Pemphigus Vulgaris Antigen, a Desmoglein Type of Cadherin, Is Localized Within Keratinocyte Desmosomes

Sarolta Kárpáti, Masayuki Amagai, Ronald Prussick, Kerstin Cehrs, and John R. Stanley
National Cancer Institute, Dermatology Branch, Bethesda, Maryland 20892

Abstract. Pemphigus vulgaris antigen (PVA) is a member of the desmoglein subfamily of cadherin cell adhesion molecules. Because autoantibodies in this disease cause blisters due to loss of epidermal cell adhesion, and because desmoglein is found in the desmosome cell adhesion junction, we wanted to determine if PVA is also found in desmosomes. By immunofluorescence, PV IgG bound, in a dotted pattern, to the cell surface of cultured human keratinocytes induced to differentiate with calcium, suggesting junctional staining. However, by preembedding, immunogold electron microscopic studies only slight labeling could be detected in desmosomes, presumably because of difficulty in gold penetration of intact desmosomes. We therefore treated the keratinocytes with 0.01% trypsin in 1 mM calcium, conditions known to preserve cadherin antigenicity but that caused slight separation of desmosomes, before immunogold staining. In this case there was extensive labeling of the extracellular part of desmosomes but not of the interdesmosomal cell membrane which was stained with anti-β2-microglobulin antibodies. To confirm the specificity of this binding we showed that antibodies raised in rabbits against the extracellular portions of PVA also bound desmosomes in these cultures. In intact mouse epidermis we could also show slight, but specific, immunogold desmosomal labeling with PV IgG. Furthermore, neonatal mice injected with PV IgG affinity purified on PVA showed desmosomal separation with the IgG localized to desmosomal cores. These results indicate that PVA is organized and concentrated within the desmosome where it presumably functions to maintain the integrity of stratifying epithelia.

Pemphigus vulgaris (PV) is an autoimmune disease in which blisters of skin and mucous membranes result from loss of epithelial cell-to-cell adhesion, a pathologic process called acantholysis (31). Immunofluorescence studies demonstrate that PV patients have IgG bound on their epidermal cell surfaces in vivo and also have circulating IgG that binds to the cell surface of normal stratified squamous epithelia (5, 6). These PV autoantibodies have been shown to be pathogenic by both clinical and experimental observations (for review see reference 29).

The antigen (PVA) recognized by PV autoantibodies has been characterized by immunochemical methods. Immunoprecipitation of cultured keratinocyte and normal human epidermal extracts demonstrates that PV sera bind a 130-kD glycoprotein which forms a stoichiometric complex with plakoglobin (22), a plaque protein of intercellular adhering junctions (i.e., desmosomes and adherens junctions) (8).

Recent cDNA cloning has demonstrated that the PVA is a member of the cadherin supergene family (3). The originally described or classical cadherins (called E-cadherin or uvomorulin, L-CAM, N-cadherin, and P-cadherin) are a family of cell adhesion molecules with certain functional and structural similarities (7, 36, 37). They are transmembrane molecules that mediate calcium-dependent, homophilic adhesion and share the interesting property that they are protected from trypsin degradation by calcium. The classical cadherins have been described to be found concentrated in adherens junctions.

Another subfamily within the cadherin superfamily is the desmogleins. Desmoglein was originally identified as a transmembrane glycoprotein of desmosomes (12, 25, 26). Recent cDNA cloning studies have shown that desmogleins are actually a gene family of their own (7, 19, 20) and have homology to the originally described cadherins, both in their extracellular amino-terminal region and in part of their cytoplasmic region (11, 21, 23, 39). The amino acid sequence of PVA is more closely related to the desmogleins than to the classical cadherins, both in the cytoplasmic region (which contains the "desmoglein-specific" repeating motif and cysteines) and in the extracellular domains (3).

Because antibodies against PVA cause loss of epidermal cell adhesion and because PVA is closely related to desmogleins, we speculated that PVA, like desmoglein, might be organized into the desmosome type of cell adhesion junc-
tion. Previous localization studies with PV antibodies have been contradictory. Immunofluorescence studies of mouse keratinocytes in culture have suggested that PV antibodies might bind desmosomes (16). In addition, immunoblot studies of bovine desmosomes demonstrate that a ~130-kD antigen is detected with PV sera (13, 16). However, by immunoperoxidase staining of PV patients' skin, IgG is distributed diffusely along the keratinocyte cell membrane (40). In addition, ultrastructural localization of PVA by indirect immunoferritin on trypsinized guinea pig keratinocytes, by immunoperoxidase staining of skin from neonatal mice injected with PV IgG, and by immunogold staining of bovine tongue with a rabbit antibody raised against a presumptive PVA from bovine tongue desmosomes all revealed PVA distributed diffusely along the cell membrane and not concentrated in desmosomes (17, 35, 38). However, contrary ultrastructural localization results, that showed PV IgG predominantly in desmosomes, were obtained by immunoferritin detection of IgG on patients' skin (14) and by immunogold localization of PV serum binding on cultured squamous cell carcinoma keratinocytes (1).

The purpose of this study was to definitively determine if PVA is predominantly found in desmosomes. We took advantage of the information from recent cloning of the PVA cDNA in order to perform ultrastructural localization studies. Because of the unique property of cadherins to resist trypsinization in the presence of calcium, we studied keratinocytes whose desmosomes were made more accessible to gold-labeled probes by this treatment. In addition, for increased specificity we affinity purified human antibodies, and raised rabbit antibodies, with extracellular portions of the PVA, and used these antibodies for immunolocalization. Our results show that PVA is found predominantly in keratinocyte desmosomes.

**Materials and Methods**

**Patient Sera, Antibodies, and Rabbit Antiserum**

Sera from five patients with PV were used. Each serum immunoprecipitated the characteristic PVA complex (containing the 130-kD antigen) from extracts of normal epidermis or cultured keratinocytes (9, 33). This immunoprecipitation has been shown to be specific for PV patient sera (9, 33). Antibodies from the sera of two of the PV patients were affinity purified on β-galactosidase fusion proteins made, as previously described, from cDNA cloned into the pUEX 1 plasmid and encoding all of the two most amino-terminal extracellular domains of PVA, as well as part of the third domain (2). This region of PVA is referred to as EC1-2 (see reference 2 for details).

Antibodies were raised in rabbits against β-galactosidase fusion proteins containing EC1-2 or the most amino-terminal extracellular domain (termed ECl). The production of these fusion proteins has been previously described in detail (2). Rabbits were immunized subcutaneously at multiple sites with ~500 µg of insoluble fusion protein mixed with complete (first injection) or incomplete Freund's adjuvant every 2 wk for a total of five injections. For the last two boosts the fusion proteins were first solubilized in 8 M urea then dialyzed against 10 mM Tris-HCl, pH 7.6.

A rat mAb, ECD-2, against the extracellular region of E-cadherin and a mouse mAb, HECD-1, against human E-cadherin were kindly provided by Dr. M. Takeichi (27, 28). An affinity purified rabbit antibody against β2-microglobulin was purchased (Boehringer-Mannheim Corp., Indianapolis, IN). A rabbit polyclonal antibody against desmoglein isolated from bovine snout was kindly provided by Dr. M. S. Steinberg (10), and a mouse mAb, DG3.10, against desmoglein was obtained commercially (IBL Research, Cambridge, MA) (25).

**Keratinocyte Cell Culture and Fixation**

Normal human epidermal keratinocytes were cultured in media containing 0.15 mM calcium (Clonetics, San Diego, California) and subcultured into cell culture chamber slides (Lab-Tek, Nunc Inc., Naperville, IL). When subcultures were almost confluent, the calcium concentration was raised to 2 mM for 24 h to induce synthesis of PVA (3, 34). In some experiments, subcultures were then incubated at room temperature with Hanks-Hepes solution (Gibco-BRL, Grand Island, NY) containing 0.03% trypsin (1:250) (Gibco-BRL, 1 gm digests 250 gm casein at 25°C for 10 min, pH 7.6) and 1 mM Ca, for 20-30 min, in order to separate desmosomal junctions to allow penetration of immunogold reagents. Both trypsin-treated and untreated cells were fixed in 2% paraformaldehyde in PBS for 10 min. Cells were then processed for immunofluorescence or immunoelectron microscopic studies.

**Immunoblotting and Immunoprecipitation**

Immunoblotting was performed, as previously described (3), on extracts of cultured human keratinocytes induced to differentiate with high calcium, as described above.

Immunoprecipitation was performed on cell extracts of these keratinocytes after metabolic labeling with [35S]methionine/cysteine (NEN Express, Dupont, Boston, MA) for 2-24 h. In some experiments cells were extracted with 1% Nonidet P-40, 1% Triton X-100, 2 mM CaCl2, in TBS (10 mM Tris-HCl, 0.15 M NaCl, pH 7.4). After adding BSA to a final concentration of 0.1%, these extracts were preabsorbed with normal human and mouse sera and protein G sepharose (Sigma Chemical Co., St. Louis, MO). Immunoprecipitation was performed with 10 µl of serum or 10 µg of mAb incubated overnight at 4°C, then 25 µl of protein G incubated for 1 h. Immunoprecipitates were washed 5 times with 0.5% Triton X-100 in TBS, and precipitated complexes were eluted in SDS gel sample buffer and electrophoresed as previously described (32). In some experiments, the labeled cell extracts were extracted with 1% SDS, 8 M urea in TBS. These extracts were dialyzed against 0.3% Nonidet P-40, 2 mM CaCl2 in TBS to remove the denaturants, then immunoprecipitation was performed with protein A as previously described (32).

**Immunofluorescence**

Fixed cultured human keratinocytes were incubated for 45 min at room temperature with human sera, rabbit antibodies or mAbs diluted 1:10 to 1:50 in PBS. After washing extensively with PBS, the appropriate second antibody, diluted in PBS, was applied for 30 min. Second antibodies included: FITC- or biotin-conjugated affinity purified F(ab')2 anti-human, anti-rabbit or anti-rat IgG (Tago, Burlingame, CA). Biotinylated antibodies were developed with streptavidin-Texas red (Tago) or streptavidin-FITC (Vector Laboratories, Burlingame, CA).

**Neonatal Mouse Model of Pemphigus Vulgaris**

Two newborn Balb/c mice were injected subcutaneously with IgG precipitated with ammonium sulfate from PV sera, and three mice were injected with PV IgG affinity purified on ECl-2, as recently described (2).

**Immunoelectron Microscopy**

Cultured keratinocytes that were not treated with trypsin were fixed, as described above, then incubated overnight at 4°C with PV antibodies. After washing extensively with PBS the cells were incubated with 1 nm gold-labeled goat anti-human IgG for 2 h at room temperature, washed, and postfixed in Karnovsky fixative. The 1-nm gold was developed with silver enhancement (24). Cultured keratinocytes that were treated with trypsin and fixed were incubated for 4 h or overnight with antibodies, washed, and incubated for 3 h at room temperature with the appropriate biotinylated second antibody. After additional washing, the chambers were incubated for 3 h at room temperature with 5 nm streptavidin gold (Biocell, Ted Pella, CA). In some experiments, cells were doubly labeled by using gold-labeled (5 and 10 nm) anti-rabbit and anti-human antibodies (Biocell). All of these labeled cells were then washed, dehydrated in ethanol, and embedded in Epon 812 (EM Sciences, Washington, PA).

Skin from neonatal mice injected with PV IgG was cut into small pieces (<1 × 1 mm), incubated with biotinylated anti-human IgG (Vector Laboratories) for 5 h at room temperature, washed with PBS, and incubated overnight at 4°C with 5 nm gold-streptavidin (18). This method is referred to
as "en bloc." Alternately, some skin was embedded in OCT (Miles, Naperville, IL) then cut in a cryostat into 6-µm sections before incubation with 5 nm gold-goat anti-human IgG for 2 h at room temperature. This method is referred to as "cryosectioning."

Skin from normal newborn mice was fixed with 2% paraformaldehyde, then cut into small pieces and incubated with PV IgG followed by 1 nm gold goat anti-human IgG as described above (18). Gold was visualized by silver enhancement.

Thin sections were mounted on uncoated copper grids, stained in 2% uranyl acetate and Reynold's lead citrate, and examined with a Philips transmission electron microscope (model 400) operated at 80 kV.

Results

Immunofluorescence of Differentiated Cultured Keratinocytes with PV Sera

Normal human keratinocytes, cultured in high calcium to induce differentiation and PVA expression (3), were stained after fixation by indirect immunofluorescence with 5 PV sera and 2 normal human sera. PV IgG bound to these cells in a cell surface pattern that in some areas was resolved into a dot-like pattern, suggesting junctional staining (Fig. 1 a).

These findings confirm previous impressions of pemphigus antibody staining of cultured mouse keratinocytes (15, 16). Because we wanted to separate the desmosomes of these cells in order to permit gold penetration for immunoelectron microscopy (see below), we wanted to determine if PV antibodies would still bind after the keratinocytes were treated with 0.01% trypsin in the presence of 1 mM calcium, conditions known to maintain intact the classical cadherins. We first confirmed that antibodies to E-cadherin bound the cell surface of these keratinocytes whether or not they were treated with trypsin/calcium (data not shown). Similarly, the staining pattern with PV sera of keratinocytes treated with trypsin/calcium was the same as that of keratinocytes without such treatment (compare Fig. 1, a and b). Normal human sera, as controls, showed no staining of these cells.

Immunoelectron Microscopy with Human PV Antibodies on Cultured Keratinocytes

Human keratinocytes cultured for 24 h in high calcium were fixed with 2% paraformaldehyde before being used for immunogold electronmicroscopy to localize the PVA with PV serum or PV IgG affinity purified on the ECl-1-2 extracellular domains of PVA. Although there was excellent binding of the PV antibodies by immunofluorescence with these fixation conditions (see Fig. 1 a), there were few gold particles as detected by immunoelectron microscopy. What gold binding that was seen, however, was limited to desmosomes (data not shown). No staining of desmosomes was seen with normal human serum or IgG.

We speculated that the reason we saw such limited staining by immunogold in the face of such readily demonstrable PV antibody binding was that the gold particles penetrated poorly into desmosomes where we hypothesized the PVA was predominantly located. To circumvent this potential problem, we exposed the keratinocytes to 0.01% trypsin in 1 mM calcium. These conditions preserved the PV immunoreactivity (Fig. 1 b) and resulted in markedly increased immunogold binding to PVA in association with desmosomes that were often separated (Figs. 2, 3). Normal human sera, used as a control, did not show desmosome binding (Fig. 2 b). Another control, antibodies against human β2-microglobulin, showed binding to the cell surface between desmosomes, but not within desmosomes (Fig. 3). For these immunoelectron microscopic studies we used 4 PV sera, 2 normal human sera, and one rabbit antisera against β2-microglobulin.

These immunoelectron microscopic results suggested that PVA is localized and concentrated in desmosomes.

Production and Characterization of Rabbit Anti-PVA Antibodies

To confirm the specificity of our antibody staining with hu-
Figure 2. PVA is localized by immunogold staining to the surface of separated desmosomes in cultured keratinocytes treated with trypsin/calcium before fixation. (a) PV serum; (b) normal human serum. Note gold localization to desmosomal cores, but not along the extradesmosomal membranes in a. (Arrow in a) Shows a "missing" half-desmosome that is presumably turned out of the plane of section but is labeled by gold particles. (Arrows in b) Show a few gold particles nonspecifically binding the cell membrane. Bars, 0.15 μm.

Figure 3. Double immunogold localization of PVA and β2-microglobulin to cultured keratinocytes with desmosomes slightly separated by trypsin/calcium. (a) 5 nm gold indicates PVA; 10 nm gold indicates β2-microglobulin. (b) 5 nm gold indicates β2-microglobulin; 10 nm gold indicates PVA. Inserts in upper right corner show separated desmosomes. Note that PVA is found in desmosomes but β2-microglobulin is found along the extradesmosomal cell membrane. Bars, 0.1 μm.

Figure 4. Immunoblots of extracts of cultured keratinocytes demonstrate that rabbit antibodies raised against extracellular regions of PVA bind the 130-kD PVA but not the homologous desmoglein. (a) Rabbit antibodies against ECl and ECl-2 (αEC1, αEC1-2) bind the 130-kD PVA (arrow) identified by PV serum. (b) Rabbit antibodies bind the 130-kD PVA (arrows) but do not bind the 160-kD desmoglein (arrowheads) stained by anti-desmoglein (αDG) antibodies.

Immunolocalization of Rabbit Anti-PVA Antibodies

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Figure 6. Rabbit antibodies raised to the extracellular regions of PVA have the same ultrastructural localization to keratinocyte desmosomes as does human PV IgG. Cultured human keratinocytes had the desmosomes separated with trypsin/calcium. (a) Almost intact desmosomes stained with rabbit anti-EC1 antibodies (5 nm gold). (b) Slightly separated desmosome stained with rabbit anti-EC1 (5 nm gold) and PV serum (10 nm gold). (c) Separated desmosome stained by rabbit anti-EC1 (5 nm gold) and PV serum (10 nm gold). (d) Totally separated desmosome stained with rabbit anti-EC1-2 (5 nm gold) and PV serum (10 nm gold). Bars, 0.2 μm.

Figure 5. Immunoprecipitation of extracts of cultured keratinocytes demonstrate that PV IgG, affinity purified from patient serum, and rabbit anti-PVA antibodies bind PVA, but not E-cadherin or desmoglein. (a) Nonionic detergent extracts. (Lane 1) affinity purified PV IgG; (lane 2) HECD-1 monoclonal antibody against E-cadherin (open circles show, from top, E-cadherin, α and β catenin); (lane 3) DG3.10 monoclonal anti-desmoglein (open circle shows desmoglein); (lane 4) rabbit anti-EC1; (lane 5) preimmune rabbit serum; (lane 6) monoclonal anti-κ control; (lane 7) normal human serum. (Arrows show PVA and plakoglobin). (b) To increase the amount of desmoglein (and other desmosomal proteins) extracted, the cells were extracted with SDS and urea, which was subsequently dialyzed out (see Material and Methods). (Lane 1) affinity purified PV IgG; (lane 2) rabbit anti-EC1-2; (lane 3) rabbit anti-EC1; (lane 4) rabbit anti-desmoglein; (lane 5) normal human serum; (lane 6) preimmune (for anti-EC1-2) rabbit serum. (Arrow shows desmoglein, arrowhead shows PVA).

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Immunoelectron microscopy of cultured keratinocytes indicated that these rabbit antibodies and human PV antibodies, colocalized to the desmosomes (Fig. 6). Preimmune rabbit sera gave negative results in these immunofluorescence and immunoelectron microscopic procedures.

Localization of PVA to Desmosomes in Mouse Skin

The above studies demonstrate the localization of PVA in the desmosomes of cultured keratinocytes. To determine if PVA is also localized to desmosomes in vivo, we performed immunogold electron microscopy on normal neonatal mouse skin. As in cultured cells, 5 or 10 nm gold did not penetrate into intact desmosomes of normal skin. However, to try to show PVA in intact mouse skin we used PV IgG affinity purified on EC1-2 and localized it by 1 nm gold with silver enhancement, as we did with cultured keratinocytes whose desmosomes were intact. As with cultured keratinocytes, we...
saw minor binding of gold, but what binding we did see was in desmosomes (data not shown).

To increase access of gold to desmosomes, we used a neonatal mouse model of PV. Mice injected with the total IgG that was precipitated with ammonium sulfate from human PV sera developed severe suprabasilar blistering, as previously reported (2, 4). Electron microscopy revealed extensive suprabasilar acantholysis with basal cells maintaining their attachment to basement membrane. Immunolocalization of this IgG showed that gold particles did not penetrate to areas that were not blistered, but were concentrated on separated desmosomes as well as on internalized desmosomes (data not shown). However, gold was also detected in clumps floating freely in the widened intercellular spaces as well as in vesicles surrounding internalized desmosomes. This type of gold localization, unassociated with any structures, suggested that extensive damage may have released PVA from cell surfaces or may have resulted in nonspecific aggregation of IgG.

Therefore, in order to minimize the extensive damage caused to the epidermis and to increase the specificity of the antibodies injected in these mice, we injected mice with PV IgG affinity purified on the E1–2 domain of PVA. This affinity purified IgG immunoprecipitated PVA but not E-cadherin or desmoglein from extracts of cultured human keratinocytes (Fig. 5 a, compare lane 1 with lanes 2 and 3; Fig. 5 b, compare lane 1 with lane 4). Affinity purification greatly reduces the injection of IgG that is not directed against PVA and results in much less extensive blistering as judged by clinical examination and histology of the skin lesions (2). Again, suprabasilar acantholysis was seen, but in general there was less extensive separation of cells and better preservation of desmosomes. In this case, immunolocalization of PV IgG showed gold on the surface of separated desmosomes but essentially none on extrasomal membranes or in the space between separated keratinocyte membranes (Fig. 7). Again, there was no penetration of gold in areas where desmosomes remained intact.

These in vivo studies, like the in vitro studies, show PVA localized in desmosomes.

Discussion

We used both an in vitro and in vivo system as well as several different antibodies and conditions to ultrastructurally localize PVA. In all cases we found it within desmosomes.

A potential problem with PVA immunolocalization studies is that PVA and cadherins, particularly desmoglein, are closely related molecules (3). However, in this study we not only used patients' sera, but also used IgG from such sera affinity purified on PVA as well as rabbit sera raised against PVA. By immunoblotting and immunoprecipitation these antibodies bind PVA but not E-cadherin or desmoglein, and localize to the desmosome by immunoelectron microscopy.

It should be noted that with almost any tissue preparation and fixation procedure it can be argued that there might be a selective loss of the antigen from extrasomal membranes but not from desmosomes. If such a loss did occur, the results still imply that PVA is organized into desmosomes and thus protected from destruction. However, we think such a selective loss is unlikely because: (a) we used two different methods to open desmosomes—trypsin/calcium for keratinocytes and PV IgG-induced separation in neonatal mice; (b) in neonatal mice injected with affinity purified PV IgG, PVA was clearly localized to desmosomes in the early lesions of limited disease; (c) in culture and epidermis, even without causing separation of desmosomes, gold tended to localize to desmosomes and did not localize to interdesmosomal membranes.

These studies extend our concept of the desmoglein family of cadherins, which includes PVA. These molecules make up a family not only because of shared amino acid sequence homology, but because they share other interesting similarities. The two known desmogleins (dsg1 and dsg2) and PVA are all found on chromosome 18 (7). Both desmoglein and PVA bind plakoglobin, which is found in cell adhesion junctional plaques (22). In humans, autoantibodies against both desmoglein, in pemphigus foliaceus, and PVA, in PV, cause blisters of the epidermis due to loss of cell adhesion (30). Finally, as shown here, PV, like desmoglein, is localized predominantly in desmosomes. These similarities suggest a shared cell biologic function, probably related to cell adhesion in desmosomes, of this subfamily of cadherins.

Figure 7. Immunolocalization by cryosectioning (a) or en bloc method (b, c) of human PV IgG affinity purified on PVA E1–2 and injected into neonatal mice. (a) Note extensive labeling on the surface of a split desmosome but not on the adjacent extrasomal membrane (arrows). (b) Note the gold stained half desmosome at the right and a few gold particles at the edges of the closed desmosomes (arrows), but no gold on the adjacent extrasomal cell membranes. (c) Gold labeling of half desmosomes but no labeling of the adjacent cell membranes. Bars, 0.2 μm.

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