Acantholysis in pemphigus
van der Wier, Gerda

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Introduction

G. van der Wier

Centre for Blistering Diseases,
Department of Dermatology,
University of Groningen,
University Medical Centre Groningen,
Groningen, The Netherlands
The term pemphigus is derived from the Greek word pemphix, which means blister. Pemphigus is a group of chronic mucocutaneous blistering diseases caused by autoantibodies directed against the desmosomal cadherins desmoglein (Dsg)1 and/or Dsg3. Pemphigus is characterized macroscopically by blisters and erosions of the skin and/or mucous membranes and microscopically by acantholysis.\textsuperscript{1,2}

**Epidemiology**
Pemphigus has a worldwide incidence of 0.76–5 cases per 1,000,000 per year.\textsuperscript{1} The incidence of the different subtypes of pemphigus varies from country to country. Pemphigus vulgaris (PV) and the sporadic form of pemphigus foliaceus (PF) are most common in Europe and the USA. The incidence of PF in these countries is about a fifth to a tenth of that of PV.\textsuperscript{3} Endemic PF is frequently diagnosed in rural areas of Brazil and other underdeveloped areas of the world.\textsuperscript{4} Pemphigus affects all races but is diagnosed more often in people of Ashkenazi Jewish, Greek and Indian descent.\textsuperscript{5} The expression of various HLA alleles may play a role in the epidemiology of pemphigus.\textsuperscript{2} PV is associated with HLA–DRB1*0402 in Ashkenazi Jews and DRB1*1401/1404 and DQB1*0503 in non–Jewish patients of European or Asian descent.\textsuperscript{3–7} Sporadic forms of PF are associated with HLA DRB1*0102 and 0404\textsuperscript{3,4}, while endemic PF is associated with HLA DRB1*0102, 0404, 1402, and 1406.\textsuperscript{8} The mean age of onset of the disease is approximately 50–60 years.\textsuperscript{1} However, pemphigus is also described in adolescents, children and the elderly.\textsuperscript{2} The endemic form of PF mostly affects teenagers and individuals in their 20s.\textsuperscript{4} The male–to–female ratio is almost equal, however in puberty, girls are more often affected than boys.\textsuperscript{2}

**Pemphigus subtypes**
Pemphigus can be divided into two major forms, based on the level of the blistering in the epidermis: PF and PV. Other members of the pemphigus group include paraneoplastic pemphigus (PNP), drug induced pemphigus and IgA–pemhigus (Table 1).
### Pemphigus subtypes

<table>
<thead>
<tr>
<th><strong>Pemphigus foliaceus (PF)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>– sporadic PF (Cazenave type)</td>
<td></td>
</tr>
<tr>
<td>– endemic PF (fogo selvagem, Brazilian pemphigus)</td>
<td></td>
</tr>
<tr>
<td>– pemphigus erythematosus (Senear–Usher syndrome)</td>
<td></td>
</tr>
<tr>
<td>– pemphigus herpetiformis</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Pemphigus vulgaris (PV)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>– mucosal-dominant PV</td>
<td></td>
</tr>
<tr>
<td>– mucocutaneous PV</td>
<td></td>
</tr>
<tr>
<td>– pemphigus vegetans</td>
<td></td>
</tr>
<tr>
<td>– Neumann type</td>
<td></td>
</tr>
<tr>
<td>– Hallopeau type</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>IgA–pemphigus</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>– subcorneal pustular dermatosis (SPD)</td>
<td></td>
</tr>
<tr>
<td>– intraepidermal neutrophilic (IEN) IgA dermatosis</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Paraneoplastic pemphigus (PNP)</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug–induced pemphigus</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 – Pemphigus subtypes

**Pemphigus foliaceus**
The superficial forms of pemphigus are grouped under pemphigus foliaceus, including sporadic PF (Cazenave type), endemic PF, pemphigus erythematosus, and pemphigus herpetiformis.

**Sporadic pemphigus foliaceus**
Pemphigus foliaceus was described for the first time in 1844 by Cazenave. Patients with PF develop multiple, pruritic, scaling, nummular lesions. The lesions usually have a seborrhoic distribution and prefer the face, scalp and upper trunk (Figure 1a). The lesions are present on healthy looking skin and have been described as cornflakes or puffed pastry (Figure 1b). The level of blistering in PF is so superficial (subcorneal) that intact blisters are rarely present and only the resultant scaling and crust are seen. The disease often starts with a few transient lesions which may be mistaken for impetigo, seborrhoic dermatitis or actinic keratoses. The disease
may stay localized for years or it may progress rapidly. In some cases the disease generalizes and evolves into an erythroderma. The Nikolsky sign is positive. It is extremely rare for patients with PF to develop mucosal involvement.1,2,4 Generally patients with PF are not severely ill, but they can complain of itching, burning and pain of the skin lesions.

**Pemphigus erythematosus**

Pemphigus erythematosus (PE) is also called the Senear–Usher syndrome, according to the names of the authors who first described this variant.9 The disease is localized on the face and is characterized by erythematous, scaly, hyperkeratotic or crusted lesions often in a butterfly distribution that resembles lupus erythematosus2,4 (Figure 1c). In addition to the intercellular deposits of antibodies that are present in all forms of pemphigus there are often granular deposits of immunoglobulin and/or complement present at the dermal–epidermal junction.2,4

**Endemic pemphigus foliaceus (Fogo selvagem, Brazilian PF)**

This form of pemphigus occurs endemically in Brazil and in certain rural areas of the world and is clinically, histologically and immunopathologically indistinguishable from PE.2,4

**Pemphigus herpetiformis**

Pemphigus herpetiformis (PH) is one of the less common forms of pemphigus, which was described for the first time by Jablonska in 1975.10 Most patients have a variant of PF and the remainder may have a variant of PV.1 Skin lesions are similar to dermatitis herpetiformis, including erythematous, vesicular, bullous or papular lesions, which are often grouped and are severely pruritic. PH often presents with annular lesions, probably resulting from centrifugal spread of inflammatory processes. PH resembles other autoimmune bullous diseases like linear IgA dermatosis and IgA pemphigus. The affected sites are the trunk and proximal extremities, while mucous membranes are spared in most cases.11 PH is histologically characterized by eosinophilic spongiosis, subcorneal pustules and minimal acantholysis.10 Direct immunofluorescence (DIF) shows autoantibodies directed against Dsg1 in most cases and against Dsg3 in the rest.12

**Pemphigus vulgaris**

The deep forms of pemphigus are pemphigus vulgaris and its variant pemphigus vegetans.
**Pemphigus vulgaris**

Almost all patients with PV have painful erosions of the oral mucosa. Patients with mucocutaneous PV (more than half of the patients) also develop blisters and cutaneous erosions. In mucosal-dominant PV, there are only oral lesions present (Figure 1e).

The mucous membranes are often affected first. The blisters rupture easily and lead to erosions in the oral cavity. Although the erosions may be seen anywhere in the oral cavity, the most common sites are the buccal mucosa, palate and tongue.\(^1,2,4\) The erosions extend peripherally and may spread to involve the pharynx and larynx with difficulty in eating and drinking and hoarseness of the voice.\(^1,2\) Also the nasal cavity can be involved.\(^4\) Involvement of other mucosal surfaces can be present including the conjunctiva, oesophagus, vulva, cervix, urethra and rectal mucosa.\(^1,2,4\)

After weeks to months, the disease progresses with lesions appearing on the skin (Figure 1d). Sometimes, skin lesions are the first sign of the disease.\(^4\) The first lesion of the skin is a blister that is filled with a clear fluid, on a normal or erythematous skin, which breaks easily resulting in painful erosions. The fluid within the blisters may become hemorrhagic, turbid or even seropurulent.\(^1\) When there is no treatment, the erosions enlarge to form large denuded areas, which may become crusted and may lead to complications as infections or metabolic disturbances or both. Before systemic corticosteroids became available, about 75% of patients who developed PV died within a year.\(^4\) Blisters may be present anywhere, but the scalp, upper chest and back are most often affected. The face and neck may also be involved. Although relatively rare, periungual and nail involvement must not be missed.\(^2,4\)

A characteristic feature of all forms of active and severe pemphigus is the Nikolsky sign, produced when lateral pressure is applied adjacent to a lesion leading to separation of the epidermis. The lack of cohesion of the skin may also be demonstrated with the bulla–spread phenomenon (Asboe–Hansen sign).

With treatment, the lesions of PV generally heal with crusting followed by re–epithelialisation. There is no scarring, although transient residual hyperpigmentation may be present. This hyperpigmentation may be present for many months. Mild forms of the disease can even regress spontaneously. Most patients with PV eventually enter a phase of complete remission in which they can be maintained lesion–free with minimum doses of corticosteroids or without any therapy. As medications are tapered, flares in disease activity with development of new lesions and itching are not uncommon.
Figure 1 – Clinical features of pemphigus foliaceus (PF) and pemphigus vulgaris (PV). (a) Erosions on the skin in a patient with PF. (b) Puffed pastry like scaling in PF. (c) Erythema and crusts on the face in a patient with pemphigus erythematosus. (d) Extensive erosions on the back of a patient with mucocutaneous PV. (e) Erosions of the oral mucosa in a patient with mucosal-dominant PV. (f) Pemphigus vegetans with pustules in the inguinal region.
Pemphigus vegetans

Pemphigus vegetans is a rare variant of PV in which healing is associated with vegetating plaques on the skin (Figure 1f). Lesions usually appear in intertriginous areas (axillae, groin and inframammary area) and on the scalp or face. Pustules characterize early lesions, but these soon progress to vegetative plaques. The tongue may show cerebriform–like changes.1 Two subtypes are recognized: the papillomatous Neumann type and the pustular Hallopeau type. Because of their location the lesions are generally secondarily infected. The vegetative response may occasionally also be seen in lesions of PV that tend to be resistant to therapy and remain for long periods of time in one location.1

Desmosomes

Desmosomes are intercellular junctions that are present in epithelia and some nonepithelial tissues such as cardiac muscle and the meninges. They are present in large numbers in tissues that withstand mechanical stress such as the epidermis. Their most important function is to provide strong intercellular adhesion and to link the intermediate filament cytoskeleton into a tissue–wide scaffolding giving strength to tissues.13

History

In 1864, Giulio Bizzozero, described desmosomes for the first time.14,15 He examined the stratum spinosum by light microscopy and noted small nodules at the cell–cell contact points, which were named “nodes of Bizzozero”.15,16 He interpreted these nodes as contact points between two adjacent separately contributing cells.15 Later, in 1920, the term desmosome was introduced by Josef Schaffer. This term comes from the Greek word desmos, meaning “bond” and “some” meaning “body”.15–17 In that time Schaffer and others, thought that desmosomes were intercellular bridges filled with cytoplasm.14 Ninety years later, electron microscopy made it possible for Porter to confirm Bizzozero’s observation that desmosomes are contact points between adjacent cells.14,15 Improvement of electron microscopic techniques showed the lack of continuity between adjacent cells and electron–dense plates separated by a light space, with fibers extending from the membrane to the inside of the cell.15

In 1958, George Odland described these electron dense plates for the first time. Odland reinterpreted the nodes of ‘Bizzozero’ and described that they had seven layers with different densities.15 The layers occupied a space of 350 Å and had fibrils terminating at the attachment
At the end of the millennium an international group of cell biologists reported the molecular map of the desmosome.\textsuperscript{18}

**Ultrastructure**

Desmosomes are oval shaped junctions with a diameter of about 0.2–0.5 µm, but they may be as small as 0.1 µm or as large as several micrometers.\textsuperscript{14,16} The desmosomes are composed of two electron–dense plaques, one in each of the two adjacent cells, separated by an intercellular space of 24–30nm (Figure 2).\textsuperscript{14,16} The intercellular space is penetrable by water and ions and filled with electron–dense material, the desmoglea or extracellular core domain (ECD).\textsuperscript{13,16} In mature desmosomes a distinct line can be seen in the middle, which is named the dense midline (DM).\textsuperscript{16} The plaques are 15–20 nm in size and are separated by a 10– to 20–nm gap.\textsuperscript{16} Thus, the total
thickness of the desmosomal plaque is 40–50 nm and the total thickness of a desmosome of the order of 130 nm. Within the plaques, an outer dense plaque (ODP) that is adjacent to the plasma membrane can be distinguished from a less dense inner plaque (IDP) facing the cytoplasm. The inner dense plaque is connected to the intermediate filaments.

**Composition of the desmosome**
Desmosomes are composed of members of at least three protein families: 1) cadherins, 2) armadillo proteins, and 3) plakins. Transmembrane members of the cadherin family, the desmogleins and desmocollins, form the intercellular adhesive interface. Proteins from the armadillo and plakin family form the plaques. The cytoplasmic tail of desmogleins and desmocollins binds with plakoglobin (PG) which then binds to desmoplakin. Desmoplakin is connected to the intermediate filament cytoskeleton. The interactions are stabilized laterally by plakophilin (Figure 3).

**Figure 3** – Schematic drawing of a desmosome and an adherens junction. The autoantigens desmoglein 1 and desmoglein 3 are depicted in blue. Drawing by M.F. Jonkman.
Cadherins
The desmosomal members of the cadherin family are single-pass transmembrane glycoproteins, which provide adhesion in a Ca²⁺-dependent manner. In humans there are seven desmosomal cadherins, four desmogleins (Dsg1–4) and three desmocollins (Dsc 1–3). The Dsc genes give rise to two isoforms, Dsc a and b.

The amino-terminal extracellular domain of desmosomal cadherins consists of four cadherin repeats (EC1–4) which are each about 110 amino acids followed by a membrane-proximal domain (EC5) or extracellular anchor (EA) domain. Within the cell, both desmocollin ‘a’ and ‘b’ proteins have an intracellular anchor (IA) domain but only ‘a’ form proteins have an intracellular cadherin-like sequence (ICS) domain, which provides a binding site for other desmosomal components such as PG. The b isoform is therefore unable to bind PG but binds with plakophilin 3 instead.

Other domains in the cytoplasmic tails of desmoglein are the intracellular proline-rich linker (IPL) domain, a repeat unit domain (RUD) and a glycine rich desmoglein terminal domain (DTD).

Simple epithelia express only Dsg2 and Dsc2. Stratified epithelia such as the epidermis, express Dsc1, Dsc3, Dsg1 and Dsg3. Low levels of Dsg2/Dsc2 are present in the basal layers and small amounts of Dsg4 are expressed in the granular and cornified layers.

Most desmosomes exhibit calcium-independent adhesion and are “hyperadhesive” desmosomes according to the hypothesis of Garrod. Hyperadhesion seems to be associated with an ordered arrangement of the extracellular domains of the desmosomal cadherins, which results in the intercellular midline visible by electron microscopy. Protein kinase C downregulates hyperadhesion.

Armadillo proteins
Armadillo family members are characterised by the presence of a central domain containing a variable number of 42 amino acid repeats (arm repeats). Armadillo proteins found in desmosomes are PG and the plakophilins, of which there are three (PKP1–3).

Plakoglobin is the only desmosomal component which is also found in adherens junctions.

Plakoglobin contains 12 arm repeats. Plakoglobin binds to the intracellular ICS domain of desmogleins and desmocollins with its first three armadillo repeat domains. Plakoglobin also interacts with other desmosomal components like desmoplakin and plakophilins and with cytokeratin filaments. Plakoglobin may be involved in regulating lateral association between
other desmosomal components and desmosome size.19 Besides having a structural role in desmosomes and adherens junctions, PG plays a role in intracellular signal transduction14,19 and there is evidence that PG is important in regulating cross-talk between desmosomes and adherens junctions.19

The plakophilins contain 9 arm repeats with a flexible insert between repeats 5 and 6 that introduces a major bend in the overall structure.19 There are two isoforms of plakophilins 1 and 2, a shorter a form and a longer b form, which are formed by alternative splicing.14,19 Plakophilins can directly interact with all other desmosomal components and the intermediate filaments through the aminoterminal head domain.14

Plakophilins 1–3 are expressed in the epidermis where they have differentiation-specific patterns of expression.19 All three plakophilins can be present in both desmosomes and the nucleus. PKP1b is only localized in the nucleus.14,19

Plakophilin 1 is responsible for bringing desmosomal components to the cell membrane and increasing size and number of desmosomes and is therefore an important protein which induces desmosome assembly.14 Besides its function in the regulation of desmosome assembly, plakophilins may also regulate signalling mechanisms, both at cell borders as well as in the nucleus.14

Plakins

Proteins from the plakin family form the link between the cytoskeleton and cell–cell or cell–matrix contacts. There are two splice variants of desmoplakin. Desmoplakin is a very important component of the desmosomal plaque and therefore is the most important protein of the plakin family. Other members such as plectin, envoplakin en periplakin are also found in desmosomes, but it is not clear how important they are for the structure and function of desmosomes.14 Desmoplakin is composed of three parts, a globular head or plakin domain, a coiled–coil rod domain and a tail domain.19 The globular head is an important region for protein–protein interactions.19 Desmoplakin can interact with all other desmosomal proteins like PG, plakophilins and Dsc1a.14 The central coiled–coil rod domain is important for dimerization.14,19 The C-terminal tail domain consists of three plakin repeat domains (PRDs) named A, B and C,19 which serve as linkers for different types of intermediate filaments14 Desmoplakin is the main linker protein between the cadherin–plakoglobin complex and the intermediate filaments.14 Experiments have shown that it may also be involved in regulating microtubule organisation.19
Adherens junctions

Adherens junctions are found at the lateral surface of epithelial cells as well as sporadically along the surface of stratified squamous epithelial cells.\textsuperscript{21} Adherens junctions are composed of: 1) classical cadherins (such as E–, N– and P–cadherin), 2) armadillo proteins (such as β catenin and PG) and 3) cytoskeleton adaptor proteins (such as a catenin) (Figure 3).\textsuperscript{22} E–cadherin and P–cadherin are classical cadherins expressed in the epidermis. E–cadherin is expressed in all layers of the epidermis, whereas P–cadherin is only expressed in the basal cell layer. Adherens junctions are necessary for desmosome formation. PG and E–cadherin recruit plakophilin 3 a component of desmosomes to the cell border to start desmosome formation. Cross–talk exists between adherens junctions and desmosomes for the regulation of cell–cell adhesion in keratinocytes.\textsuperscript{23} Vasioukhin et al. describe the zipperfunction of adherence junctions. Neighbouring cells form filopodia which slide along each other and project into the opposing cell’s membrane. Embedded tips of filopodia are stabilized by puncta, which are transmembrane clusters of adherence junction proteins. Once initial filopodia embed, this anchorage seems to enhance the probability that additional filopodia will make functional contact, extending the zone of contact between two neighbouring cells. Desmosomes then form in the flanking regions of contact that are brought together by filopodia embedding.\textsuperscript{24}

Pathogenesis of pemphigus

Desmogleins as pemphigus antigens

In 1964 Beutner and Jordan observed circulating antibodies directed against the cell surface of keratinocytes in the sera of patients with PV.\textsuperscript{25} Later it was demonstrated that autoantibodies in pemphigus are pathogenic and induce blister formation in skin organ culture systems\textsuperscript{26} and in neonatal mice.\textsuperscript{27} In 1982 Stanley et al. characterized the PV antigen at the molecular level by immunoprecipitation using cultured keratinocytes extracts as a substrate. All the PV sera identified a glycosylated 130 kDa glycoprotein.\textsuperscript{28} Two years later the PF antigen was characterized using immunoblot analyses of normal human epidermal extracts and demonstrated that about one–third of the PF sera identified a polypeptide of about 160 kDa. Later they showed that all PF sera bind to the 160 kDa glycoprotein.\textsuperscript{29} Koch et al. isolated a cDNA clone for Dsg1 from bovine muzzle epithelium and soon afterwards cDNA for human Dsg1 from human stratified squamous epithelia or human cultured keratinocytes was isolated.\textsuperscript{30} In 1991 Amagai et al. isolated a cDNA clone for the PV antigen by immunoscreening a human keratinocytes expression library with autoantibodies prepared
from the sera of patients with PV. Analysis of the deduced amino acid sequences of the cDNA clones revealed the nature of pemphigus antigens. Both Dsg1 and PV antigen are cadherin type adhesion molecules that occur in desmosomes. PV antigen is termed Dsg3 because PV antigen is more closely related to Dsg1 than to other cadherins.\textsuperscript{31}

**Non desmoglein proteins as possible pemphigus antigens**

Several studies suggested a role for target antigens other than desmogleins in pemphigus. Bedane et al. compared the localization of immune deposits in patients with PV and PF by both direct and indirect immunoelectron microscopy. They showed that in PV immune deposits were situated both on the extracellular part of the desmosomes and along large portions of the keratinocytes without desmosomal structures in most of the studied samples, while in PF the immune deposits were located on the extracellular part of desmosomes only. This led to the idea that the target antigen in PV is not always a component of the desmosomes but can also be a component of other focal adhesions.\textsuperscript{32} Later Nguyen et al. injected Dsg3–lacking mice with PV IgG that did not cross react with Dsg1 and observed suprabasal acantholysis and staining in a fishnet–like pattern. This led to the hypothesis that that mucocutaneous lesions in pemphigus could be caused by non desmoglein antibodies.\textsuperscript{33} Studies in mouse models showed that loss of Dsc3 leads to intra epidermal blistering.\textsuperscript{34} IgG directed against Dsc3 purified from Dsc3–reactive sera causes loss of adhesion of epidermal keratinocytes \textit{in vitro}.\textsuperscript{35} Muller et al. studied the presence of IgG and IgA autoantibodies against Dsc1, Dsc2 and Dsc3 in a cohort of patients with bullous diseases and found that IgG and IgA reactivity against Dsc is restricted to cases of PNP, IgA pemphigus and atypical pemphigus.\textsuperscript{36} Besides being present in PNP, desmoplakin antibodies have also been found in PV,\textsuperscript{37} however these desmoplakin antibodies are probably caused by an epitope–spreading phenomenon. Also antibodies directed against E–cadherin have been described in PF and PV although probably most of these antibodies cross react with Dsg1.\textsuperscript{38}

**Theories on the pathomechanism**

The exact mechanism by which pemphigus IgG induces acantholysis has been a subject of debate since the discovery of pemphigus autoantibodies by Beutner and Jordan. Since then acantholysis has been explained by several theories: 1) steric hindrance, 2) deranged cell signalling, 3) impairment of desmosome assembly and increased desmosome disassembly
Steric hindrance
One of the first concepts to explain acantholysis in pemphigus was “steric hindrance”. This theory is based on the idea that there is direct interference of pemphigus IgG with the amino-terminal extracellular domain of desmogleins which form the trans-adhesive interface between keratinocytes. This concept is supported by several observations. Sekiguchi et al. described that the dominant autoimmune epitopes recognized by pemphigus antibodies are located in the amino-terminal adhesive region of desmogleins.³⁹ Later it was shown that monoclonal antibodies directed against the amino-terminal adhesive region of Dsg3 induce a phenotype similar to mucosal-dominant PV in a mouse model.⁴⁰ By electron microscopy split desmosomes were shown in the lesional mucosa of a PV mouse model⁴¹ and also in lesional skin of patients with mucocutaneous PV, mucosal-dominant PV and PF.⁴² Heupel et al. showed by using atomic force microscopy, that PV-IgG directly interferes with homophilic Dsg3.⁴³ However, PF-IgG did not interfere with homophilic Dsg1 transinteraction.⁴³ Later, the same group showed that PF IgG causes dissociation of Dsg1 containing junctions without blocking Dsg1 transinteraction.⁴⁴

Cell signalling
Signalling pathways involving p38MAPK, RhoA, PKC, PG and c-myc have been shown to play a role in the pathogenesis of pemphigus. Also the pathways leading to apoptosis have been implicated to play a role in acantholysis.

P38 MAPK
There are three subfamilies of the mitogen activated protein kinases (MAPK): 1) p38, 2) ERK (extracellular signal-regulated kinase) and 3) JNK (c-jun amino-terminal kinase). The p38 MAPK family members can be activated by environmental stress and regulate the expression of inflammatory cytokines.²¹ Berkowitz et al. observed that p38MAPK and heat shock protein 27 (HSP27) were rapidly phosphorylated after PV IgG binding to Dsg3 in human keratinocyte cell cultures. Inhibition of p38MAPK activity prevented PV IgG-induced HSP27 phosphorylation, keratin filament retraction and actin reorganization.⁴⁵ Later they also showed phosphorylation of both p38MAPK and HSP25 (murine HSP27 homolog) in the skin of PF IgG-treated mice and that p38MAPK inhibitors prevent both PF and PV blistering in a mouse model system.⁴⁶,⁴⁷ The observations made in human keratinocyte cell cultures and mouse models were enforced by the finding of increased phosphorylation of p38MAPK and HSP27 in PF and PV patient skin.⁴⁸ Jolly et al. later found that
p38 MAPK signalling and Dsg3–internalization are linked, as cell surface Dsg3 internalization and depletion from both detergent soluble and detergent–insoluble fractions were blocked by the p38 MAPK inhibitor.49 However, it is thought that p38 MAPK activation is probably secondary to the loss of intercellular adhesion. Mao et al. showed that PV monoclonal antibodies that do not dissociate keratinocytes because of compensation by Dsg1, do not activate p38. Whereas, the same monoclonal antibodies in combination with exfoliative toxin to inactivate Dsg1 but not exfoliative toxine alone activate p38 MAPK. Also mice with a targeted deletion of p38alfa in the epidermis show loss of intercellular adhesion after passive transfer of PV monoclonal antibodies. However, p38MAPK may function downstream to induce blistering through Dsg3 endocytosis.50

**RhoA**
Rho, Rac and Cdc42 are some of the best known members of the family of over 20 known Rho small GTPases, which regulate mitosis, cytoskeletal reorganization, cell polarity, cell cycle regulation, morphogenesis and cell migration. Rho family GTPases affect adherens junction assembly and disassembly through various pathways that regulate clustering of cadherin receptors on the cell surface, actin recruitment to junctions and endocytosis. Both Rho and Rac are required for the formation of adherens junctions with Rac localizing to new areas of intercellular contact.21 Waschke et al. suggested that PV IgG and PF IgG induce skin blistering by interference with RhoA signalling.51

**Plakoglobin**
A possible role for cell signalling involving PG was suggested from the observation that in keratinocytes cultures from PG knockout mice incubated with PV IgG no keratin retraction and loss of adhesion was observed. However reintroduction of PG into the cells restored the responsiveness to PV IgG.52 The signalling cascade is started by binding of PV antibodies to Dsg3. This temporarily enhances the turnover of PG at the plasmamembrane and results in reduced availability of PG in the nucleus. As a result PG cannot function as a transcriptional repressor of the proto–oncogene c–myc, leading to accumulation of c–myc in the nuclei.53 C–myc inhibitors prevented formation of lesions induced by PV antibodies in the neonatal mouse pemphigus model.53 In patients there is a correlation between disease progression and regression and fluctuations in the levels of c–Myc.54
Apoptosis

Apoptosis is an important general principle to regulate tissue homeostasis and remodeling as well as protection against diseases such as cancer, infection and autoimmune disorders. The two main pathways of apoptosis are extrinsic and intrinsic. In the intrinsic pathway, increased expression of pro-apoptotic proteins as Bax, Bak, and Bim result in the increase of mitochondrial permeability and finally activation of caspases 3 and 6 leads to cell death. In the extrinsic pathway, activation of plasma membrane death receptors leads to reactions and ultimately gives rise to activation of executioner caspases 3 and 6. Eventually this results in the fragmentation of cellular DNA which can be evaluated by the TUNEL staining.

While the morphological characteristics of apoptosis are not observed in hematoxylin and eosin (H&E) stainings of lesional pemphigus skin, evidence has been found for the role of apoptosis in the pathogenesis of pemphigus. Most evidence comes from studies that have shown an increased incidence of apoptosis by staining of TUNEL in perilesional, early lesional and lesional PV skin. Also in cultured keratinocytes incubated with PV serum or IgG, TUNEL reactivity was observed. However, the TUNEL technique in detecting apoptosis is not specific and rarely stains necrotic cells as well. Also, apoptosis is not always associated with DNA fragmentation.

Several studies also investigated proteins involved in the pathways of apoptosis. Lesional PV skin showed expression of Fas ligand, p53, Bax and activation of caspase 8. PV sera showed high levels of Fas ligand, and skin organ cultures incubated with pemphigus IgG showed activation of Fas ligand, Fas receptor and p53, and activation of caspase 1, 3 and 8. However, most studies were done in cultured keratinocytes.

Fewer studies have been done on apoptosis in PF skin, but evidence has been found for apoptosis in lesional PF skin. Acantholysis could be prevented by inhibiting apoptosis by adding anti-Fas ligand, caspase 1 inhibitor and caspase 8 inhibitor. There has been much debate about whether apoptosis is an early phenomenon, which leads to acantholysis or not. Most researchers believe that apoptosis occurs secondary to acantholysis. According to this point of view, acantholysis results in anoikis and later apoptosis occurs. Anoikis is the concept that loss of cell adhesion ends in apoptosis. Some researchers believe that apoptosis is an early phenomenon which is related to acantholysis and probably there is a relationship between them, but it is not necessarily a causal relation. Recently the 'apoptolysis' theory was proposed by Grando et al. that explains the functional relationship between apoptosis and acantholysis, which is mediated by the same set of cell death enzymes.
Assembly and disassembly

PV IgG leads to depletion of non–junctional Dsg3 in cell culture systems as shown by immunoblot and immunofluorescence.50,67–70 Non–junctional Dsg3 is depleted by endocytosis50,69 probably through a dynamin– and clathrin–independent mechanism.71 Immuno–electron microscopy shows that the endocytosed Dsg3 is present in simple clusters without keratine intermediate filament attachment.72 Some researchers found that together with the loss of cell surface Dsg3 there is also a loss of desmoplakin.50 Eventually, the depletion of non–junctional Dsg3 results in prevention of desmosome assembly. Mao et al. treated keratinocytes cultures with pathogenic PV monoclonal antibodies and observed less desmosomes by electron microscopy.50 Depletion of junctional Dsg3 is observed after 24–30 hours of incubation with PV IgG.50,70 In contrast to Aoyama et al., Mao et al. observed not only depletion of junctional Dsg3, but also of Dsc3 and PG. It is suggested therefore that prevention of Dsg3 incorporation into the desmosome leads to destabilization of the desmosomal structure, with subsequent turnover of various desmosomal molecules.50 Expression of exogenous Dsg3 using an adenoviral delivery system prevented the downregulation of Dsg3 and loss of adhesion in PV IgG treated cells.69 Besides binding of PV IgG to non–junctional Dsg3, it might also be possible that PV IgG binds to Dsg3 integrated in the core domain of desmosomes. This leads to exclusion from the desmosomes and possible internalization into endosomes.67 Unfortunately not much research has been done on the effect of anti–Dsg1 antibodies on desmosome assembly and disassembly. Cirillo et al. showed that anti–Dsg1 antibodies induced temporary internalization of Dsg1 and reduced the adhesion strength among keratinocytes.73 However, binding of IgG to Dsg1 did not lead to early depletion from the adhesion complexes but reduced the amount of Dsg1 found in the Triton X–100 soluble pool of proteins.73,74

Diagnosis

The diagnosis of pemphigus is based on three criteria: clinical features, histopathological findings and immunological tests.

Clinical features

As mentioned above the classic clinical findings are multiple flaccid blisters on healthy skin and/or multiple oral ulcers and a positive Nikolsky sign.4
Histological findings

Pemphigus foliaceus

Blisters in PF are localized in the upper layers of the epidermis, within the granular or upper spinous layer (Figure 4a). As the blisters are superficial and fragile, it is often difficult to find an intact lesion for histological examination. Therefore, acantholysis is sometimes difficult to detect, but usually a few acantholytic keratinocytes can be found attached to the roof or floor of the blister. These superficial blisters are histologically indistinguishable from those seen in staphylococcal scalded skin syndrome or bullous impetigo, because Dsg1 is targeted in both diseases. Sometimes the blister cavity contains numerous acute inflammatory cells, particularly neutrophils. The dermis shows a moderate number of inflammatory cells, among which eosinophils are often present.1

Pemphigus vulgaris

The characteristic histological finding in PV is an intraepidermal blister usually just above the basal layer (suprabasilar acantholysis) (Figure 4b). A few rounded–up acantholytic keratinocytes (acanthocytes) as well as clusters of epidermal cells are often seen in the blister cavity. Although the basal cells loose lateral desmosomal contact with adjacent keratinocytes, they maintain attached to the basement membrane via hemidesmosomes, thus giving the appearance of a row of tombstones. The acantholytic process may also involve the hair follicles. The dermal papillary outline is usually maintained and, often, the papillae protrude into the blister cavity. The blister cavity may contain a few inflammatory cells, notably eosinophils, and in the dermis there is a moderate perivascular mononuclear cell infiltrate with eosinophils.1

Figure 4 – Histopathology and immunofluorescence of cutaneous lesions in pemphigus. (a) Pemphigus foliaceus: intraepidermal split at the level of the granular layer (hematoxylin and eosin (H&E)). (b) Pemphigus vulgaris: suprabasal acantholysis level with apico–lateral detached basal cells (‘tombstones’) (H&E)). (c) Direct immunofluorescence microscopy of pemphigus vulgaris: dotted or granular staining of IgG in an epithelial cell surface (ECS) pattern.
Desmoglein compensation theory
Mahoney et al. developed the desmoglein compensation theory for the explanation of the location (skin or mucous membranes) and level of blisters in pemphigus patients. This theory states that coexpression of Dsg1 and Dsg3 in keratinocytes protects against acantholysis due to antibody–induced dysfunction of Dsg1 or Dsg3 alone (Figure 5).75 In skin, Dsg1 is expressed throughout the whole epidermis, but more intense in the superficial layers. Expression of Dsg3 is limited to the basal and suprabasal layers. In mucosa, Dsg1 is expressed in the superficial layers, while Dsg3 is expressed throughout the whole epidermis. According to the desmoglein compensation theory blistering in PF can be explained as follows: In PF, anti–Dsg1 antibodies cause blisters in the superficial epidermis only because in this area Dsg1 is present without coexpression of Dsg3. Although the Dsg1 antibodies bind to mucosa, no blisters are formed because of the coexpression of Dsg3. In mucosal-dominant PV, Dsg3 antibodies cause blistering of the mucous membranes only, because in skin there is sufficient Dsg1 present to compensate for Dsg3. In mucocutaneous PV there is suprabasal blistering of both the skin and the mucous membranes, as Dsg1 and Dsg3 cannot compensate for one another anymore.

Basal cell shrinkage hypothesis
Bystryn and Grando proposed the basal cell shrinkage hypothesis for acantholysis in PV. This hypothesis states that binding of pemphigus antibodies to basal keratinocytes causes changes in their cytoskeletal structure with consequent collapse and shrinkage of the cells. Keratinocytes separate because they shrink more than they can be held together by desmosomes and not because of a primary defect in the function of desmosomes. The shrinkage is limited to the basal cells because these cells are less rigid and shrink more readily when their cytoskeleton is altered than their suprabasal counterparts. The cytoskeletal structure of the basal cells is affected to a greater extent by the signalling event and/or a different set of signalling events is triggered.76

Immunological tests
All forms of pemphigus are associated with the presence of skin–fixed and circulating antibodies against keratinocytes cell surface antigens.2

Direct immunofluorescence
Tissue–fixed intercellular antibodies are present in lesions and adjacent healthy skin in about 90% of patients with pemphigus and are detected by direct immunofluorescence.2 These antibodies are very rare in other diseases and are more sensitive and specific for the diagnosis of pemphigus than circulating antibodies. They are usually IgG, but IgM and IgA with or without complement may also be deposited (Figure 4c).2
Figure 5 – Desmoglein compensation hypothesis. (a) Normal distribution of desmoglein (Dsg)1 and Dsg3 in the epidermis and mucous membrane. (b) In pemphigus foliaceus, IgG directed against Dsg1 causes subcorneal blistering in skin because in the lower layers Dsg3 compensates for the loss of function of Dsg1. In mucosa however anti-Dsg1 antibodies do not cause blistering, because there is sufficient Dsg3 present throughout all the layers to compensate for Dsg1. (c) In mucosal-dominant pemphigus vulgaris (PV), IgG directed against Dsg3 does not cause blistering of the skin because Dsg1 compensates for the loss of function of Dsg3. However there is suprabasal blistering of the mucous membranes because there is not sufficient Dsg1 present to compensate for Dsg3. (d) In mucocutaneous PV antibodies directed both Dsg1 and Dsg3 cause blistering of the skin and the mucous membranes.
Indirect immunofluorescence
Circulating intercellular antibodies are detected by indirect immunofluorescence assays of serum using human skin, monkey oesophagus or guinea pig oesophagus as a substrate. Circulating antibodies are present in about 80% of patients. There is a correlation between the titre of intercellular antibodies and the activity of the disease. Serial determinations antibody titres may be useful in guiding therapy, since a rise in their titre usually precedes a recurrence in disease activity, while they usually decrease with successful treatment and disappear in patients in remission.²

ELISA
Enzyme–linked immunosorbent assays (ELISA) are available to detect antibodies directed against Dsg1 and Dsg3. The presence of antibodies directed against Dsg3 sometimes together with those against Dsg1 is associated with PV, whereas antibodies directed against Dsg1 alone are associated with PF. ELISA kits are available with the ectodomain of desmoglein produced in insect cells or in human cells. The latter has the advantage of containing the mature protein only and not the proprotein as well.⁷⁷ It is thought that pathogenic antibodies are directed against conformational epitopes only and these epitopes are present in the mature desmogleins, while non–pathogenic antibodies recognize both mature and proprotein isoforms, correlating with binding of nonconformational epitopes.⁷⁸

Aims of the study
The aim of this thesis is to gain more insight into the pathogenesis of pemphigus by studying acantholysis in pemphigus patient skin using histology, immunohistochemistry and electron microscopy. Most investigators use in vitro cell models and mouse models to study the pathogenesis of pemphigus. In this thesis we will focus on patient biopsies and organ cultures of human skin. In chapter 2 we will give an overview of the human model systems that have been used in the past to study the pathogenesis of pemphigus. Furthermore we will discuss to what extent these models represent the in vivo situation and how suitable they are to study the pathogenesis of pemphigus.

In chapter 3 we will describe the distribution of IgG and the desmosomal proteins in PF and PV patient skin. Chapter 4 will give a description of the ultrastructure of PF patient skin. We have used this old technique to look at pemphigus skin with the knowledge we have gained in the last decennium. In chapter 5 the differential effect of Dsg1 autoantibodies and Dsg3 autoanti-
bodies on desmosomes is studied in a morphometric study on the different types of pemphigus patient skin. In chapter 6 we searched for evidence of apoptosis in pemphigus skin by immunofluorescence and electron microscopy.
References


