Pemphigus
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Subclinical pathology in pemphigus foliaceus mucosa

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Submitted
Abstract
Pemphigus foliaceus (PF) is an autoimmune blistering disease of the skin, characterized by anti-
desmoglein 1 (Dsg1) IgG autoantibodies that induce acantholysis. Although these autoantibodies
also bind to the mucosal epithelium, this tissue is clinically unaffected. In this study we examined
if there are subclinical changes in oral PF mucosa. We performed immunofluorescence analysis
(IF) for bound IgG and desmosomal components, and electron microscopy of parallel biopsies of
the oral mucosa from PF patients (n=4) and normal human controls (n=4). IF showed clustering
of IgG and Dsg1 along the epithelial cell surface of mainly the lower spinous cell layers in PF
mucosa, while Dsg3 and E-cadherin were unaffected. A significant decrease in desmosome size
and a trend of increased intercellular widening was seen in the lower spinous layers of PF mucosa.
The number of desmosomes was not reduced compared to controls. Our findings show that PF
IgG interferes with Dsg1 distribution in oral mucosa, resulting in reduced desmosome size and
intercellular widening, that does does not progress to acantholysis or clinical pathology.
Introduction

Pemphigus comprises a group of chronic autoimmune blistering diseases clinically characterized by flaccid blisters of the skin and/or mucosal epithelium. The disease is evoked by autoantibodies against the epithelial cell surface (ECS), which induce loss of intercellular adhesion, i.e. acantholysis. The pathogenic autoantibodies are directed against desmogleins (Dsg); transmembrane glycoproteins that are located in desmosomes, but also exist in a non-desmosomal form in the cell membrane. The isoforms Dsg1 and Dsg3 are the main autoantigens in pemphigus. In the skin, Dsg1 is expressed throughout the epidermis, with strongest expression in the superficial cell layers, whereas Dsg3 is expressed in the basal and first few suprabasal cell layers. In mucosa however, Dsg3 is expressed throughout the epithelium, whereas Dsg1 is expressed at a much lower level and is absent in the basal cell layer. The two ends of the pemphigus spectrum are pemphigus foliaceus (PF), in which patients have anti-Dsg1 antibodies and blistering of only the skin, and mucosal dominant pemphigus vulgaris (mPV), in which patients have anti-Dsg3 antibodies and involvement of only the mucous membranes. The involvement of skin or mucosa is explained by the desmoglein compensation theory. It postulates that antibody induced functional loss of one Dsg isoform can be compensated by another Dsg isoform. Therefore, Dsg1 antibodies in PF lead to subcorneal skin blisters, since Dsg3 is not expressed in the granular and corneal layers. Mucous membranes are not clinically affected, because transepithelial Dsg3 compensates for functional loss of Dsg1. In contrast, Dsg3 antibodies in mPV induce suprabasal mucosal blisters because there is no Dsg1 in the basal layer to compensate for loss of Dsg3. The skin is, however, unaffected as the Dsg1 expressed is sufficiently high in all layers. In mucocutaneous pemphigus vulgaris (mcPV), blistering of both skin and mucous membranes occurs, as compensation mechanisms are compromised by the dual presence Dsg1 and Dsg3 antibodies.

There are several theories regarding the cellular mechanism by which autoantibodies induce acantholysis that results in blistering. The steric hindrance theory proposes that anti-Dsg autoantibodies, by binding to trans-adhesive epitopes, directly inhibit desmoglein trans-interaction. Secondly, autoantibody binding may alter cell-signaling pathways that influence cellular adhesion or the homeostasis of desmosomes. Desmosomes are dynamic structures in which desmogleins are continuously being built in and discarded, and pemphigus autoantibodies may induce desmosomal disassembly or may interfere with desmosomal assembly. In support of the latter, we showed recently that in PF skin IgG co-localizes with Dsg1 and plakoglobin, but not with other desmosomal components, aggregating into clusters along the cell surface of keratinocytes. This clustering starts at the basal cell layers and progresses upwards during later stages of disease. In addition electron microscopy showed intercellular widening, and reduction in size and in number of desmosomes in skin with active PF. Together, these findings suggest that PF IgG sequesters Dsg1 into clusters, thereby eliminating Dsg1 from desmosomes or inhibiting the incorporation of Dsg1 into desmosomes. Acantholysis occurs if desmosome size and number drops below a critical minimum, and this minimum is not reached in PF skin without disease activity. In view of this, we wondered if the same holds true for...
clinically unaffected PF mucosa. Therefore, in the current study we investigated the distribution of desmosomal components, desmosome size, and epithelial cell approximation in PF mucosa.

Materials and Methods

Patient samples
Mucosal biopsies of four patients with PF (PF1-4), and four normal human controls were included. The diagnosis PF was based on clinical and histological findings and enzyme-linked immunosorbent assay (ELISA) (MBL, Nagoya, Japan) demonstrating only anti-Dsg1 IgG in PF. The anti-Dsg1 IgG Elisa titers were as follows: PF1, 150; PF2 >150; PF3 >150; PF4 >150. As a measure for disease activity, blisters were induced by rubbing on healthy appearing skin, which is called a Nikolsky sign type I. The Nikolsky’s sign type I at the biopsy visit was retrieved from the patient files. From each individual, two biopsies in the same region of the intact buccal mucosa were taken: one 3 mm punch biopsy for immunofluorescence microscopy, and one 1.5 mm punch biopsy for electron microscopy. All human samples were collected after informed consent.

Immunofluorescence microscopy
Specimens were snap frozen, and immunostainings were performed on 4 µm thin cryosections, as previously described. (Vodegel et al, 2004) Primary antibodies used were a monoclonal anti-desmoglein 1 rabbit IgG, diluted 1:500 (EPR6766, Abcam), a monoclonal anti-desmoglein 3 mouse IgG, diluted 1:40 (G194, Progen, Immuno-diagnostika), and a monoclonal anti-E-cadherin mouse IgG, diluted 1:100 (NCH-38, Dako). Secondary antibodies used were FITC-conjugated goat anti-human IgG (Protos Immunoresearch), FITC conjugated donkey anti-rabbit antibody (Jackson Immunoresearch), and Alexa568 conjugated goat anti-mouse antibody (Molecular Probes, Invitrogen) Sections were viewed under a Leica DMRA fluorescence microscope, and images were recorded with a Leica DFC350 FX digital camera (Leica, Solms, Germany).

Electron microscopy
Specimens were fixated using 2% glutaraldehyde in 0,1mol/L phosphate buffer, and post-fixated using 1% osmium tetroxide 1,5% potassium ferrocyanide in 0,1mol/L sodium cacodylate buffer. Subsequently, biopsies were dehydrated in ethanol and embedded in epon. Ultrathin sections were stained with uranyl acetate and Reynolds lead citrate. Sections were viewed under a Philips CM100 transmission electron microscope. Electron microscopy images of the sections were taken of representative regions of the basal cell layer, lower, 1st-4th, spinous cell layers and higher, 5th – 11th, spinous cell layers. Per stratum, the area analyzed ranged between 300 and 600 µm². In these areas, all desmosomes were counted and their length was measured using Adobe Photoshop software. Intercellular widening was quantified by measuring the area of extracellular space as a percentage of the total area assessed.
Statistical analysis
The Mann-Whitney U test was used to compare the number of desmosomes per µm² and the percentage of intercellular space in the PF-group versus the control group. A mixed model analysis was used to compare the desmosome length between the control group and the PF group. For all statistical analysis, SPSS software was used.

Results
IgG depositions correlate to Dsg1 clustering and intercellular widening in PF mucosa
In control mucosa, no IgG depositions were present along the epithelial cell surface (ECS). Dsg1 showed a smooth expression along the ECS in all layers of the mucosa except the basal layer where no Dsg1 was detected (Fig. 1, first column). In PF mucosa, IgG depositions were clustered along the ECS predominantly in the lower spinous cell layers, reaching the higher spinous layers in patient PF4 with active disease (Fig. 1, 2nd-5th column). Parallel to IgG, Dsg1 was also clustered along the ECS in PF mucosa, again mostly in the lower spinous layers. The degree of Dsg1 clustering varied among patients, being least in Nikolsky negative (N-) PF1 and most in Nikolsky positive (N+) PF4. In contrast, Dsg3 and E-cadherin distribution was not altered and remained in a smooth ECS pattern in PF mucosa (Fig. 2.)

Morphometry of the amount of intercellular space in the epithelium (Fig. 3A) showed that the three patients with most pronounced Dsg1 clustering (PF2, 3 and 4), had an increase of the interdesmosomal intercellular space, in the lower spinous cell layers, compared to the control group. PF1 had minor Dsg1 clustering and showed no clear intercellular widening. The mean amount of intercellular space in the lower spinous layers was 17.9% (range 5.3%-24%), in the PF group, compared to 9% in the control group (range 3.9%-13.4%). The N+ PF mucosa group had a mean intercellular space of 21% (range 19.7%-22.7%). This trend of intercellular widening in PF mucosa as compared to the control mucosa, was statistically not significant (p=0.133) due to small sample size (n=8).

Smaller desmosomes in the lower spinous layers of PF mucosa
In the lower spinous cell layers of the PF mucosa, desmosomes were significantly smaller (average length 0.18 µm; p=0.000) than in the normal control (average length 0.26 µm) (Fig. 3B). In the basal and upper spinous layers, with the exception of PF4 in the upper layers, desmosomal size was not altered to control (0.15 µm, and 0.16 µm respectively). There was no statistical difference in desmosome number in the PF group as compared to the control group (Fig. 3C).
IgG depositions, as visualized by immunofluorescence microscopy, are absent in control mucosa (first row, first column), and present in predominantly the lower spinous cell layers of PF mucosa (first row, second to last column). In PF4 IgG depositions extend towards the higher cell layers (first row, last column). Immunostainings for Dsg1 (second row), reveal a smooth expression pattern along the epithelial cell surface in control mucosa (second row, first column), and a varying degree of clustering in PF mucosa, with least clusters in PF1 (second row, second column) and most in PF4 (second row, last column). Electron microscopy (third row) shows no increase in intercellular space in PF1 (third row, second column) compared to the control (third row, first column). In PF2, PF3 and PF4, intercellular widening is seen in the lower spinous cell layers. N+: positive Nikolsky sign; N-: negative Nikolsky sign; the dashed lines depict the BMZ. White scale bars represent 20 µm, black scale bars represent 2 µm.
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Figure 2. Distribution of E-cadherin, Dsg1 and Dsg3 in PF mucosa
E-cadherin (first column) is smoothly distributed along the epithelial cell surface in PF mucosa. Double staining for Dsg1 and Dsg3 reveal a similar smooth distribution of Dsg3 (third column and overlay), while Dsg1 has a clustered distribution (second column and overlay). Normal human mucosa had a smooth Dsg1 (figure 1, second row, first column), E-cadherin and Dsg3 distribution (not shown). The dashed lines depict the BMZ. Scale bars represent 20 µm. Shown are the immunostaining results for PF4, which, with the exception of Dsg1 distribution that varies between patients, as show in figure 1, were representative for all 4 PF patients.

Figure 3. Quantification of intercellular widening, and desmosome length and number in PF mucosa
Morphometrical analysis was performed for the four controls (white bars, each bar represent 1 control) and the four PF patients (grey bars, each bar represents 1 patient. PF1 to 4 are arranged from left to right). In the lower spinous cell layers, an increase in extracellular space (i.e. intercellular widening) was seen in PF2, PF3 and PF4, as compared to the control group (A). The mean desmosome length was significantly smaller in the lower spinous cell layers of PF mucosa (B). No significant difference in desmosome number was seen between the PF and control group (C).
Discussion
Our study shows that subclinical pathology occurs in the oral mucous membranes of PF patients, manifested by clustered Dsg1 distribution, and a decrease in desmosome size and a trend of intercellular widening mainly in the lower spinous cell layers where IgG depositions were most abundant. Intercellular widening by EM was also found in mucosa of endemic PF patients.22 Our study pursues this further by correlating these changes to IgG depositions and desmosomal protein distribution. We show that the extent of widening varies between patients, and may be dependent on disease activity as manifested in the skin, since intercellular widening in the mucosa was more prominent in PF patients with N+ skin, the group that also had more pronounced mucosal IgG depositions and Dsg1 clustering. Larger studies would be needed to confirm this.

Cellular adhesion not only depends on the presence of mature desmosomes but also on adherens junctions. Previous studies have suggested that in PF, IgG may be directed against E-cadherin, an important adherens junction protein.23,24 However, our results show that the intercellular widening seen PF mucosa is not likely due to these antibodies, as we found an unaltered distribution of E-cadherin. Instead, our findings suggest that after IgG binds to Dsg1 it induces clustering of an interdesmosomal Dsg1 fraction, resulting in widening of the interdesmosomal intercellular space. Future immunoelectron microscopy studies investigating the ultrastructure of Dsg1 distribution and IgG binding in PF mucosa would be needed to prove this hypothesis.

We also found that desmosomes are smaller in predominantly the lower spinous cell layers of PF mucosa, similar as we found previously for non-lesional N+ PF skin.19,21 In contrast to PF skin, the number of desmosomes appeared not to be reduced in PF mucosa.19,21 We think that reduction of desmosome size precedes reduction of desmosome number in PF, and that desmosome alteration in PF mucosa lags behind that in skin. One explanation might be that mucosa may have more compensatory expression of Dsg225 or of Dsg3.26 Desmosome size may be reduced due to the IgG induced depletion of Dsg1 from desmosomes. Evidence for depletion of Dsg3 from desmosomes in PV, has been provided by various studies.27-29 Further immunoelectron microscopy studies investigating the relation between desmosome size and desmosomal Dsg1 content in PF mucosa, would be needed to prove desmosomal depletion in PF.

The ultrastructural changes were most abundant in the lower spinous cell layers because, as IgG diffuses into the mucosal epithelium from the underlying lamina propria, it first binds to these layers. The findings are in line with studies of skin in PF, where desmosome alterations were first seen in the lower cell layers of N+ skin.19,21 The abundant and unaltered presence of Dsg3 in the mucosa of PF patients, safeguards the maintenance of a sufficient number of large enough desmosomes, thereby preventing acantholysis.
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