Ultrastructure of acantholysis in pemphigus foliaceus re-examined from the current perspective.

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Abstract

Background Pemphigus foliaceus (PF) is a chronic cutaneous autoimmune blistering disease that is characterized by superficial blistering of the skin, and according to the current perspective is caused by autoantibodies directed against desmoglein (Dsg)1.

Objectives To examine early acantholysis in the skin of patients with PF at an ultrastructural level.

Patients/Methods Two Nikolsky-negative (N-), five Nikolsky-positive (N+) and two lesional skin biopsies from immunoserologically defined patients with PF were studied by light and electron microscopy.

Results We found no abnormalities in N- PF skin, whereas all the N+ skin biopsies displayed intercellular widening between desmosomes, a decreased number of desmosomes and hypoplastic desmosomes in the lower epidermal layers. Acantholysis was present in two of five N+ biopsies, but only in the upper epidermal layers. The lesional skin biopsies displayed acantholysis in the higher epidermal layers. Hypoplastic desmosomes were partially (pseudo–half–desmosomes) or completely torn off from the opposing cell.

Conclusions We propose the following mechanism for acantholysis in PF: initially PF IgG causes a depletion of non-junctional Dsg1, leading to intercellular widening between desmosomes starting in the lower layers and spreading upwards. Depletion of non-junctional Dsg1 impairs the assembly of desmosomes, resulting in hypoplastic desmosomes and a decreased number of desmosomes. In addition, antibodies might promote disassembly of desmosomes. In the upper layers of the epidermis, where Dsg3 is not expressed and cannot compensate for Dsg1 loss, ongoing depletion of Dsg1 will finally result in a total disappearance of desmosomes and subsequent acantholysis.

Introduction

Pemphigus is a chronic mucocutaneous autoimmune blistering disease, characterized by blistering of the skin and/or the mucous membranes due to autoantibodies directed, among others, against the desmosomal cadherins, desmoglein (Dsg)1 and/or Dsg3.1 The two main forms of pemphigus are pemphigus vulgaris (PV) and pemphigus foliaceus (PF). In mucosal-dominant PV, patients have suprabasal blistering of the mucous membranes and autoantibodies against Dsg3 only. In patients with mucocutaneous PV there is suprabasal blistering of both skin and mucous membranes, in combination with autoantibodies against both Dsg1 and Dsg3. PF presents as superficial blistering or erosive exfoliation of the skin and the autoantibodies are directed against Dsg1.
The level and localization of blisters in pemphigus is explained by the Dsg compensation hypothesis, which states that one Dsg isoform can compensate for loss of the other Dsg isoform. In human skin, Dsg1 is present throughout the whole epidermis and its expression increases upwards from the basal layer. Dsg3 is absent in the subcorneal layers and its expression decreases from the lower to the higher layers. In mucosa, Dsg3 is expressed in all layers, while Dsg1 is expressed at low levels in higher layers. This implies that, in PF, blistering will occur only in the subcorneal layers of the skin where Dsg3 is absent. Vice versa, in mucosal-dominant PV, blistering will occur suprabasally in the mucosa where Dsg1 is absent. In mucocutaneous PV, both skin and mucosa will blister suprabasally due to loss of both Dsg1 and Dsg3.

By applying lateral pressure with a finger on normal–appearing pemphigus skin it is sometimes possible to remove the epidermal sheet. This is called a positive direct Nikolsky sign (N+), while skin from which no epidermal sheet can be removed is called Nikolsky negative (N−). We consider a positive direct Nikolsky sign on normal–appearing skin to be the earliest stage of clinical pathology in pemphigus.

There are several theories on the pathogenesis of acantholysis in pemphigus. The steric hindrance theory suggests that acantholysis is the result of direct inhibition of the adhesive function of desmogleins by autoantibodies, which would lead to lengthwise splitting of desmosomes. Alternative theories state that the autoantibodies interfere with the assembly and/or disassembly of desmosomes or that acantholysis is the result of outside–in signalling.

In the past, a small number of electron microscopic studies on the skin of patients with PF were performed. Since then, the knowledge on pemphigus antibodies and their targets has greatly increased, as well as the concepts on the mechanism of acantholysis. Therefore, in this study we investigated the ultrastructural changes in the skin of patients with PF from the current perspective. Moreover, we investigated the early stages of the acantholytic process by studying normal–appearing N- and N+ PF skin.

Material and methods

Patients

Skin biopsies of six patients with PF, three men and three women, were included (Table 1). The age of the patients varied between 19 and 92 years. The diagnosis PF was proven by histology, direct and indirect immunofluorescence of skin and serum, and by enzyme–linked immunosorbent assay (ELISA) that demonstrated exclusively anti–Dsg1 antibodies. Two patients were treated with systemic glucocorticosteroids at the time of biopsy. All patients had skin lesions when biopsies were taken.
### Table 1 – Patient characteristics. ELISA, enzyme–linked immunosorbent assay; Dsg, desmoglein; DIF, direct immunofluorescence; EM, electron microscopy; N+, Nikolsky positive; N–, Nikolsky negative; ICS, intercellular substance.

#### Biopsies
Nine biopsies were included, two from N– skin, five from N+ skin, and two from lesional skin (Table 1). Rubbing for the Nikolsky sign was performed juxtaposed to the biopsy site at a distance of 2 cm, so that the actual biopsied normal appearing skin remained undisturbed by the rubbing finger. We will refer to this skin as unrubbed N+ skin. Two biopsies from normal human skin served as reference controls.

#### Light and electron microscopy
The biopsy specimens were fixed in 2% glutaraldehyde in 0.1 mol L⁻¹ phosphate buffer, post-fixed with 1% osmium tetroxide in 0.1 mol L⁻¹ sodium cacodylate buffer with 1.5% potassium
ferrocyanide and dehydrated with ethanol. Subsequently, the specimens were embedded in Epon (Hexion Specialty Chemicals Inc., Danbury, CT, U.S.A.), and semithin sections were cut for light microscopy and ultrathin sections for electron microscopy. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands). In selected biopsies the number of desmosomes per µm² and the length of a number of desmosomes was measured.

**Results**

**Nikolsky–negative pemphigus foliaceus skin**

We found no abnormalities by light microscopy of the semithin sections of the N- biopsies (Figure 1a), although there was intercellular deposition of IgG as shown by immunofluorescence staining of IgG in biopsies taken from the same location. The ultrastructure also appeared normal (Figure 2b). Intercellular widening was not present, the desmosomes were normal in size and number, and no retraction of the cytoskeleton was observed.

**Unrubbed Nikolsky–positive pemphigus foliaceus skin**

Light microscopy of three of the five biopsies taken from N+ skin showed no acantholysis (Figure 1b), while two showed acantholysis at the level of the spinous layer (Figure 1c). Electron microscopy showed intercellular widening without acantholysis in the basal and lower spinous layers (Figure 2c). We found prominent interdigitation and many filopodia in the widened intercellular spaces between keratinocytes. In the basal and spinous layers of N+ PF skin desmosomes were decreased in size. Basal layer desmosomes in N+ skin were $0.14 \pm 0.05 \mu m$ vs. $0.25 \pm 0.09 \mu m$ in N- skin and $0.26 \pm 0.11 \mu m$ in control skin ($p = 0.004$). At the spinous layer in N+ skin the desmosomes measured $0.20 \pm 0.09 \mu m$ vs. $0.28 \pm 0.09 \mu m$ in N- skin and $0.31 \pm 0.14 \mu m$ in control skin ($p = 0.013$). In N+ PF skin the number of desmosomes was also decreased at the spinous layer. While control skin contained 0.24 desmosomes per µm² and N- skin 0.23 desmosomes per µm², N+ skin contained only 0.10 desmosomes per µm².
Figure 1 – Light microscopy of stages in pemphigus foliaceus (PF) skin. Nikolsky-negative (N-) PF skin (a) shows no abnormalities and immunofluorescence staining (inset) shows intercellular deposition of IgG. Nikolsky-positive (N+) PF skin reveals subtle intercellular widening in the lower layer (b) and immunofluorescence staining (inset) shows intercellular deposition of IgG. (c) Some biopsies of N+ PF skin showed acantholysis at the mid stratum spinosum (asterisks). (d) Lesional PF skin with transepidermal intercellular widening and complete subcorneal acantholysis (asterisk). Scale bars = 200 nm.
Figure 2 – Electron microscopy of stages in pemphigus foliaceus (PF) skin. No intercellular widening was observed in human control skin (a) and Nikolsky-negative (N-) PF skin (b). In unrubbed Nikolsky-positive (N+) PF skin without acantholysis (c) intercellular widening between desmosomes was present at the level of the basal and suprabasal layers. In lesional acantholytic PF skin (d) increased intercellular widening with prominent filopodia could be seen in all the layers below the blister. Scale bars = 5 μm. (a–d insets) Details (arrow) of control skin (a) and N) PF skin (b) show normal-sized desmosomes. Detail (arrow) of N+ PF skin (c) shows small desmosomes and detail of lesional PF skin (d) shows tiny desmosomes. Scale bars = 200 nm. Dotted line, basal membrane zone.
In the two N+ biopsies with acantholysis a reduction in the number of desmosomes was evident at the sites facing the acantholytic area. Here, desmosomes were severely hypoplastic. We observed plaque–like structures at the cell surface of keratinocytes (Figure 3a–c). Some of these structures contained one cell membrane thus resembling tiny half–desmosomes or early immature desmosomal nucleations (Figure 3a). Rarely, we found a normal–sized half–desmosome (Figure 3b). We also found plaque–like structures with a double cell membrane (Figure 3c), pseudo–half–desmosomes, which are torn–off desmosomes resulting from an intracellular split between the plasma membrane and plaque in the opposite cell. Retraction of the keratin cytoskeleton was not observed.

Lesional skin
Both lesional skin biopsies contained subcorneal blisters (Figure 1d). Prominent intercellular widening between desmosomes was seen in all layers underneath the blister (Figure 2d). Filopodia were prominent in the widened intercellular spaces. Keratinocytes that were almost detached from their neighbours (pre–acanthocytes) had plasma membranes that were stretched to their limits (Figure 3d). The desmosomes here were still attached but started to tear off. Torn–off desmosomes were visible with the point of breakage at the cytoplasmic site of the desmosomal plaque (Figure 3e).

In both biopsies, desmosomes were decreased in size and number in the lower layers as well as in the upper epidermis (Figure 2d). Acantholytic keratinocytes that had almost lost attachment with their neighbouring cells showed some retraction of the cytoskeleton (Figure 3d). We found no half desmosomes in lesional PF skin.
Figure 3 – Sequelae of desmosomes in pemphigus foliaceus (PF) skin. Nikolsky–positive (N+) unrubbed PF skin with (a) a hypoplastic halfdesmosome (arrowhead), (b) a plaque density representing a half–desmosome (arrowhead) and a ruptured cell membrane (double arrow). (c) This ruptured cell membrane is situated opposed to pseudo–half–desmosome plaques (arrows). These pseudo–half–desmosomes are attached to tonofilaments topped with a second cell membrane torn off from the opposing cell. Asterisk, blister cavity; scale bars = 200 nm. (d) In lesional PF skin, a decreased number of desmosomes (arrows) in a preacanthocyte is accompanied by peripheral retraction of tonofilaments (open circle). The cytoskeleton of the opposing cell (open rectangle) is not (yet) retracted. Intercellular widening between desmosomes is present between intact desmosomes, which are stretched to their limits (arrowheads). Scale bar = 2 μm. (e, f) Details from (d): (e) nonsplit desmosome torn off from the cell body; (f) some desmosomes look like torn–off desmosomes, but may be tangential sections of filopodia (double arrow); scale bars = 200 nm.
Discussion

The first ultrastructural sign of pathology that could be observed in PF was intercellular widening between desmosomes in the basal and lower spinous layers in normal–appearing unrubbed N+ skin. In lesional skin, this intercellular widening became more profound and could also be seen in the upper epidermal layers.

Widening between basal cells was described by Wilgram et al. in 1964 as the earliest visible change in skin of patients with PF, and has later been confirmed by others. Sotto et al. reported that, in skin of patients with endemic PF, acantholysis started with the separation of nonspecific junctions before desmosomes became disrupted. In line with our observations in patients, the spreading of intercellular widening between desmosomes from the lower to the upper layers has also been observed in a mouse model of endemic PF. Intercellular widening in the lower layers was seen 1–3 h after injection of patient IgG and, after 6–12 h, widening also occurred in the spinous layer.17 Intercellular widening has also been described as one of the first events in the pathogenesis of acantholysis in mucocutaneous PV,15,18 although others have suggested that it is not specific for pemphigus.14

From cell experiments with PV sera, it is known that PV IgG binds non-junctional Dsg3, leading to its endocytosis.6,8,9 Also in PF, antibodies caused depletion of non-junctional Dsg1 in cultured cells.19 Although it has long been assumed that little or no Dsg1 is expressed in the basal epidermal layer, in normal human skin we observed a complete transepidermal staining pattern of Dsg1 with an increasing expression upwards from the basal layer. In skin of patients with PF, clustering of Dsg1 clearly starts in the basal layer.20 We propose that, in PF, depletion of non-junctional Dsg1 is responsible for the intercellular widening between desmosomes, starting in the lower layers as observed in normal–appearing N+ skin and spreading towards the upper layers as observed in lesional skin. This explains the ‘spongiosis’ often seen in the histopathology of PF skin.21 Like transadhesion between junctional cadherins, there might also be transadhesion between non-junctional cadherins providing for zipper–like approximation of opposite keratinocytes.22 This transadhesion can be either homo– or heterophylic. In case of heterophylic binding, loss of non-junctional Dsg1 will result in intercellular widening between desmosomes in PF. Additionally, or alternatively, adherens junctions can be weakened by antibodies directed against E–cadherin which may be present in patients with PF, and might result in intercellular widening between desmosomes as well.

As a consequence of binding of PV IgG to non-junctional Dsg3, newly synthesized Dsg3 cannot be incorporated into the desmosomes anymore, leaving Dsg3–depleted desmosomes.6,8,9 Based on the changes in Dsg1 distribution in skin of patients with PF we previously hypothesized that
binding of PF IgG to Dsg1 can also deplete Dsg1 from desmosomes.\textsuperscript{20} With regard to the Dsg compensation hypothesis\textsuperscript{2} one could hypothesize that in the upper layers of the epidermis, where Dsg3 is not expressed, the depletion of Dsg1 will result in smaller and eventually a total disappearance of desmosomes, and subsequent acantholysis. In the lower layers where Dsg3 is present to compensate for the loss of Dsg1, Dsg1 depletion might result in smaller desmosomes, but not in acantholysis. We indeed observed a decrease in size and number of desmosomes in the lower epidermal layers in N+ skin. The subtle abnormalities of desmosomes at the level of the basal cell layer in early PF were also noted in the initial study by Wilgram et al.,\textsuperscript{11} but were not mentioned in later studies by others.\textsuperscript{12–15}

Acantholysis may be present in the lower spinous layers of unrubbed N+ skin, and may be evoked by the friction of the biopsy procedure itself (‘scissor–Nikolsky’). The lower spinous layers are depleted of the non-junctional and junctional Dsg1 at this stage of the pathogenesis, and therefore are the locus minoris resistentiae. We hypothesize that the direct Nikolsky sign performed on normal–appearing skin has a lower split than the marginal Nikolsky on the edge of a lesion.

In one of the N+ biopsies with acantholysis we observed dense plaque–like structures in the cell wall at the end of tonofilament bundles that might resemble the so–called half-desmosomes observed in pemphigus skin\textsuperscript{14} and in mouse models of endemic PF\textsuperscript{17} and of PV.\textsuperscript{24} Half–desmosomes are explained by the steric hindrance hypothesis, that states that pemphigus antibodies bind to the extracellular domain of Dsg and block the transadhesion between the half–desmosomes.\textsuperscript{25} These free plaques were observed only in the cell wall facing the blister cavity. However, a part of the plaques was covered by a second plasma membrane from the opposing half of the desmosome; therefore these structures represent pseudohalf–desmosomes, as the cell membrane was torn off from the opposing plaque of the desmosome. These pseudo–half-desmosomes were also previously described in PV.\textsuperscript{15} Torn–off desmosomes are also seen when plaque components such as desmoplakin are lacking because of hereditary disease, and desmosomes cannot hold onto the keratin filaments.\textsuperscript{26} Apparently in pemphigus, the decrease of intercellular adhesion between desmosomes and the reduction in size and number of desmosomes leads to such transcellular forces that remaining desmosomes are stretched out and finally break between inner plaque and keratin filaments. The weakening behind the plaque might simply be the result of desmosome hypoplasia, but may also be due to cell signalling events occurring after IgG binding to desmogleins resulting in phosphorylation of plakoglobin (PG) and compromising the adherence between plaque and the intermediate filaments.\textsuperscript{10} The desmosomes are not torn off because of collapse of the intermediate filament cytoskeleton and cell shrinkage as explained
by the ‘basal cell shrinkage theory’, as we observed tonofilament retraction to a limited extent after acantholysis was almost complete, and this postacantholytic shrinkage was not confined to the cells in the basal layer.

In conclusion, based on the results of this ultrastructural study, we propose the following model for acantholysis in PF: IgG causes a depletion of non-junctional Dsg1 leading to intercellular widening between desmosomes. This widening starts in the lower epidermal layers and spreads towards the upper layers. Later, the depletion of non-junctional Dsg1 impairs the assembly of desmosomes, which results in a decrease in size and number of desmosomes. In addition, pemphigus antibodies might promote disassembly of desmosomes. In the upper epidermal layers, where Dsg3 is absent, ongoing depletion of Dsg1 will finally result in a total disappearance of desmosomes, and subsequent acantholysis.
References


