Pemphigus pathogenesis
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Chapter 3

Desmoglein 1 in pemphigus foliaceus patient skin is depleted from desmosomes, clustered in interdigitating double membrane structures and sequestered in large cytoplasmic vesicles

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Abstract

Pemphigus foliaceus (PF) is an autoimmune blistering skin disease where IgG autoantibodies against the desmosomal cadherin desmoglein 1 (Dsg1) cause loss of cell-cell adhesion (acantholysis) in the upper part of the epidermis. IgG deposits in the epidermis in a coarse clustered pattern especially in the lower layers. Large scale electron microscopy has shown an abundant presence of so-called double membrane structures in PF patient skin. In order to get more insight into PF pathogenesis we applied correlative light and electron microscopy (CLEM), immunolabeling for Dsg1 in electron microscopy and large scale electron microscopy (nanotomy). By CLEM we show that the clusters of Dsg1 and immunoglobulin present in PF patient skin are the same as the double membrane structures seen by electron microscopy. We furthermore found that the amount of Dsg1 incorporated into desmosomes is severely decreased in the layers that contain double membrane structures. Also desmosomes were smaller. In the higher levels of the epidermis Dsg1 was present in the membranes of large unique cytoplasmic double membrane vesicles. Depletion of Dsg1 from desmosomes in PF patient skin is likely the main cause of acantholysis.
Desmoglein 1 is depleted from desmosomes in pemphigus foliaceus patient skin

Introduction

In pemphigus foliaceus (PF) autoantibodies against desmoglein 1 (Dsg1) cause loss of cell-cell adhesion (acantholysis) in the subcorneal layers of the epidermis (Dasher et al., 2008; Stanley and Amagai, 2006) resulting in erosive exfoliation.

Dsg1 is one of the four Dsg isoforms, which are transmembrane calcium dependent adhesion molecules of the cadherin family that together with desmocollins mediate adhesion by binding to its opposites in extracellular region of desmosomes (Garrod et al., 2002). The armadillo proteins plakoglobin and plakophilin mediate further adhesion by linking the cadherins to desmoplakin, which in turn bind to the intermediate filaments (Garrod and Chidgey, 2008). Desmosomes interconnect intermediate filament networks of neighboring cells and thereby provide strong intercellular adhesion (Garrod and Chidgey, 2008; Delva et al., 2009). The ultrastructural organization of desmosomal components can be easily recognized as two opposite, mirror image, inner and outer dense plaques with an extracellular core (ECD) domain in between these plaques. For yet unknown reasons desmosomes change their protein composition as they differentiate moving upwards from the basal layer, what leads to a different distribution of Dsg1 and Dsg3 over the distinct cell layers of the epidermis. This change of protein composition has led to one of the most influencing visions on the pathogenesis of pemphigus, which is called the compensation hypothesis (Mahoney et al., 2006; Mahoney et al., 1999), resulting from the notion that the two major forms of pemphigus, namely pemphigus vulgaris (PV) and PF have different antibody profiles resulting in a different level of acantholysis.

While in PV antibodies to Dsg3 are always present, accompanied by anti-Dsg1, PF is characterized by antibodies to Dsg1 only. The pemphigus compensation hypothesis finds its foundation in the assumption that desmosomes will only be functional as either Dsg1 or Dsg3 is available and that antibodies to either of the Dsgs compromise their function in desmosomes. Dsg1 being present in all layers while Dsg3 is absent in subcorneal layers (Mahoney et al., 2006). Loss of desmosomal Dsg1 in the subcorneal layers for that reason, would therefore inevitably lead to loss of desmosomes, ultimately resulting in acantholysis. Similarly, in PV loss of Dsg3 in the basal layer of the mucosa, where Dsg1 is absent would lead to mucosal blistering.

The exact pathomechanism of acantholysis is still unknown. While the simplest assumption would be that the pathomechanism is similar in PV and PF, any scientific proof of such a kind of mechanism is so far unexplored.

Data on PF tissue and experimental models are scarce. However PF hallmarks, apart from subcorneal acantholysis, include the initial observation of double membrane structures, called curvicircular structures, and endocytosis of Dsg1, IgG, plakoglobin and
connexin43 (Cx43) in patient’s skin (Tada and Hashimoto, 1996; Iwatsuki et al., 1999b). Recently we confirmed these observations using large-scale electron microscopic analysis, termed nanotomy, and found an abundance of double membrane structures in patient skin cells, as well as by immunofluorescence analysis revealing endocytosis of Dsg1, IgG and plakoglobin but not Cx43 in lesional skin (Oktarina et al., 2011; Sokol et al., 2015). Other observations include clustering of Dsg1, IgG and plakoglobin in patient skin, accompanied by intercellular widening and shrinkage of desmosomal size and number (Oktarina et al., 2011; van der Wier et al., 2014). Our hypothesis is that in patient skin desmosomes become depleted of essential Dsg, resulting in acantholysis.

We set out to understand the nature of the IgG/Dsg1 clusters, the double membrane structures, and the endocytosis in pemphigus foliaceus skin. Here we demonstrate, using correlative light and electron microscopy (CLEM) (Sjollema et al., 2012), that desmosomes indeed are depleted of Dsg1 in patient skin, that Dsg1 is trapped between cells in double membrane structures, and that from the suprabasal layers onwards Dsg1 is internalized in large double membrane vesicles that localize perinuclear

Results

Localization of desmoglein 1 in pemphigus foliaceus patient skin

To obtain information on the near-molecular localization of Dsg1 in PF patient skin we performed CLEM. Dsg1 of a perilesional PF skin section was labeled with FluoroNanogold Alexa 488 that can be detected both on the light [Figure 1a,c green] and EM level [Figure 1b,c black] (Sjollema et al., 2012). The immunofluorescence showed an even distribution of Dsg1 around the cells of the higher epidermal layers while Dsg1 was clustered in the lower epidermal layers. Electron microscopy showed that in the higher layers the FluoroNanogold Alexa 488 was heavily present on desmosomes [Figure 1d,e]. In contrast, in the lower layers Dsg1 in the clusters was not present in desmosomes but instead on non-desmosomal structures that have an intertwined double membrane appearance [Figure 1f,g]. Hardly any label was found here on desmosomes. Consecutively cut EM sections of double membrane interdigitations in PF patient skin revealed that these structures were continuous invaginations of once cell into its neighbor [Figure 2].
**Desmoglein 1 is depleted from desmosomes in pemphigus foliaceus patient skin**

*In pemphigus foliaceus patient skin desmosomes are depleted of desmoglein 1*

To investigate if the amount of Dsg1 differs in the higher and lower epidermal layers of PF patient skin compared to the healthy skin. Dsg1 was immuno-labeled in sections of three perilesional PF patient skin biopsies and control skin biopsies using 10nm gold. Dsg1 label was decreased in desmosomes of PF patient skin compared to the controls [Figure 3a, b]. We counted the number of labels per desmosome in respectively the basal, suprabasal, low spinous, high spinous and the granular layer [Figure 3c]. In perilesional PF skin a decrease in desmosomal Dsg1 was measured with a residual amount of approximately 15% desmosomal Dsg1 from the basal layer to the spinous layer. In the higher epidermal layers more desmosomal Dsg1 was present but only up to 50% of its normal amount. The size of the desmosomes in PF skin, especially in the basal and spinous layer, was smaller than in control skin [Figure 4].

**Desmoglein 1 is present in large cytoplasmic vesicles in the higher epidermal layers of perilesional pemphigus foliaceus skin**

A large scale EM dataset of Dsg1 immuno-labeled perilesional PF skin was analyzed to determine the fate of Dsg1 through the epidermal layers (Figure 5a and http://www.nanotomy.org/PW/Sokol2014). In the lower layers of perilesional PF skin Dsg1 was present between cells in interdigitating double membrane structures that were depleted of desmosomes [Figure 5b, c]. In the higher layers Dsg1 was found in double membrane vesicles that localize perinuclear [Figure 5d]. Typically these vesicles, up to 3 µm large, were grouped in clusters. Dsg1 was located close to the membranes of these cytoplasmic vesicles but not in their lumen, suggesting that Dsg1 was still membrane bound. In the corneal layer rudimentary flattened double membrane vesicles containing Dsg1 label were present [Figure 5e]. The intermembrane distance in the double membrane vesicles of the upper layers was smaller, around 22 nm [Figure 6a, b], than the intermembrane distance between interdigitations in the lower layers, which was around 40 nm [Figure 6c, d].
Figure 1. In higher epidermal layers of perilesional pemphigus foliaceus patient skin desmoglein 1 is present in desmosomes, while in the lower in the double membrane structures. a) Cross-section of perilesional PF patient skin immunolabeled for Dsg1 (FluoroNanogold Alexa 488) with part of dermis and all epidermal layers. Note that in the lower layers of the epidermis Dsg1 is clustered, while in the upper layers Dsg1 has normal cell border pattern. b) Corresponding EM. c) Overlay. Pseudocoloured orange and purple nuclei and red arrow indicate the areas enlarged in panels d and f. while yellow, red and pink nuclei and yellow arrow indicate the area that is enlarged in panel f and g. Note that the dermis in panel b and c is pseudocoloured. d) EM of keratinocytes from the upper epidermal layers from panels a-c. The black box is enlarged in panel e. e) Enlarged region from panel d showing concentrated Dsg1 label on desmosomes. f) EM of keratinocytes from the lower layers of the epidermis from panels a-c. The black box is enlarged in panel g. g) Enlarged region from panel f showing concentration of Dsg1 label in interdigitating double membrane structures. Bars: (a-c) 50 µm, (d, f) 2.5 µm, (e, g) 1 µm.
Desmoglein 1 is depleted from desmosomes in pemphigus foliaceus patient skin

Figure 2. Double membrane structures are continuous interdigitations between two cells in pemphigus foliaceus patient skin. Reproduction of interdigitating structure composed of membranes of two neighbouring keratinocytes through consecutive EM sections of PF patient skin. Cell membranes are coloured red.
Figure 3. Desmoglein 1 is depleted from desmosomes in perilesional pemphigus foliaceus patient skin. a) Dsg1 on desmosomes of healthy control skin. b) Dsg1 on desmosomes of perilesional PF patient skin. Note that the distribution of the gold labels fit the specificity of the used monoclonal for the cytoplasmic domain of Dsg1. c) Quantification of Dsg1 label per desmosomes in control skin and perilesional patient skin in the basal, suprabasal, low spinous and high spinous and granular layer. Note reduction of Dsg1 label in PF skin control to the controls. NHS- normal human skin 1,2; Pt A,B,C- patient skin A,B,C. D- desmosome. Bar: (a) 100nm, b is at the same magnification as a.
Desmoglein 1 is depleted from desmosomes in pemphigus foliaceus patient skin

**Figure 4. Desmosomes are smaller in perilesional pemphigus foliaceus skin.** Correlation between desmosomal length and Dsg1 label in the a) basal b) spinous and c) granular layers of normal human and PF patient skin. Note that desmosomes are smaller in size (red, orange and green circles) compared with controls (black circles). The spinous layer of patient A had only few cells and was therefore omitted from b.
Figure 5. Fate of desmoglein 1 through epidermal layers of perilesional pemphigus foliaceus patient skin. a) Immuno-labeled perilesional PF patient skin with upper dermal layers and all epidermal layers. Red, blue, yellow, orange boxes are enlarged regions in panels b, c, d and e. b) Double membrane structure enriched in Dsg1 label from the suprabasal region. Red box in panel a. c) Interdigitating double membrane structure enriched in Dsg1 label from the suprabasal region. Blue box in panel a. d) Intracytoplasmic double membrane vesicles containing Dsg1 label from the granular layer. Yellow box in panel a. e) Double membrane vesicles in the corneal layer containing Dsg1. Orange box in panel a. SB- stratum basale, SS- stratum spinosum, SG- stratum granulosum, SC- stratum corneum. Bars: (a) 50 µm and (b-d) 500 nm. The interactive data set can be found on www.nanotomy.org.
Desmoglein 1 is depleted from desmosomes in pemphigus foliaceus patient skin

Figure 6. Desmoglein 1 containing double membrane structures and vesicles in perilesional pemphigus foliaceus patient skin labeled for desmoglein 1 have different intermembrane distances. Data is taken from large scale electron microscopy of figure 4 and http://www.nanotomy.org/PW/Sokol2014. a) Intracytoplasmic double membrane vesicle from the granular layers. The black box is the region enlarged in panel b. b) The distance between the two membranes is 22 nm. c) Double membrane structure from the suprabasal layer. The black lined box is the region enlarged in panel d. d) The distance between the two membranes is 40 nm. Bars: (a, c) 500 nm and (b, d) 100 nm.
Discussion

In PF patient skin the Dsg1/IgG/PG clusters reported before (Oktarina et al., 2011) represent double membrane structures that we recently also detected using nanotomy (Sokol et al., 2015). In perilesional PF skin Dsg1 is depleted from desmosomes. Dsg1 depletion is most profound in lower epidermal layers where the clusters, now revealed as double membrane structures are abundant. The average size of Dsg1 depleted desmosomes is smaller than in healthy skin. Together with the reported decrease in number and size of desmosomes, and a complete absence of desmosomes in acantholytic areas of PF patient skin, this fits the prediction that desmosomes ‘melt’ away in PF skin (Sokol et al., 2015; van der Wier et al., 2014). The non-desmosomal Dsg1 in double membrane structures is derived from desmosomal disassembly, or from newly synthesized Dsg1 transported to the cell membrane ready for desmosomal assembly, and trapped by pathogenic anti-Dsg1 patient IgG either way. In line with the desmoglein compensation hypothesis this would result in loss of desmosomes and acantholysis in the subcorneal layers where Dsg3 is not expressed (Mahoney et al., 2006). Recently we found by immunofluorescence microscopy that in lesional PF patient skin the clusters of IgG/Dsg1/PG become internalized together with the early endosomal antigen EEA-1 and localize perinuclear in seemingly large vesicular structures (Oktarina, submitted/ not shown). Here by EM we support these findings by revealing that Dsg1 is present in up to 3 μm wide vesicles that appear to have a double membrane. Vesicles with double membranes are rare and in the literature only few other examples are described, being internalized gap junctions, tubulolbar complexes, autophagosomes and trogocytosis (Piehl et al., 2007; Guttmann et al., 2004; Yokota, 1993; Dopfer et al., 2011). How these double membrane vesicles form in PF skin remains elusive. It is unlikely that the double membrane vesicles are the cause of acantholysis, since in experimental PF acantholysis can be evoked by Fab fragments that do not induce Dsg1 clustering. Yet double membrane vesicles are the result of the pathologic process in PF, as their remnants are present in the corneal layer not seen in normal skin. We envision that these vesicles in non-lesional skin will be eventually lost through shedding of corneocytes. The primary cause of acantholysis in PF however is loss of Dsg1 from desmosomes. In summary, we show here the depletion of Dsg1 from desmosomes in PF patient skin that is the most likely the cause of acantholysis. We furthermore demonstrate that Dsg1 is endocytosed in double membrane vesicles by a yet to be solved unique mechanism.
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Materials and methods

Skin biopsies
Three biopsies were obtained from perilesional Nikolsky-negative skin of three different PF patients. Pemphigus was diagnosed on the basis of clinical appearance and confirmed by histology, positive immunofluorescence and anti-Dsg1 ELISA. A control skin biopsy from healthy individual was obtained from redundant skin after plastic surgery. Written informed consent was obtained.

Sample preparation for cryo electron microscopy
The 2-mm biopsies were fixed in 2% formaldehyde in 0.1 M phosphate buffer pH 7.4 and then rapidly cut into 300-400 µm perpendicular slices using a scalpel. The tissue remained in 2% formaldehyde overnight at 4 °C and was then transferred to 0.1 M glycine in phosphate buffered saline (PBS). This was followed by a series of concentration increments of glycerol in PBS (5-15%). The tissue was then plunge frozen in liquid propane and stored in liquid nitrogen.

Cryo substitution
The tissue was transferred to a Leica CS-automachine (Leica, Wetzlar, Germany) at -90°C in methanol and incubated overnight in 0.2% uranyl acetate in methanol at -90°C for ten hours and warmed up to -45°C at 5°C/h. Tissue then washed with methanol and the concentration of Lowicryl HM20 (EMS, Hatfield, PA, USA) in methanol was gradually increased to 100%. Polymerisation of HM20 was achieved with UV light at -45 °C and post-polymerisation at -20 °C.

Sectioning, antigen retrieval and post embedding immuno-labelling
Sections of 70 nm were cut and placed on formvar coated nickel grids. Antigen retrieval was by 10 mM citrate buffer pH 6.0 in an EMS 820 microwave oven (EMS, Hatfield, PA, USA) at 40% of maximum power for five minutes. Blocking was performed in incubation buffer (PBS with 1% BSA, 5% normal goat serum and 1% cold water fish gelatin at 4°C in a humidified chamber. Mouse monoclonal anti-Dsg1 B11 (Santa Cruz Biotechnology, Dallas, TX, USA) was used as primary antibody. As secondary antibodies either 10 nm gold conjugated goat-anti-mouse IgG or Alexa Fluor 488 FluoroNanogold conjugated goat anti-mouse IgG (Nanoprobes, Yaphank, NY, USA). GoldEnhance (Nanoprobes, Yaphank, NY, USA) was used to visualize the 1.4 nm FluoroNanogold for EM.
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Correlative light and electron microscopy
Ultrathin sections stained with FluoroNanogold Alexa 488 were first imaged with a Leica DMRA fluorescence microscope and thereafter with a CM-100 electron microscope. Data of the two modalities were overlaid using Adobe Photoshop CS5 software (San Jose, CA, USA) as were additional annotations that were added for illustrations.

Large-scale electron microscopy
Image acquisition was performed using a Zeiss supra 55 electron microscope (Zeiss Oberkochen, Germany) and ATLAS software developed by Fibics (Ottawa, Ontario, Canada) as extensively been described before (Sokol et al., 2015b). For illustrations, the data set was exported with ATLAS VE viewer as TIFF file downscaled to 10 nm per pixel and additional annotations were added using Adobe Photoshop.

Quantification of Dsg1 label
TEM images of representative areas of the basal, suprabasal, spinous and granular cell layers were analyzed using Photoshop software. For each layer, the number of gold labels per desmosome was counted and averaged. Also the size of the desmosomes was measured.

Supplementary data
The large-scale EM data (nanotomy) at full resolution is currently available at: http://www.nanotomy.org/PW/Sokol2014/ and password protected:
Login: pemphigus
Password: blister
Upon publication the dataset will become open access available online via: www.nanotomy.org

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