: 556–558.
37. Senyürek I, Kempf WE, Klein G, Maurer A, Kalbacher H, Schäfer L, Wanke I, Christ C, Stevanovic S, Schaller M, Rousselle P, Garbe C, Biedermann T, Schittek B. Processing of laminin α chains generates peptides involved in wound healing and host defense. *J Innate Immun* 2014; **6**: 467–484.
38. Matsui C, Wang CK, Nelson CF, Bauer EA, Hoeffler WK. The assembly of laminin-5 subunits. *J Biol Chem* 1995; **270**: 23496–23503.
39. Turcan I, Jonkman MF. Blistering disease: insight from the hemidesmosome and other components of the dermal-epidermal junction. *Cell Tissue Res* 2015; **360**: 545–569.
40. Seltmann K, Cheng F, Wiche G, Eriksson JE, Magin TM. Keratins stabilize hemidesmosomes through regulation of β4-integrin turnover. *J Invest Dermatol* 2015; **135**: 1609–1620.
41. Snider NT, Omary MB. Post-translational modifications of intermediate filament proteins: mechanisms and functions. *Nat Rev Mol Cell Biol* 2014; **15**: 163–177.
42. Boisnic S, Ouhayoun JP, Branchet MC, Frances C, Béranger JY, Le Charpentier Y, Szpirglas H. Alteration of cytokeratin expression in oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1995; **79**: 207–215.
43. Koster J, Geerts D, Favre B, Borradori L, Sonnenberg A. Analysis of the interactions between BP180, BP230, plectin and the integrin α6β4 important for hemidesmosome assembly. *J Cell Sci* 2003; **116**: 387–399.
44. Seltmann K, Roth W, Kröger C, Loschke F, Lederer M, Hüttelmaier S, Magin TM. Keratins mediate localization of hemidesmosomes and repress cell motility. *J Invest Dermatol* 2013; **133**: 181–190.
45. Cooper SM, Prenter A, Allen J, Dean D, Wojnarowska F. The basement membrane zone and dermal extracellular matrix in erosive lichen planus of the vulva: an immunohistochemical study demonstrating altered expression of hemidesmosome components and anchoring fibrils. *Clin Exp Dermatol* 2005; **30**: 277–281.
46. Marren P, Dean D, Charnock M, Wojnarowska F. The basement membrane zone in lichen sclerosus: an immunohistochemical study. *Br J Dermatol* 1997; **136**: 508–514.
47. Al-Refu K, Goodfield M. Basement membrane changes in lichen planopilaris. *J Eur Acad Dermatol* 2009; **23**: 1289–1293.
48. Giannelli G, Brassard J, Foti C, Stetler-Stevenson WG, Falk-Marzillier J, Zambonin-Zallone A, Schiraldi O, Quaranta V. Altered expression of basement membrane proteins and their integrin receptors in lichen planus: possible pathogenetic role of gelatinases A and B. *Lab Invest* 1996; **74**: 1091–1104.
49. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol* 2001; **13**: 555–562.
50. Chiarugi P, Giannoni E. Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol* 2008; **76**: 1352–1364.
51. Baldo M, Bailey A, Bhogal B, Groves RW, Ogg G, Wojnarowska F. T cells reactive with the NC16A domain of BP180 are present in vulval lichen sclerosus and lichen planus. *J Eur Acad Dermatol* 2010; **24**: 186–190.
52. Buijsrogge JJA, Hagel C, Duske U, Kromminga A, Vissink A, Kloosterhuis AJ, van der Wal JE, Jonkman MF, Pas HH. IgG antibodies to BP180 in a subset of oral lichen planus patients. *J Dermatol Sci* 2007; **47**: 256–258.
53. Herrero-González JE, Parera Amer E, Segura S, Mas Bosch V, Pujol RM, Martínez Escala ME. Epithelial antigenic specificities of circulating autoantibodies in mucosal lichen planus. *Int J Dermatol* 2016; **55**: 634–639.
54. Shipman AR, Cooper S, Wojnarowska F. Autoreactivity to bullous pemphigoid 180: is this the link between subepidermal blistering diseases and oral lichen planus? *Clin Exp Dermatol* 2011; **36**: 267–269.
55. Fukuda A, Himejima A, Tsuruta D, Koga H, Ohyama B, Morita S, Hashimoto T. Four cases of mucous membrane pemphigoid with clinical features of oral lichen planus. *Int J Dermatol* 2016; **55**: 657–665.
56. Schmidt T, Sitaru C, Amber K, Hertl M. BP180- and BP230-specific IgG autoantibodies in pruritic disorders of the elderly: a preclinical stage of bullous pemphigoid? *Br J Dermatol* 2014; **171**: 212–219.

Supporting Information

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

Figure S1. Hypothetical model of the assembly and disassembly of hemidesmosomes in basal keratinocytes.

Table S1. Details of the primary antibodies.