Acantholysis in pemphigus
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General discussion and future perspectives

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The aim of this thesis was to gain more insight in acantholysis in pemphigus by studying pemphigus skin using histology, immunohistochemistry and electron microscopy. Most investigators have used in vitro cell models and mouse models to study the pathogenesis of pemphigus. In this thesis we will focus on actual patient skin biopsies and organ cultures of human skin. In the Center for Blistering Diseases at the University Medical Center Groningen we see about 10 new patients per year with pemphigus vulgaris (PV) and pemphigus foliaceus (PF) referred to us from all over the Netherlands. Therefore we have been able to collect a number of well documented patient biopsies and serum samples. We have the unique opportunity to conduct translational medical science by using clinical material for basic science. Using both PF and PV biopsies and samples enabled us to study the effects of both desmoglein (Dsg)1- and Dsg3 autoantibodies on human skin cells.

In chapter 2 we give an overview of the human models that have been used in the past to study the pathogenesis of pemphigus, i.e. organ cultures of human skin, cultured human monolayer keratinocytes, reconstituted skin and human skin grafted on mice. The organ cultures of human skin that we used are based on the organ culture systems of Michel and Ko. In this model human skin biopsies were either placed on a transwell such that the bottom of the biopsy contacts the solution containing patient IgG or they were submerged in a solution containing IgG. The latter approach enables to incubate several biopsies in one and the same volume of medium with added pemphigus IgG or Fab fragments. The biopsies can be harvested at any time and processed for light microscopy, immunofluorescence or electron microscopy. Submerged culturing induces shifts in the expression of the different cadherins, but this manifests only after culturing longer than 24 hours. The major advantage of our explant model is that it is actual human skin with the correct architecture and desmosomal make–up of all epidermal layers. The level of blistering induced by pemphigus IgG is comparable to that in patient skin i.e. suprabasal splits are induced by PV IgG and subcorneal acantholysis is induced by PF IgG.

The majority of the past studies have been conducted with in vitro monolayers of cultured cells. In chapter 2 we also described the expression of desmosomal components by the various used monolayers. When cultured in high calcium, monolayers of normal human epidermal keratinocytes (NHEKs) express Dsg3 and after several days, the differentiating cells also start expressing Dsg1. Furthermore, they also express Dsg2 and as such differ from the cells of the basal layer of the native epidermis. Since acantholysis is only present in cells that either express Dsg3 alone (basal layer of oral mucosa) or Dsg1 alone (granular layer of epidermis), cultured cells therefore
do not represent an ideal model system to study PF and mucosal-dominant PV acantholysis. To answer future questions concerning the pathogenesis of pemphigus, one should use patient skin, human models and mouse models in a complementary fashion. It is important to realize that the results might not be representative for the in vivo situation, and that data derived from for example cell culture studies cannot be extrapolated to skin.

In chapter 3 we describe the distribution of IgG and the desmosomal proteins in pemphigus patient skin. In PF skin, clustering of IgG, Dsg1 and plakoglobin (PG) was observed. The aggregation process starts low in the basal layer and when more IgG becomes available aggregation also spreads to the layers above. In skin of mucosal-dominant PV patients, Dsg3 was clustered and the overall Dsg3 fluorescence intensity was reduced compared to normal human skin. IgG and also some PG colocalized with the clustered Dsg3. Clusters composed of IgG, Dsg1 and PG and of IgG, Dsg3 and PG were present in the skin of patients with mucocutaneous PV. By using a human in vitro organ culture model in which blistering was induced by pemphigus Fab fragments, we demonstrated that clustering is caused by crosslinking of Dsg by bivalent IgG. The clusters reveal that the targeted non–junctional Dsg becomes sequestered from desmosomal components.

The degree of Dsg1 clustering is correlated with the degree of intercellular widening. Light microscopy showed that in PF skin with profound Dsg1 clustering, keratinocytes exhibited subtle intercellular widening, especially between the basal cells and to a lesser degree also between the suprabasal cells. In lesional skin, widening also extended to higher layers. Biopsies with little IgG deposition and minimal Dsg1 clustering had no visible widening. By electron microscopy it was shown that intercellular widening was present in the basal cell layer of a Nikolsky–positive (N+) PF skin biopsy. Light- and electron microscopy of clinically unaffected mucosal-dominant PV skin showed no intercellular widening and the desmosomes were normal of size and number. Intercellular widening was also present in the non–lesional skin of patients with mucocutaneous PV which showed substantial Dsg1/PG aggregation.

Based on the data of chapter 3 we formulated the following nonassembly depletion hypothesis for acantholysis in pemphigus skin. This model follows the rule of the desmoglein compensation hypothesis that states that desmosomes need minimal one desmoglein, either Dsg1 or Dsg3, to remain functional. In pemphigus, IgG first depletes the non–junctional desmoglein. The depleted non–junctional desmoglein cannot be incorporated anymore into newly forming desmosomes and therefore the desmosomes will be depleted of the targeted desmoglein. As the IgG diffuses upwards from the dermis into the lower layers of the epidermis and further towards the upper
layers of the epidermis the effect is first visible in the lower layers, also in PF skin. However, as the targeted desmoglein in PF is Dsg1, there is no acantholysis in the lower layers as enough Dsg3 is present here to compensate for the loss of Dsg1. When the IgG spreads further upwards, depletion of Dsg1 will lead to melting away of desmosomes and acantholysis in the upper layers, where no Dsg3 is present to compensate for the loss of Dsg1. When there are antibodies present directed against Dsg3 only, there is no acantholysis as all layers contain sufficient Dsg1 to completely compensate for the loss of Dsg3. In patients with antibodies against both Dsg1 and Dsg3 the lower layers become depleted of both desmogleins and therefore acantholysis in mucocutaneous PV is suprabasal.

When using Fab fragments to induce acantholysis in the in vitro model, we showed that these do not induce clustering of the targeted desmoglein. Therefore, it is likely to think that Fab fragments induce acantholysis by another mechanism than IgG. That pemphigus antibodies can have a different effect on desmosomes has been described by Saito et al., who showed that polyclonal antibodies induce clustering and smaller desmosomes in cultured keratinocytes, while in cells incubated with monoclonal antibodies there was no clustering and the desmosomes remained normal sized. However, it is also possible that that Fab fragments induce acantholysis through nonassembly depletion. To confirm this it is necessary to study organ cultures incubated with Fab fragments by electron microscopy to see whether there is also interdesmosomal widening, smaller desmosomes and finally melting away of desmosomes.

We furthermore observed that in PF and in mucocutaneous PV, but not in mucosal-dominant PV, subtle intercellular widening between the cells was present that seemed to correlate with the presence of clustered Dsg1.

To investigate this observation at the ultrastructural level we performed electron microscopy of PF patient skin biopsies in chapter 4. Most electron microscopy studies of pemphigus patient skin date back to the 1960s and 70s. Findings might have been overlooked or misinterpreted due to lack of today's knowledge on antigen specificity and desmosome remodelling.

We studied Nikolsky negative (N-), N+ and lesional PF patient skin. N- PF biopsies showed no intercellular widening, whereas N+ PF biopsies showed intercellular widening at the level of the basal and lower spinous layers. Also lesional PF skin showed intercellular widening between the desmosomes in all layers under the blister. We hypothesized that this widening is the result of depletion of non-junctional desmoglein. Like transadhesion between junctional Dsg, we suggest that is it likely that transadhesion also exists between non-junctional Dsg. Immuno-electron microscopy studies have already indicated that non-junctional Dsg3 exists, however for Dsg1
this has not been investigated. Depletion of Dsg3 does not seem to result in interdesmosomal widening, what suggests that Dsg1 and Dsg3 may have different functions in transadhesion. Further immuno–electron microscopic studies will be needed to demonstrate which proteins provide adherence at the interdesmosomal plasma membrane.

In acantholytic N+ and lesional PF skin we observed five types of desmosomes: 1) Hypoplastic desmosomes, which are the result of nonassembly depletion. 2) Normal sized half desmosomes were sporadically seen. Half–desmosomes have been described before in PF and PV skin9 and might be the result of steric hindrance. However, they have also been observed in cultured cells and are assumed to be an intermediate in the assembly process of desmosomes.10 We therefore cannot exclude here that half–desmosomes in patient skin in fact represent an attempt of keratinocytes to restore intercellular adhesion. 3) These intermediate desmosomes might be represented by the hypoplastic half desmosomes which we observed frequently. 4) Torn off desmosomes, with the point of breakage at the cytoplasmic site of the desmosomal plaque. 5) Pseudohalf–desmosomes, which are plaque like structures representing torn–off desmosomes resulting from an intracellular split between the plasma membrane and the plaque of the opposing cell.

In N– PF skin the desmosomes were normal in number. N+ PF skin showed less desmosomes at the level of the spinous layer. Acantholytic N+ and lesional PF skin also showed less desmosomes in the acantholytic area.

To confirm and quantify the differences in desmosome size and -number in pemphigus skin, we performed a morphometric electron microscopy study on PF, mucosal-dominant PV and mucocutaneous PV skin in chapter 5. Desmosomes in the lower layers of N+ PF and N+ mucocutaneous PV skin indeed appeared to be smaller in size than those in normal control skin.

Interestingly, what our study also showed is that desmosomes in mucosal-dominant PV skin are not changed, neither in size nor in number. Thus, depletion of Dsg3 also does not seem to affect desmosomes in contrast to depletion of Dsg1. The challenge for the coming years will be to understand why depletion of Dsg1 affects desmosomes in such a different way than depletion of Dsg3. To answer this question it is necessary to know what the exact molecular composition of desmosomes is and what the function of each molecule is. How much of each cadherin is present in the desmosomes in the different layers of the epidermis? At present little is known about this. Maybe desmosomes contain much more Dsg1 than Dsg3. That would explain why depletion of Dsg3 in mucosal-dominant PV skin has no influence on desmosome size. Another possibility is that Dsg1 is doing extremely well in compensating for the loss of Dsg3. Immuno–
electron microscopy studies and real time studies are needed to gain further insight into the mechanism of compensation by Dsg1. What also should be investigated is the possible role of plakoglobin (PG) in the desmosomal depletion process. In mucosal-dominant PV skin Dsg3 is clustered but only a very limited amount of PG is found in these clusters. In PF skin however, PG intensely co-clusters with Dsg1. It might be that depletion of PG accounts for the shrinkage of the desmosomes. Smaller desmosomes have also been described in lethal congenital epidermolysis bullosa due to a homozygous nonsense mutation in the junction plakoglobin gene (JUP).\textsuperscript{11} Immuno-electron microscopy has to show if the PG content of the desmosomes in PF is indeed changed.

Whether apoptosis plays a role in the pathogenesis of pemphigus is a constantly recurring question. Research had mainly been conducted in cell models. In chapter 6 we therefore looked for evidence of apoptosis in patient skin. We studied different types of pemphigus patient skin by immunofluorescence for activation of markers of both the intrinsic and extrinsic pathways of apoptosis. We used TUNEL staining and also performed electron microscopy for signs of apoptosis but could not find any evidence. Therefore we conclude that apoptosis is not involved in acantholysis in pemphigus. We consider this as an important observation as the possible involvement of apoptosis or part of the apoptic pathways in the mechanism of acantholysis was an ongoing part of the discussion until now. The absence of apoptosis in pemphigus strengthens other explanations for acantholysis, such as our nonassembly depletion hypothesis.
References


