Summary, discussion and future perspectives

Angelique M. Poot
In this thesis, the disease pemphigus has been investigated from several angles. Firstly, in chapter 2 we analyzed the composition of the so-called 'lupusband' in pemphigus erythematosus (PE). We found that the lupusband is composed of the desmoglein 1 (Dsg1) ectodomain, and propose that in the skin of our patients, Dsg1 has undergone UV-induced cleavage, releasing the ectodomain which in turn has formed immune complexes with anti-Dsg1 IgG. These immune complexes have subsequently precipitated along the basement membrane zone (BMZ). The skin biopsies available for this study were taken from pemphigus foliaceus (PF) patients that had acquired a PE phenotype after UV-treatment. The question therefore arises if these patients are comparable to idiosyncratic PE patients who have not undergone UV treatment. Studies that analyze the skin of idiosyncratic PE patients would be needed to confirm the BMZ composition in PE.

One could question if the idiosyncratic PE patient exists, as it is also possible that the PE phenotype always arises from an exogenous trigger, but that this exogenous trigger is not always recognized. And if so, is this trigger always UV, or could it also be due to other factors that induce apoptosis? Interestingly, a recent case report of a patient that developed an exacerbation of PE due to the use of statins supports the role of apoptosis in the development of PE, as statins also induce pro-apoptotic effects. Therefore, the BMZ depositions in PE may reflect the apoptotic mechanism that has been activated.

Another question is which mechanism is involved in the UV induced exacerbation of acantholysis in our patients. Is it the cleavage of Dsg1 that leads to the disruption of cell-cell adhesion, comparable to the toxin-mediated cleavage seen in staphylococcal scalded skin syndrome? Or does the released Dsg1-ectodomain interfere with adhesive function of desmosomes and desmogleins? This could be by binding to half-desmosomes or non-desmosomal Dsg1, thus sterically hindering the adhesion of the half-desmosome or Dsg with their opposing counterparts. Lastly, it may be the UV induced activation of the apoptosis-signaling pathway that facilitates acantholysis, as has also been suggested by various other in vitro studies and in vivo pemphigus mouse models. Evidence for this in patient skin is however lacking. Future studies using for example an ex-vivo skin explant pemphigus model or in vivo mouse pemphigus models exposed to UV, could be used to support our hypothesis that UV-exposure induces the Dsg1 ectodomain-containing BMZ depositions in PE. Furthermore, exposing these models to caspase- and metalloprotease inhibitors could shed more light on the cellular mechanism involved in the induction of these BMZ depositions and the exacerbation of acantholysis. Future studies to assess the functional properties of a cleaved Dsg1 ectodomain, may be performed using a keratinocyte disassociation assay, whereby cultured keratinocytes are exposed to the Dsg1-ectodomain to determine if this reduces intercellular adhesion.

Recently, the role of UV exposure in PE has been addressed by a recent case-report of a patient with PE that clinically showed exacerbation of her symptoms after UV exposure. A biopsy of UV irradiated skin showed complement depositions along the BMZ, and IgG depositions in an epidermal cell surface (ECS) pattern, but not along the BMZ. This is discrepant with our study, as these authors did not find any IgG along the BMZ. This difference may be due to the shorter interval between the UV exposure and biopsy in this case report, as compared to our patients. The discrepancy may also be due to differences in patient population as, in contrast to our study, the case report may concern a idiosyncratic PE patient.
In chapters 3 and 4 we investigated the value of direct immunofluorescence microscopy (DIF), and an array of serological techniques in the diagnosis of paraneoplastic pemphigus (PNP), and concluded that the detection of anti-envoplakin and -periplakin, and/or -alpha-2-macroglobulin-like 1 (A2ML1) antibodies by immunoprecipitation is most sensitive. However we also proposed that indirect immunofluorescence on rat bladder and immunoblot could be the combination of choice to confirm the diagnosis PNP, due to its equally high sensitivity and specificity and because these techniques are more readily available and easier to perform than immunoprecipitation. Importantly, this combination will not diagnose PNP patients with solitary anti-A2ML1 antibodies. Future studies aimed at developing an A2ML1 enzyme-linked immunosorbent assay (ELISA) may therefore be useful, as this technique would be faster, cheaper and easier than immunoprecipitation. This ELISA would also differentiate between lower-titer TEN patients and higher-titer PNP patients. 

In our PNP population, two patients showed negative IB and IP results for PNP-specific plakins, but positive rat bladder staining. It is unclear which autoantigens are responsible for these stainings, and we cannot rule out that urothelium contains yet undiscovered PNP autoantigens. In future studies urothelial extracts could be employed in immunoprecipitation or immunoblotting assays to answer this question. Previous studies have suggested that a subset of seronegative PNP patients exists. This subset may be missed by the techniques used in chapters 3 and 4. This seronegativity may due to therapy or natural disease course as suggested by our study. Furthermore, a T-cell type or 'lichenoid' PNP subset has been described in the literature and a portion of these patients have been reported to be seronegative for autoantibodies. Up to date, five such seronegative lichenoid PNP cases have been published, although not all of these cases were tested by the combination of rat bladder IIF and IB or IP, and some of the patients had received immunosuppressive therapy prior to testing. Despite these limitations, it is plausible that a truly seronegative PNP population exists and therefore a further adjustment of the diagnostic algorithm for PNP is in place, to include this subset of seronegative T-cell type PNP patients (figure 1). Diagnostic markers for this subset may include clinical and histological findings of an interface or lichenoid dermatitis, or specific serum markers associated with increased cellular immunity. Interleukin 6 (Il-6) has also been proposed as a putative serum marker, as this interleukin has been found to be elevated in PNP compared to other autoimmune diseases. However, it remains to be seen if IL-6 is specific enough for PNP, as it has also been reported to be elevated in patients with toxic epidermal necrolysis and graft versus host disease, conditions that may all mimic PNP. In addition it is unknown if the autoantibody-negative subset of PNP patients also has increased IL-6 serum levels. Therefore, further studies are needed to identify diagnostic markers for this subset of PNP patients.

In chapter 6 we investigated the ultrastructure of Dsg1 clusters in PF skin. We found that these Dsg1 clusters are double membrane structures, of which there seem to be two types. First, in the lower epidermis these structures were continuous finger-like projections of one cell into its neighbour. Secondly, in the upper epidermis these double membrane structures disappear and instead large intracellular double membrane vesicles are found. Importantly, we found that the
membrane-bound Dsg1 in these projections and vesicles was located outside of desmosomes, while desmosomes in PF skin were depleted of Dsg1. Our findings confirm the results of Oktarina et al., who showed by immunofluorescence microscopy that in PF and pemphigus vulgaris (PV) patient skin, Dsg clusters do not contain all desmosomal components but are composed of only IgG, Dsg and plakoglobin. Desmosomal depletion may be due to the IgG mediated inhibition of desmosomal assembly by binding to non-desmosomal Dsg1, or due to the induction of desmosomal disassembly via binding to desmosomal Dsg1, which is then expelled out of desmosomes. Our findings could fit with both options. Either way, the binding of IgG to Dsg1 results in depletion of desmosomes. Although lesional skin biopsies were not available for our study, it is expected that in acantholytic skin, further desmosomal depletion results in a reduced number and even absence of desmosomes and subsequently acantholysis, as also suggested by van der Wier et al. and Sokol et al. (www.nanotomy.org). Data from Bedane et al. questions the existence of non-desmosomal Dsg1. In their study, they used direct and indirect immunoelectron microscopy (IEM) to investigate the ultrastructural binding sites of IgG in PV and PF skin. They found that for PV, IgG binds to desmosomes but also to non-desmosomal parts of the keratinocyte cell membrane in patient skin. However, for PF, this study showed the binding of IgG to be restricted to desmosomes. This does not support our observations. This difference may be explained by differences in fixation and visualization techniques. It may be that some IgG epitopes were damaged during their fixation steps, or that their one-step peroxidase mediated visualization was less effective than our two-step nano-gold staining, resulting in lower sensitivity to detect bound IgG. Indeed, in 3 of their 7 studied PF patients they found no in vivo bound IgG at all, while of the 4 patients with IgG depositions they reported that in some the depositions were restricted to the upper part of the epidermis. We, by immunofluorescence microscopy in forty biopsies, have never seen that IgG deposition was lacking in any of the lower layers. (Dr. H.H. Pas, personal communications) Furthermore, their study included subclinical acantholytic skin, in which interdesmosomal Dsg1 may already has been depleted. In addition, the focal density of non-desmosomal Dsg1 in healthy skin may be lower than in PF skin due to the lack of clustering, and this would explain why non-desmosomal bound IgG could not be detected in normal skin by indirect IEM. The double-membrane interdigitating structures described in chapter 6, could have been formed as a result of crosslinking of opposite Dsg1 molecules. Atomic force experiments have shown that polyclonal PF IgG is able to increase the binding force between two opposing Dsg1 molecules, in a cell-free system. This effect was not observed when using PF Fab fragments or a monoclonal anti-Dsg1 antibody instead of IgG. In line with this, pemphigus patient IgG, but not Fab fragments induces Dsg clustering in an ex vivo skin explant pemphigus model. These findings suggest that polyclonal PF IgG has cross-linking activities, and in PF patient skin this may result in the cross-linkage of two opposing cell membranes, thereby forming double membrane structures. The projections and invaginations of these double membranes between neighbouring cells may reflect attempts of internalization and degradation of this surface bound IgG, however the cellular mechanisms of how projections and invaginations form are at still completely unknown. Possible mechanisms involved in the formation of these projections may be changes in the actin cytoskeleton, endocytosis and the activation of signaling pathways such as that of p38 mitogen.
activated protein kinase (p38MAPK), as has been suggested to occur in cultured keratinocytes and skin exposed to pemphigus vulgaris IgG. Interestingly, inhibition of p38MAPK prevented the formation of Dsg3 clusters in cultured keratinocytes exposed to polyclonal PV IgG. In addition this pathway has been linked to Dsg3 internalization and cytoskeletal reorganization. This signaling pathway may also play a role in PF, as suggested by Lee et al., who found an upregulation of p38MAPK in mouse skin exposed to PF IgG, and the prevention of blister formation when these mice were exposed to a p38MAPK inhibitor. Interestingly, Dsg1 clustering may vary between different patients and in different stages of disease (chapter 5 this thesis). Future studies investigating the activation of p38MAPK signaling in PF patient skin and correlating this to the extent of clustering could provide more insight in the cellular mechanisms involved in cluster formation.

The formation of these Dsg clusters and projections is probably not a prerequisite for acantholysis, as monovalent anti-Dsg Fab fragments, and monoclonal anti-Dsg antibodies induce acantholysis in the absence of these clusters. Also, the biopsies used in our study, and those in chapter 5 (see below) were not lesional but nevertheless had an abundance of Dsg1 clusters. The question therefore still remains, which cellular mechanisms actually lead to acantholysis in clustered pemphigus patient skin compared to non-clustered acantholytic Fab-exposed skin. Previous studies have suggested that steric hindrance of Dsg trans-interaction is the main mechanism mediating acantholysis in keratinocytes without Dsg clusters and exposed to monoclonal anti-Dsg antibodies while cell signaling pathways would mediate acantholysis in keratinocytes with Dsg clusters and exposed to PV or PF IgG. Our data and that of others favors the ‘melting’ desmosomes theory, thus the total disappearance of desmosomes as observed in the acantholytic PF biopsy (www.nanotomy.org). However, this desmosomal ‘melting’ probably also involves cell-signaling pathways, which is not mutually exclusive to the desmosome ‘melting’ hypothesis.

Besides investigating PF skin, the investigation of clinically unaffected tissue such as PF mucosa, can also provide us with important clues on determinants of pathogenicity. In chapter 5 we found that the desmosomes in PF mucosa are smaller than those of normal human mucosa, and that this is related to Dsg1 clustering. Furthermore our data suggests that Dsg1 clustering correlates to intercellular widening, which may be caused by PF IgG that interferes with the adhesive role of non-desmosomal Dsg1. This is in line with previous reports on widening in PF and mcPV skin and in cultured keratinocytes exposed to PF IgG. We propose that the reduced desmosomal size in PF mucosa is due to Dsg1 depletion, as we have shown in chapter 6 to occur in PF skin. Future studies using immuno-electron microscopy to quantify Dsg1 content in desmosomes of PF mucosa are needed to prove desmosomal Dsg1 depletion in this tissue. In addition, future studies investigating the further composition and ultrastructure of the Dsg1 clusters should reveal if, like in PF skin, these mucosal clusters are double-membraned projections that contain non-desmosomal Dsg1 that has been rendered unavailable for incorporation into desmosomes by PF IgG. Interestingly, our findings contrast
with van der Wier et al.’s findings of clinically unaffected skin of mucosal dominant PV patients in which, despite clustering of Dsg3, desmosome size was found to be normal. This could be because Dsg1 is more important in determining desmosome size than Dsg3, as Dsg1 binds more plakoglobin than does Dsg3, and thus depletion of Dsg1 would result in the exclusion of larger desmosomal protein complexes than the depletion of Dsg3.

Recently, studies of PF patient skin have shown the disruption of plakoglobin distribution, co-localizing with Dsg1 in clusters along the keratinocyte cell surfaces. Plakophilin-3 was not disrupted, but plakophilin-1 was not investigated. Plakoglobin and plakophilin-1 are important regulators of desmosomal assembly, and disruption of their distribution may affect desmosomal size. It is unknown if plakoglobin and plakophilin-1 distribution is disrupted in PF mucosa. Therefore, future studies investigating these proteins in PF mucosa may provide further insights in why desmosomal size is altered in this tissue.

We showed that in PF mucosa, even the N-PF patients showed significantly smaller desmosomes than the control mucosa. In contrast, van der Wier et al showed that only the Nikolsky positive (N+) PF patients have smaller desmosomes in their skin, while the N-PF patients have normal sized desmosomes. Apparently, mucosa is more sensitive to the depletive effects of PF IgG than skin. It is unknown what accounts for this difference. It could be due to the differential desmosomal composition between the two tissues. Previously it has been show that, compared to skin, mucosal desmosomes contain less Dsg1 and therefore these desmosomes may be quicker depleted of Dsg1 by PF IgG. It is also possible that there is relatively more interdesmosomal Dsg1 in mucosa than in skin. Mucosal tissue has a higher turnover rate than skin, and therefore mucosal desmosomes may also have a higher turnover rate. A larger non-desmosomal pool of Dsg1 in the mucosal cell membrane would facilitate this fast desmosomal renewal. Therefore, if PF IgG mainly exerts its effects through the binding to non-desmosomal Dsg1, mucosa would be more susceptible to the initial effects of PF IgG than skin. Future immunoelectron microscopy studies investigating the quantity of desmosomal and non-desmosomal Dsg1 in skin compared to mucosa would shed more light on this.

The composition of depleted desmosomes of suprabasal mucosal cells in PF mucosa resembles that of the basal cells, whereby the predominant Dsg is Dsg3. Does this shift in Dsg1/Dsg3 balance also have phenotypical consequences? For example suprabasal cells in PF mucosa might adopt basal cell characteristics such as higher proliferation and intercellular widening as these changes have previously been found in the skin of transgenic mice that over-expressed Dsg3 in suprabasal cells. Future studies investigating the proliferative activity of the mucosa of PF patients would therefore be interesting to understand possible non-adhesive functions of desmogleins.

Besides desmosome size and number, changes in desmosomal composition and adhesional state may have consequences for epidermal and epithelial integrity. A marker for hyperadhesive desmosomes has been proposed to be the presence of a dense midline. It is currently unknown if this hyperadhesion is negatively affected in pemphigus. Future electron microscopy studies investigating the influence of pemphigus IgG on desmosomal hyperadhesion, by looking at the presence or absence of a dense midline would therefore also be of interest.
Besides providing new insights on desmosomal depletion, this thesis also touches on the role of signaling pathways in pemphigus pathogenesis. In chapter 7 we failed to observe a protective effect in the diseased oral mucosa of three pemphigus patients after two weeks of topical sirolimus treatment. Sirolimus has been used as inhibitor of the mammalian target of rapamycin (mTOR) signaling pathway to treat various diseases. Previous reports have suggested that its topical application is effective in improving psoriasis 29 and oral erosive lichen planus 30 whereby local immunosuppression is thought to be the main mode of action. Pretel et al. used sirolimus in an experimental pemphigus mouse model, to show that mTOR signaling precedes acantholysis. 31 This effectivity of sirolimus in preventing acantholysis has been proposed to occur at the local level of the keratinocyte, instead of a systemic immunosuppressive level. 32 The lack of effects observed in our three patients due to the inadequate penetration of the topical solution in the mucosal epithelium can have various reasons. Sirolimus is highly lipophilic and our patients were only exposed to sirolimus shortly, so exposure time could have been a problem. It is also possible that mTOR signaling is not involved in mediating acantholysis in humans. This thesis does not provide an answer to these questions. Actual evidence of increased mTOR signaling in human pemphigus skin and mucosa is lacking today. Therefore, quantitative studies that investigate whether or not mTOR signaling is upregulated in pemphigus patient tissue should be performed in the future. As mTOR signaling may also be a dynamic transient event, comparable to that of p38MAPK 22, additional experimental models using human skin, such as human skin explant models or mouse models with grafted human skin, can be used to monitor the effects of pemphigus IgG on mTOR signaling over time. In such models sirolimus can be used to assess possible role of mTOR signaling in pemphigus acantholysis in human tissue.
Figure 1. Revised algorithm for the laboratory diagnosis of PNP.
References


8. Summary, discussion and future perspectives
