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### Pemphigus pathogenesis

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*Chapter 5*

# **Discussion and future perspectives**

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## Introduction

The pathomechanisms of autoimmune diseases are in detail unknown despite of all the ongoing research. Pemphigus is a model of autoimmune disease for a single organ, for which the target, skin and mucosa, are easy accessible thus allowing it to be studied on actual patient tissue where the pathogenesis takes place. Furthermore certain aspects of pemphigus pathogenesis can be studied in different experimental models using patient autoantibodies as involvement of other components of the immune system is not required. This makes pemphigus one of the most approachable autoimmune disorders to study. How autoantibodies against desmosomal proteins are able to induce loss of cell-cell adhesion and which mechanisms are involved is still a trilling question that fascinated scientists over the last 50 years. This question is also the subject of this thesis. Here we combined the clinical and scientific expertise and the patient sample availability of the Center for Blistering Diseases with newly developed imaging techniques of the Imaging Center in the Department of Cell Biology of the University Medical Center Groningen to gain more insights into the pemphigus pathomechanism. We also combined studies on actual pemphigus patient tissue with those on cultured primary keratinocytes using patient autoantibodies. To visualize pathological induced changes we used the correlative light and electron microscopy (EM) on the same specimen, immuno-labeling, confocal and time lapse imaging and large scale electron microscopy. By combination of these techniques we discovered novel findings which contribute to the current understanding of pemphigus pathogenesis.

### **Approach to the pathogenesis: pemphigus models and techniques used**

When studying pemphigus pathogenesis choice of experimental model and techniques and understanding its limitations is crucial, since most of the differences in views on pemphigus pathogenesis originate from the approach. The most valuable object to study is the skin of the pemphigus patient (Chapters 2 and 3) since that contains information on the actual pathogenesis. However it is necessary to reflect on the stage of the pathogenesis at the site of collecting the patient tissue, different specificities of autoantibodies, different titers and clinical differences between patients. For instance the level of IgG/Dsg clustering in tissue samples obtained for diagnostics can vary between patients (data not shown).

In Chapter 2 large scale microscopy approach was used which enables unbiased EM observation and data sharing. All EM data from Chapter 2 are now as open source publicly available. One of the most interesting findings were the abundant double membrane structures present in pemphigus skin. Apart from one observation in 1996 (Tada and Hashimoto, 1996) these have been missed by conventional EM. In Chapter 3 correlative light and electron microscopy was implemented to identify the IgG clusters

seen in patient skin. Furthermore the same large scale approach was used as in Chapter 3 to follow the fate of Dsg1 in PF skin through the epidermal layers.

In Chapter 4 we changed to primary human keratinocytes, a model easy to handle and manipulate, however the composition of the desmosomes in keratinocyte monolayers differs from epidermis or mucosal epithelium as they contain also Dsg2. Therefore, in accordance with desmoglein compensation theory (Mahoney et al., 1999) actual acantholysis cannot be induced by patient autoantibodies on keratinocyte monolayers, yet the effects of anti-Dsg3 IgG on Dsg3 can be studied. Time lapse imaging was used to follow the fate of fluorescently labeled PV IgG and additionally transfected this model with mCherry keratin to enable visualization of cell borders. For observation of IF images combination of wide field microscopy and confocal microscopy was used. In keratinocyte culture model Dsg1 is not visibly expressed on 1 day after induction of desmosomes, but after several days it is present in differentiated cells that have flattened off. However endocytosis of Dsg1 could not be evoked by anti-Dsg1 patient IgG (not shown), therefore this model was not implemented for studying PF pathogenesis. This was in contrast with the previous finding of PF IgG internalization in primary human keratinocytes (Cirillo et al., 2007) which can be explained by difference in culture conditions.

#### **Findings through thesis chapters**

In Chapter 1b we reviewed non-adhesive functions of major desmosomal proteins and their isoforms and diseases in which these are involved. Most interestingly for the subject of this thesis are functions of Dsg1 and Dsg3 as they are the main immunological targets of pemphigus. Past studies have shown that there is relationship between the actin cytoskeleton Dsg3 regulates E-cadherin levels and interacts with actin (Tsang et al., 2010; Tsang et al., 2012a). This would be a possible explanation of the perturbed actin cytoskeleton in primary human keratinocytes after incubation with PV IgG. In this thesis the primary focus was however not on actin and as in EM the actin cytoskeleton is hardly visible so conclusions are not drawn here. However in Chapter 4 we did investigate if actin co-localized with the PV IgG induced so-called linear arrays. In contrast to an earlier claim in the literature we found no-colocalization of actin and linear arrays (Jennings et al., 2011). Actin and Dsg3 did colocalize in other structures that appeared like linear arrays but which in all probability are filopodia. It is known that Dsg3 interacts with actin and promotes filopodia formation (Tsang et al., 2012a). More proof for this should be obtained by specific staining for filopodia in combination with actin. The PF antigen Dsg1 was found to promote epidermal differentiation (Getsios et al., 2009; Harmon et al., 2013), therefore it can be expected that epidermal differentiation is disrupted in skin and mucosa of PF patients. Nothing on this however is present in the literature but from our own experience we know that hyperkeratosis is

seen in PF patient skin. We hypothesize that this is likely connected to the Dsg1 function in differentiation. A future study should address if the level of hyperkeratosis is connected with the degree of Dsg1 depletion/clustering.

In Chapter 2 10 patient datasets are open source available online by nanotomey (large scale electron microscopy, [www.nanotomey.org](http://www.nanotomey.org)). The most prominent finding in pemphigus tissue is the presence of double membrane structures which is the most abundant in lesional tissue. Distribution wise they remind of the IgG clusters seen by routine immunofluorescence of patient biopsies. The double membrane structures in some samples also contained small structures which seem as remains of desmosomal plaques. In contrast to a previous finding (Tada and Hashimoto, 1996) we also discovered double membrane structures in PV tissue. Double membrane structures can be expected in PV skin as complexes of Dsg3 and IgG. However PV patient also had a, although very low, Dsg1 ELISA titer, what might explain this finding. Therefore future study that explore more PV biopsies are warranted to elucidate this question. Why the double membrane structures have intertwined shapes remains a question for further research. In Chapter 4 a possible role for keratin in IgG induced invaginations is suggested in cultured cells, however in skin no evidence for this was found. It is clear that this is a field for further research.

As the distribution pattern of the double membrane structures was similar to the IgG/Dsg clusters observed by (Oktarina et al., 2011), we in Chapter 3 investigated if a causal relation exists between these two observations. Before we observed in an ex vivo human skin model the clusters of IgG/Dsg cannot be induced by Fab fragments, suggesting that the double membrane structures are caused by the bivalency and polyvalency of IgG molecules by which they are able to crosslink their Dsg antigens. Therefore we assume that membranes of two neighboring keratinocytes are held together by bivalent IgG molecules that link two opposite non-junctional desmogleins. Unexpectedly in PV tissue seemingly remains of desmosomes were found. Based on our previous findings we had expected a total separation of crosslinked Dsg from desmosomes. Future research has to show if desmosomes can be trapped in the crosslinked double membranes. The fate of double membrane structures is not known. When following Dsg1 through the epidermal layers in PF patient skin, large cytoplasmic vesicles containing Dsg1 were found in the upper epidermal layers. These vesicles also had double membranes but the distance between the membranes (22 nm) was smaller than in the double membrane structures (40 nm). While the double membrane structures are actually continuations of the plasma membranes of two opposing keratinocytes the cytoplasmic vesicles originate from an unknown endocytic process. Double membrane vesicles are extremely rare elements in cells (Dopfer et al., 2011; Guttman et al., 2004; Piehl et al., 2007), and here also their size that can reach 3  $\mu\text{m}$  in diameter, is astonishing. Because they contain Dsg1 they must originate from the

double membrane structures. The mechanism by which the vesicles form from the double membrane structures must be unique and represents a undiscovered ability of cells.

In our dataset we did not find a clue for the pathogenesis of double membrane vesicles as we investigated only one section. We suggest for future studies to investigate this process by 3D EM of immunolabeled biopsies. The technique of 3D EM is still in a phase of being developed and endocytic process present here would be a perfect example to demonstrate its power. Since double membrane vesicles are seen in the corneal layer we assume that they are shed from the epidermis together with corneocytes and likely are involved in the depletion of Dsg but are not primary pathogenic.

In Chapter 4 we solved the nature of the linear arrays that form in keratinocytes when these encounter pathogenic PV IgG. We showed that these structures likely arise due to the disappearance of desmosomes and that still attached keratin filaments pulls one cell into another. These arrays form at the positions of clustered IgG and have a same distance of 40 nm between opposing membranes as we found before in Chapters 2 and 3 in patient skin. These arrays contain IgG and Dsg3 but also other desmosomal components and therefore are different than the clusters seen in patient skin as these contain predominantly IgG, targeted Dsg and plakoglobin. Another difference is the presence of keratin filaments that attach to the double membrane arrays in primary human keratinocytes, a phenomenon we did not observe in patient skin. This might indicate a difference in the pathomechanism between IgG treated cultured keratinocytes and patient tissue keratinocytes. However it might also be related to the time of incubation, as primary human keratinocytes were examined 6 hours after adding IgG while patient skin receives constant IgG attacks might reflect another stage in pathogenesis. As before for skin we propose that the double membrane structures formed in cells result from crosslinking of Dsg molecules. The video presented in this thesis however also shows that besides fusing IgG clusters sometimes split. This might indicate that clusters sometimes form out of separate smaller clusters. Since keratin filaments are present on one side of the membranes we assume that arrays form as a result of loss of keratin from the membrane of the pulled in cell. When the other cell also loses its keratin the array will in all probability disappear, what would explain the change in shapes we observed over time. What Chapter 4 however clearly showed is that Dsg3 disappeared from desmosomes and that this is the most likely cause of acantholysis. However as it is unclear if the pathomechanism in cells is the same as in tissue.

### **Reflection on pemphigus pathogenesis theories**

Research on pemphigus pathogenesis over the past years resulted in several theories: steric hindrance, cell signaling and desmoglein non-assembly depletion hypothesis, which we explained in the introduction of this thesis. These theories show the diversity of pemphigus research and are results of different approaches and are mainly based on studies on cultured cells. The theory of steric hindrance prevailed out of earlier findings of half-desmosomes around the blister cavities (Wang et al., 2009) and on immune-mapping studies that showed pathogenic PV IgG to bind to N-terminal domain of Dsg that contains the trans-adhesive binding site (Amagai et al., 1992; Sekiguchi et al., 2001). In Chapter 2 of this thesis we did find half-desmosomes around the blister cavities in pemphigus tissue. According to the steric hindrance theory these half-desmosomes results of IgG blocking of the Dsg trans-adhesion interface. The half-desmosomes we found in all lesional tissue except in lesional pemphigus foliaceus skin might be caused by steric hindrance, but due to their size alternatives are newly synthesized half-desmosomes that seek an opposite partner, or remains off weakened Dsg-depleted desmosomes that split by mechanical force as wielded during biopsing. As all these three mechanisms are possible we cannot close that discussion here. In the lesional PF biopsy half-desmosomes in the acantholytic cells were not found. This could be explained by the following: being the dead of acantholytic cells or the inability of these cells to produce new desmosomes due to Dsg1, the only Dsg expressed in this area, is captured in its soluble form by the pathogenic IgG.

The cell signaling theory suggests that acantholysis is caused by IgG disturbing signaling pathways but is based on cell culture studies (Waschke and Spindler, 2014). In Chapter 2, as before, we could rule out involvement of apoptosis in the acantholytic mechanism studying patient skin (Janse et al., 2014).

Retraction of keratin filaments is claimed to be caused by MAPK kinase involvement in pemphigus (Berkowitz et al., 2005), however in Chapter 2 we here found keratin retraction only in lesional PF patient skin which is likely a secondary event instead of the primary event leading to acantholysis.

Several studies showed an beneficial effect of 38MAPK inhibitors on acantholysis (Berkowitz et al., 2006; Jolly et al., 2010) but in Chapter 4 we observed that acantholysis in cultured primary keratinocytes is prevented by Dsg2 compensation. Little is known about the effects of paralyzing signaling pathways and over interpretation on acantholysis might be present in pemphigus research. In Chapter 4 massive internalization of IgG and Dsg was seen what was used as an argument for causative involvement of pathway interference, however in our long time observations acantholysis did not occur.

The non-assembly depletion hypothesis is supported by the finding of internalization of non-desmosomal Dsg after pemphigus IgG incubation in cell monolayers and clustering of IgG and targeted desmogleins outside desmosomes in pemphigus patient skin (Oktarina et al., 2011). In this thesis we found further support for the non-assembly depletion theory as we found depletion of Dsg1 from desmosomes and clustering of Dsg1 outside desmosomes. We also found that desmosomes shrink when depleted from Dsg1 and demonstrated that Dsg3 is depleted from desmosomes when cultured cells contact PV IgG, as reported many times before (Delva et al., 2008; Jennings et al., 2011; Patel et al., 1984). Therefore we support the non-assembly depletion hypothesis.

### **Final remark**

Based on the results of this thesis we propose that desmosomes become depleted of the targeted Dsg and that this at the layers where compensation is not possible leads to the wasting away of desmosomes. Several experiments with exogenous Dsg have shown that such cells are rescued from acantholysis. This thesis focused on Dsg1 in patient skin as immunolabeling for Dsg1 for electron microscopy was successful. We were successful in finding an antibody that worked for immunoelectron microscopy procedure. What is left open is an antibody to follow the ultrastructural fate of Dsg3 in PV tissue. Successful labeling would link together 20 years of cell observations to patient skin. Furthermore, as all events in cells are under cell signaling control it is not strange that inhibitors of cell signaling pathways can also inhibit acantholysis. However, inhibiting signaling pathways to control acantholysis will therefore also easily result in unwanted side effects. If compounds can be found that selectively inhibit desmosomal Dsg depletion a new avenue for pemphigus treatment would be opened.

