Anti-desmoglein 3 (Dsg3) Monoclonal Antibodies Deplete Desmosomes of Dsg3 and Differ in Their Dsg3-depleting Activities Related to Pathogenicity*

Received for publication, August 21, 2006, and in revised form, March 9, 2007  Published, JBC Papers in Press, April 11, 2007, DOI 10.1074/jbc.M607963200

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Pemphigus vulgaris (PV) is an autoimmune blistering disease, characterized by the loss of cell-cell adhesion between epidermal keratinocytes and the presence of autoantibody against desmoglein 3 (Dsg3), which provides adhesive integrity to desmosomes between adjacent keratinocytes. We have previously shown that PV-IgG purified from patients depletes desmosomes of Dsg3. However, PV-IgG contains not only antibodies against a variety of different epitopes of Dsg3 but also against other unknown antigens. Therefore, we examined whether the Dsg3-depleting activity of PV-IgG is generated specifically by anti-Dsg3 activity in a human squamous cell carcinoma cell line (DJM-1) and normal human keratinocytes by using four different pathogenic and nonpathogenic monoclonal antibodies against Dsg3. We demonstrate that these monoclonal antibodies deplete cells and desmosomes of Dsg3, as PV-IgG does. Individual monoclonal anti-Dsg3 antibodies display characteristic limits to their Dsg3-depleting activity, which correlates with their pathogenic activities. In combination, these antibodies exert a cumulative or synergistic effect, which may explain the potent Dsg3-depleting capability of PV-IgG, which is polyclonal. Finally, although Dsg3-depletion activity correlated with AK-monoclonal antibody pathogenicity in mouse models, the residual level of Dsg3, when below ~50%, does not correlate with the adhesive strength index in the present study. This may suggest that although the Dsg3 depletion is not indicative for adhesive strength, the level of Dsg3 can be used as a read-out of pathogenic changes within the cell and that the Dsg3 depletion from desmosomes plays an important role in skin fragility or susceptibility to blister formation in PV patients.

Pemphigus is a group of an autoimmune blistering diseases that includes two major types; i.e. pemphigus vulgaris (PV), characterized by suprabasal acantholysis (the loss of cell-cell adhesion between keratinocytes) in the epidermis and autoantibodies against Dsg3 (1–4), and pemphigus foliaceus, characterized by superficial acantholysis in the granular cell layer of the epidermis and autoantibodies against Dsg1 (5, 6). Dsg3 and Dsg1 are members of the desmosomal cadherins that provide adhesive integrity to desmosomes between adjoining keratinocytes in the epidermis; the former distributes primarily in the deeper epidermis, whereas the latter is localized to the superficial epidermis. This distribution profile of these target antigens, Dsg3 and Dsg1, of PV and pemphigus foliaceus autoantibodies explains the characteristic clinical features and histopathological localization of acantholysis in their respective diseases. PV, but not pemphigus foliaceus, affects the mucous membrane, where Dsg3 is localized but Dsg1 is rarely expressed (7–10).

Recent work has suggested that two principal hypotheses emerge as pathomechanisms underlying the generation of PV blisters (4, 11). The first proposes that anti-Dsg3 antibody-dependent steric hindrance interferes with intercellular adhesive function(s) of Dsg3, leading directly to desmosomal dissociation (12–14). However, we have observed no inhibition of Ca$^{2+}$-induced desmosome formation by PV-IgG binding to surface PV-antigens (15), suggesting that PV-IgG does not directly inhibit desmosome formation, even though the antibodies may cause steric hindrance between homophilic Dsg3 interactions.

The second hypothesis is that myriad PV-IgG-induced intracellular signaling events could lead to desmosomal dissociation (11, 16). We investigated the pathomechanisms of pemphigus in terms of outside-in signaling (16), and in particular, analyzed the phosphorylation of specific desmosomal proteins (17). PV-IgG binding to the plasma membrane alone induced the phosphorylation of Dsg3 (17), an effect associated with plakoglobin (PG) dissociation from Dsg3. In addition, degradation of Dsg3 occurred within 20 min of binding, which may partially explain the Dsg3-depleted desmosomes that are subsequently formed (18). These results suggest that PV-IgG activates intracellular signaling leading to the aberrant phosphorylation of Dsg3, which appears to be linked to Dsg3-depleted desmosome formation and cutaneous blistering in PV (11). In this regard, it is of interest to note that the PV-IgG-dependent Dsg3 depletion from desmosomes is accompanied by endocytosis of Dsg3 (19, 20). In addition, we have also shown that PV-IgG activates another intracellular signaling pathway in which Ca$^{2+}$ and protein kinase C (21) are significantly involved events that are
linked to urokinase-type plasminogen activator secretion (22, 23). Thus, the signaling-related events thought to be involved in pathomechanisms of PV include not only phosphorylation of Dsg3 (17) and a Ca\(^{2+}\)/protein kinase C pathway (22), but also apoptosis signaling (24–26), as well as modulations of PG (27), p38 MAPK and heat shock protein 27 (28).

In light of these observations, the PV-IgG-dependent depletion of Dsg3 from its soluble and insoluble (desmosomal) pool(s) is probably one of the final steps directly linked to pathomechanisms leading to cell-cell detachment, because Dsg3 knock-out mice express a clinical and histopathological PV phenotype (29). On the other hand, there is a controversial report on the depletion of Dsg3 from cells that when HaCaT cells are treated with PV serum, Dsg3 half-life is reduced and de novo assembly to desmosomal sites are perturbed without reducing the Dsg3 content in the cells for 36 h (30). However, these experimental data have been produced by using PV-IgG purified from patient sera (17, 18) or sera themselves (30), which contain not only antibodies against a variety of different epitopes of Dsg3 but also other antibodies against other unknown antigens. Therefore, whether the responses to PV-IgG mentioned above are exerted by monoclonal antibodies against Dsg3, and if so, whether different antibodies to alternate Dsg3 epitopes may exert different responses with epitope specificity remains to be resolved. Furthermore, whether autoantibodies against antigens other than Dsg3 or their combination with anti-Dsg3 antibodies are involved in these responses is unclear (31). In this study, to clarify these questions, we employed four monoclonal antibodies against distinct extracellular domains of Dsg3 (AK mAbs), which are pathogenic: AK19 and AK23 mAbs that recognize residues 1–161 (the amino-terminal portion of the extracellular domain), nonpathogenic AK18 and AK20 mAbs that react with residues 195–402 (the middle portion of the extracellular domain), and 403–565 (the C-terminal portion of the extracellular domain), respectively (32). These mAbs show a cross-reactivity with human (h) Dsg3, giving compatible mapping results with domain-swapped molecules using hDsg3 and hDsg1 (32). We examined the effects of individual and combined treatments with these monoclonal antibodies on both the fate of Dsg3 and the physical binding strength between keratinocytes using both DJM-1 and normal human epidermal keratinocytes.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-Dsg3 monoclonal antibodies (AK18, 19, 20, 23) were produced by hybridoma cells from splenocytes of PV model mice, as described previously (32). Hybridoma cells were cultured in CD Hybridoma medium (Invitrogen), a protein-free, chemically defined and optimized medium for hybridoma growth and monoclonal antibody production. IgG from conditioned medium of individual hybridoma cultures was purified using a Protein G HiTrap column (GE Healthcare). Anti-desmoplakin (DKP) 1 mouse monoclonal antibody (mAb) DP-2,17 and anti-plakoglobin (PG) mouse mAb PG-5.1 were purchased from Progen Biotechnik (Heidelberg, Germany). Anti-Dsg3 rabbit polyclonal antibody AHP319, anti-α-tubulin mouse mAb B512, anti-β-catenin mouse mAb 14, and anti-desmoglein 2 mouse mAb 608 were obtained from Serotec (Oxford, GB), Sigma, BD Biosciences, and MBL (Nagoya, Japan), respectively. AK23 and AK19 mAb have been shown to have pathogenic activity as revealed by a single injection into neonatal mice. In contrast, AK18 and AK20 mAb have no pathogenic activity, as demonstrated by both a passive transfer assay and the ascites formation assay (32). As a positive control to Dsg3 depletion, PV-IgG was used, because stimulation with PV-IgG has been shown to cause a prominent depletion of Dsg3 from desmosomes in DJM-1 cells and normal human epidermal keratinocytes (18). Sera from three patients with PV and from normal volunteers without any skin disease were used for the current experiments. All sera from PV patients were confirmed to react with only Dsg3, but not with Dsg1, by antigen-specific enzyme-linked immunosorbent assay (MBL, Nagoya, Japan) using baculovirus-expressed recombinant peptides of extracellular domains of Dsg3 and Dsg1 (33). This was also confirmed by immunoblotting using total cell lysates of cultured DJM-1 cells and normal human keratinocytes (18). All sera were heated to 56°C for 30 min to inactive complement, and stored at −80°C. IgG fractions were isolated from these sera by Protein G HiTrap column (GE Healthcare). Thus, purified IgG (PV-IgG) from PV sera and normal human sera were used as positive and negative controls, and mouse IgG (mIgG) from normal mice (BALB/c) sera was also used as a negative control.

**Cell Culture**—DJM-1 cells, a cell line isolated from human skin squamous cell carcinoma (15) were seeded either in 35-mm dishes or on glass coverslips. Cells were cultured in Eagle’s minimum essential medium (MEM) (Nissui Phama, Tokyo, Japan) containing 10% fetal calf serum, 0.4 μg/ml hydrocortisone (Sigma), 20 ng/ml epidermal growth factor (TOYOBO, Osaka, Japan), 84 ng/ml cholera toxin (List Biological Laboratories, Campbell, CA), and 1.8 mM Ca\(^{2+}\).

Normal human epidermal keratinocytes (NHEKs) from neonatal foreskin were cultured first to maintain in Epilife® medium containing 0.06 mM CaCl\(_2\) and Epilife® Defined Growth Supplement. When used for experiments, they were cultured in Eagle’s MEM containing 1.8 mM CaCl\(_2\) for 24 h. They were cultured to ~40% confluence and used as described below.

**Antibody Stimulation**—To examine the effects of individual AK mAbs on the Dsg3 depletion from Triton X-100-soluble and -insoluble fractions of DJM-1 cells, AK mAbs, PV-IgG for the positive control and normal mouse IgG (mIgG) for the negative control were added to final concentrations of 0.5, 1.0, and 2.0 mg/ml in culture medium. This preliminary dose-response experiment revealed that concentrations of 0.5, 1.0, and 2.0 mg/ml AK23 mAb exerted similar results, with the maximum reduction values of the Dsg3 to PG ratio of ~40%. Therefore, we performed precise dose-response experiments to determine the concentration at which the Dsg3 depletion is initiated. For the dose-response experiments, AK23 mAb was added to final concentrations ranging from 0.0064 to 500 μg/ml (i.e. 0.0064, 0.032, 0.16, 0.8, 4.0, 20, 100, and 500 μg/ml).

To examine the effects of individual AK mAbs on Dsg3 depletion, cells were treated at a 0.5 mg/ml concentration for individual AK mAbs. In addition, to analyze the additive or synergistic effects of non-AK23 mAbs to Dsg3 depletion effects of AK23 mAb, the following combined AK mAb experiments
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

were performed. Cells were treated with control mouse IgG, single AK23 mAb, two AK mAb combinations (AK23 plus AK18, AK19, or AK20), three AK mAb combinations (AK23 plus AK18/AK19, AK18/AK20, or AK19/AK20), or a mixture of four AK mAbs for 24 h. All individual AK mAbs were used at a concentration of 0.125 mg/ml. Besides these experiments, to adjust the final concentration of IgG to the same 0.5 mg/ml, cells were treated with control mouse IgG at 0.5 mg/ml, single AK23 mAb at 0.5 mg/ml, and a mixture of four AK mAbs at 0.125 mg/ml for individual antibodies, i.e., a final concentration of 0.5 mg/ml in total for 30 min and 24 h. The culture medium was changed at 15 h for the later experiment.

Cell Fractionation and Immunoblot—For Western immunoblot analysis of whole cell lysates, cells were lysed by 2% SDS sample buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 30% glycerol, and 2% β-mercaptoethanol) and the lysates were centrifuged at 100,000 × g for 1 h. For analysis of Triton X-100-soluble or -insoluble pools, cells were lysed in 1% Triton X-100 in phosphate-buffered saline containing 5 mM iodoacetamide (Wako Pure Chemical Industries, Osaka, Japan), 10 μg/ml aprotinin (Roche Applied Science), 10 μg/ml leupeptin, 1 mM sodium orthovanadate (V) (Wako Pure Chemical Industries), 1 mM sodium fluoride (Sigma), 2 mM Pefabloc SC (Roche Applied Science), followed by centrifugation at 100,000 × g for 1 h, and the resultant supernatant was stored as the cytosol/membrane fraction. Triton X-100-insoluble pellets were solubilized in 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 30% glycerol and stored as cytoskeleton fraction, which includes whole insoluble desmosomal components. To the cytosol/membrane and cytoskeleton fractions, sample buffer for SDS-PAGE was added to a final concentration of 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 30% glycerol, 2% β-mercaptoethanol. Samples were heated at 100 °C for 5 min. Protein concentrations were determined spectrophotometrically using the DC Protein assay kit (Bio-Rad). Each fraction (10 μg of protein per lane) was subjected to reducing SDS-PAGE by the method of Laemmli (34) in 6% polyacrylamide gels. Blots were washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-Tween) and then incubated with each primary antibody, followed by horseradish peroxidase-conjugated goat anti-mouse and rabbit antibodies (DakoCytomation, Glostrup, Denmark) diluted in TBS-Tween. Blots were visualized with ECL Plus reagent (GE Healthcare). The density of the band was measured using a Lane Analyzer (version 3.0) (ATTO, Tokyo, Japan).

For semi-quantitative immunoblot analysis of Dsg3 content in Triton X-100-soluble, -insoluble, and whole cell lysates, we first examined which proteins (i.e., PG, β-catenin, α-tubulin, Dsg2, or DPKs) represented a proper internal standard. By scanning densitometry of immunoblots, the relative amount of Dsg3 was determined by probing the Dsg3 immunoblot with monoclonal antibodies to PG, β-catenin, α-tubulin, Dsg2, and DPKs. PG was found to be the most appropriate reference standard, as described under “Results.” Therefore, Dsg3 levels were normalized to PG for the remaining experiments.

Immunofluorescence Microscopy—Cells cultured on glass coverslips were stimulated with PV-IgG and AK23 mAb for 30 min, 24 h, or 48 h. The cells on the coverslips were washed with phosphate-buffered saline and fixed for 7 min in 3% paraformaldehyde at room temperature. The fixed cells were double-stained with monoclonal antibodies against Dsg3 and DPK after permeabilized with 0.5% Triton X-100, followed by staining with Alexa Fluor 488-conjugated donkey anti-mouse or Alexa Fluor 546 goat anti-rabbit secondary antibodies (Invitrogen) for 30 min. Controls for specificity of the antibodies were performed routinely. Photomicrographs were taken with a NIKON-E1000 photomicroscope (Nikon, Tokyo, Japan).

Dissociation Assay of Cell-Cell Adhesion—The adhesive strength of DJM-1 cell-cell contacts was assayed based on an adapted method that modifies a previously described method (35, 36). Cells, 5 × 10^4, were seeded onto 35-mm diameter dishes and cultured in Eagle’s MEM for 50 h. Cells were stimulated with nonpathogenic AK20 (0.5 mg/ml), most pathogenic AK23 mAB (0.5 mg/ml), the four AK mAb mixture (0.125 mg/ml for individual AK mAbs, i.e., 0.5 mg/ml in total), PV-IgG (0.5 mg/ml), and mouse IgG (0.5 mg/ml) for 6 and 24 h at 37 °C. With this culture method, cells form a number of small colonies, but not large sheets of cells, at a culture time point of 50 h (preincubation) plus 6 or 24 h (antibody stimulation). The culture cells were then washed twice with Eagle’s MEM and incubated in 1 ml of Eagle’s MEM containing 500 protein units/ml dispase (Godo Shusei, Tokyo, Japan) for 30 min at 37 °C to detach cells as separated colonies from dishes. Cells that detached from dishes as monolayer fragments of small colonies were collected carefully into 2.0-ml tubes and subject to mechanical stress by pipetting 5 times with a 1-ml pipettman (P1000). Cell suspensions were fixed by adding ethanol at a final concentration of 50%, stained by adding 4′,6-diamidino-2-phenylindole (Sigma) at a final concentration of 1 μg/ml for 15 min, and centrifuged at 2,000 × g for 5 min to recover whole fragments of cell colonies. The pellets and supernatants were re-suspended with FluorSave (CAL-BIOCHEM) reagent and placed on glass coverslips, separately. The supernatants were subsequently checked by fluorescence microscopy to determine whether all cells were recovered into the pellets leaving no cells in the supernatants. Ten photomicrographs for each sample were taken with a Nikon E1000.

To quantify the cell numbers in different sized colonies, instead of counting the numbers of cells, fluorescence intensity of the nuclei in fragments of cell colonies was measured by using Image J (37). The intensity of fluorescence was proportional to the cell number (see “Results”). Therefore, we considered the value of fluorescence intensity as an index of colony size. Dissociation scores were calculated using the number of colonies that contains over 30 cells (a) and below 30 cells (b). The ratios (a/b) of PV-IgG, AK20-mAb, AK23-mAb, and the four AK mAb mixture-stimulated colonies were compared with that of normal mouse IgG.

Statistics—Data are expressed as the mean ± S.D. from three different measurements for individual samples from three different experiments. Statistical significance of the observed effects was assessed by a Student’s t test (p < 0.05 is considered significant). Statistical analysis was done with StatMate (ATMS Co., Ltd., Tokyo, Japan).
RESULTS

AK23 mAb, a Pathogenic Anti-Dsg3 Monoclonal Antibody, Depletes Cell-Cell Contacts of Dsg3: Immunofluorescence Microscopy—To determine whether the pathogenic monoclonal antibody, AK23, depletes desmosomes of Dsg3, but not of DPKs at cell-cell contacts as PV-IgG does, double staining immunofluorescence microscopy was performed using anti-Dsg3 antibody (rabbit IgG) and anti-DPK antibody (mouse IgG) 30 min and 24 h after stimulation with AK23 mAb at a concentration of 0.5 mg/ml. AK23 mAb co-localized with Dsg3 in a punctate, linear manner at cell-cell contacts at 30 min (Fig. 1A), whereas AK23 mAb and Dsg3 disappeared from the cell and cell borders by 24 h after stimulation (Fig. 1B). However, DPK was still detected as punctate lines at cell-cell contacts, where no Dsg3 was detected 24 h after AK23 mAb stimulation (Fig. 1, B and C), suggesting that these cells retain desmosomes, although Dsg3 was depleted. The Dsg3 depletion from cell-cell contacts induced by both AK23 mAb and PV-IgG is most prominent, i.e. almost complete disappearance in a few rows of cells at periphery of the colony, but less in the central cells of colony, as determined by double immunofluorescence microscopy with anti-Dsg3 and anti-DPK antibodies (Fig. 1C). Control mouse IgG treatment did not deplete desmosomes of Dsg3 as shown by punctate lines of both DPK and Dsg3 (Fig. 1D).

Pathogenic Anti-Dsg3 Monoclonal Antibody AK23 Depletes Cells of Dsg3, but Did Not Affect the Levels of DPK and Dsg2: Semi-quantitative Immunoblot Analysis Normalized to PG, β-Catenin, and α-Tubulin—Because we have shown that stimulation with PV-IgG for 24 h causes a prominent reduction of Dsg3 from DJM-1 cells (18), we confirmed this result semi-quantitatively and examined whether the pathogenic anti-Dsg3 monoclonal antibody, AK23, similarly depletes cells of Dsg3, by semiquantitative immunoblot analysis of total cell lysates of cells exposed to AK23 mAb (0.5 mg/ml) and PV-IgG (0.5 mg/ml). Consistent with our previous study, PV-IgG treatment reduced the Dsg3 levels of total cell extracts ~90% after 24 h, whereas not at all after a 30-min treatment, by using any PG, β-catenin, or α-tubulin as an internal standard, demonstrating the robustness of this experimental system. Using this system, AK23 mAb also caused a significant decrease in the level of Dsg3, i.e. ~30% in total cell lysates after 24 h (Fig. 2A).

To determine which protein to use as an internal standard to normalize Dsg3 levels in repeated experiments using Triton X-100-soluble and -insoluble fractions, we compared the results of Dsg3 quantification normalized to PG, β-catenin, and α-tubulin using DJM-1 cells exposed to AK23 mAb (0.5 mg/ml) and PV-IgG (0.5 mg/ml) for 24 h (Fig. 2B). Normalization of Dsg3 levels to PG, β-catenin, or α-tubulin yielded nearly identical results in the Triton X-100-soluble pools (Fig. 2B), as shown in total cell lysates (Fig. 2A). However, when the samples of the Triton X-100-insoluble fraction were subjected to analysis, a faint amount of β-catenin and no α-tubulin were detected (Fig. 2B), suggesting that the size of the insoluble β-catenin and α-tubulin pools may be too small to detect compared with the levels of Dsg3. In addition, because the migrating distance for α-tubulin far exceeds that of PG, we selected PG as the internal standard to normalize Dsg3 levels for all remaining experiments. Although PG is a component of desmosomes, the amount of PG bound to Dsg3 is a small fraction of the total PG, the latter being bound to other desmosomal cadherins at desmosomes (i.e. Dsg1, Dsg3, desmocollins (Dsc) 1, Dsc2, and Dsc3), E-cadherins at adherens junctions, and distributed to soluble pools in the cell membranes and cytoplasm.

AK23 mAb Did Not Affect the Levels of DPK and Dsg2: Semi-quantitative Immunoblot Analysis—Because stimulation with AK23 mAb yielded complete loss of the punctate Dsg3 staining...
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

from cell-cell contacts (i.e., desmosomes), but did not eliminate DPK from cell-cell contacts, the effects of this mAb on another desmosomal cadherin, Dsg2, was examined by immunoblot analysis, as normalized to PG. Dsg3 was reduced 30 and 85% by treatment with AK23 and PV-IgG, respectively, whereas DPK and Dsg2 were not significantly altered. Bars represent standard deviations. Black bar, DPK/PG; gray bar, Dsg2/PG; white bar, Dsg3/PG.

Dose- and Time-dependent Effects of AK23 mAb on Dsg3 Depletion—To elucidate the dose and time dependence of the Dsg3-depleting effects of AK23 mAb, DJM-1 cells were exposed to a range of AK23 mAb concentrations, and the Dsg3 levels in the Triton X-100-soluble pools were examined at two time points (30 min and 2 h) following stimulation. The initial depleting activity is a function of concentration of AK23 mAb between 0.0 and 0.16 μg/ml (Fig. 4), whereas concentrations greater than 0.16 μg/ml did not decrease Dsg3 content proportionally; rather, Dsg3 depletion reached a plateau at ~35–40% reduction between 0.16 and 500 μg/ml. This maximum value was equivalent to that observed in cells treated with 0.5, 1.0, and 2.0 mg/ml AK23 mAb for 24 and 48 h (not shown).

The Dsg3 Depletion Plateaued at 30 Min in the Triton X-100-soluble and 24 h in Triton X-100-insoluble Pools by AK23 mAb Treatment—Previous studies have shown that Dsg3 is distributed into Triton X-100-soluble pools before being integrated into the desmosome(s), and into insoluble pools after integration into desmosomes (18, 19). In addition, we have shown that Dsg3 is already depleted to undetectable levels in the Triton X-100-soluble pools 20 min after PV-IgG treatment, whereas this is not the case in Triton X-100-insoluble pools, which required 24 h of PV-IgG treatment to deplete Dsg3 from this

DJM-1 cells were obtained after cells were exposed to AK23 mAb (0.5 mg/ml) and PV-IgG (0.5 mg/ml) for 30 min and 24 h. No significant differences were detected in the ratios of Dsg3 to PG, β-catenin (β-cat), or α-tubulin (α-tub) when total cell lysates were applied after 30-min and 24-h stimulations with AK23 mAb and PV-IgG (A). However, when cells were fractionated into Triton X-100-soluble and -insoluble fractions, α-tubulin was not detected and β-catenin was detected only with faint bands in the insoluble fraction, although they were semiquantitatively detected in soluble fraction similar to that in total cell lysates (B). Control, the ratios of Dsg3 to PG, β-catenin, and α-tubulin, respectively, when cells were treated with normal mouse IgG. Bars represent S.D. Statistical significances of differences were indicated with p (t test) in the figures.

FIGURE 2. Immunoblot to determine a proper internal standard for semi-quantitative analysis of Dsg3 after treatment with antibodies. The results of Dsg3 quantification normalized to PG, β-catenin, and α-tubulin using

FIGURE 3. Immunoblot of total cell lysates showing depletion of Dsg3 but not of DPK and Dsg2 after treatment with antibodies. The effects of 24-h treatments with AK23 mAb and PV-IgG on the levels of Dsg3, DPK, and Dsg2 were examined by immunoblot analysis, as normalized to PG. Dsg3 was reduced 30 and 85% by treatment with AK23 and PV-IgG, respectively, whereas DPK and Dsg2 were not significantly altered. Bars represent standard deviations. Black bar, DPK/PG; gray bar, Dsg2/PG; white bar, Dsg3/PG.
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

30 min

2 h

Dsg3

PG

FIGURE 4. Dose- and time-dependent effects of AK23 mAb on Dsg3 depletion. The Dsg3-depleting activity was found to be a function of AK23 mAb concentrations (between 0.0064 and 0.16 μg/ml), whereas it reaches a maximum value of ~40% reduction with antibody concentrations over 0.16 μg/ml. The % content of Dsg3 was obtained by taking a ratio of Dsg3/PG as 100% before treatments. Bars represent standard deviations.

pool (18). The levels of Dsg3 in Triton X-100-soluble pools were reduced ~45 and 90% in the cells treated with AK23 mAb and PV-IgG for 30 min, respectively (Fig. 5A, left panel). Similar reductions of Dsg3 were observed after both 30 min and 24 h (Fig. 5A, left panel), suggesting that the Dsg3 depletion from the Triton X-100-soluble pools may have reached a maximum/pla-teau level for both AK23 mAb and PV-IgG by 30 min. In contrast, the levels of Dsg3 in Triton X-100-insoluble pools were only depleted ~45% in PV-IgG-stimulated cells and no deple-
tion was detected in AK23 mAb-stimulated cells at 30 min. It should be noted, however, that at 24 h the levels of Dsg3 in the Triton X-100-insoluble pools of cells stimulated with AK23 mAb and PV-IgG for 24 h were reduced about 46 and 89%, respectively (Fig. 5A, right panel). This delay in Dsg3 depletion from the Triton X-100-insoluble pools suggests that Dsg3 depletion from plasma membrane could lead secondarily to Dsg3 depletion from desmosomes. Because the levels of Dsg3 depletion observed in all three conditions, i.e. Triton X-100-soluble pools obtained both at 30 min and 24 h, as well as the Triton X-100-insoluble pools at 24 h, were similar (Fig. 5A), the distribution of Dsg3 between Triton X-100-soluble and -insol-
uble pools in the cells stimulated with AK23 mAb and PV-IgG equilibrated between these two pools by 24 h. Comparable results were obtained with NHEK (Fig. 5B), as examined and compared in Triton X-100-soluble and -insoluble fractions from NHEK cells treated with AK23 mAb and PV-IgG for 24 h (Fig. 5A and B).

Dsg3-depleting Effect Correlates with Pathogenic Activity of Anti-Dsg3 Monoclonal Antibodies—It is of interest to know whether the Dsg3-depleting effect correlates with the pathogenic activity of anti-Dsg3 monoclonal antibodies. To answer this question, DJM-1 cells were treated with four different monoclonal anti-Dsg3 antibodies, i.e. AK18, AK19, AK20, and AK23 mAbs, of which, the AK23 mAb is the most pathogenic, with AK19 mAb being the second-most path-
ogenic; whereas AK18 and AK20 mAbs have very little pathogenic activities. The Dsg3-depleting activity correlated with the pathogenic activity in Triton X-100-sol-
able samples at 30 min after anti-
body treatments (Fig. 6, left panel), whereas no pathogenic activities for AK 18 and 20 were detected in the 24-h samples. Consistently, no Dsg3-depleting activity was de-
tected in Triton X-100-insoluble samples obtained after 30-min treatments with these antibodies, and only AK23 mAb treatment depleted Dsg3 36% after 24-h treatments (Fig. 6, right panel). These results suggest that only AK23 mAb can deplete desmo-

Combination Treatment with Different Anti-Dsg3 Mono-
clonal Antibodies Additively Depleted Dsg3—To examine the additive effects of non-AK23 mAb combined with AK23 mAb, cells were treated for 24 h with either control mouse IgG, AK23 mAb alone, two AK mAb combinations (i.e. AK23 plus AK18, AK19, or AK20), three AK mAb combinations (i.e. AK23 plus AK18/AK19, AK18/AK20, or AK19/AK20), or a mixture of four AK mAbs at a concentration of 0.125 mg/ml for each individual AK mAbs. The combination of AK23 mAb with AK18 or AK19 mAbs increased the Dsg3-depleting activity beyond that for AK23 mAb alone, whereas the combination with AK20 mAb did not alter Dsg3 depletion (Fig. 7A). In addition, AK23 mAb alone reduced Dsg3 34%, whereas the combination of AK18 or AK19 with AK23 mAbs yielded a 54 and 42% depletion from the Triton X-100-soluble pool, respectively. Moreover, the three AK mAb combination of AK23, AK18, and AK19 markedly reduced Dsg3 (~64%). However, addition of AK20 mAb with AK23 mAb or with other AK23 mAb combinations did not increase the Dsg3-depleting activity; as such, the Dsg3 depletion rates for AK23 mAb combined with AK23/AK20 mAbs show no significant differences. No significant differences in the Dsg3 depletion rates were evident for the combination of AK23/AK19 versus AK23/AK19/AK20 mAbs, or for the combination of AK23/AK18/AK19 compared with that of AK23/AK18/AK19/AK20 mAbs. Similar results also were observed in each of the Triton X-100-insoluble samples.

When cells were treated with control mouse IgG at 0.5 mg/ml, single AK23 mAb at 0.5 mg/ml, or a combination of
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

**FIGURE 5. Immunoblot of 1% Triton X 100-soluble and -insoluble fractions after treatment with antibodies.** DJM-1 cells (A) were treated with AK23 mAb, PV-IgG, and normal mouse IgG at a final concentration of 0.5 mg/ml for 30 min and 24 h. NHEK (B) were treated similarly for 24 h. The harvested cells were fractionated into the Triton X-100-soluble pool, i.e. cytosol/membrane fraction (*left panels of A and B*), and Triton X-100-insoluble pellets, i.e. cytoskeleton fraction (*right panels of A and B*). In all samples of 30-min and 24-h Triton X-100-soluble and 24-h Triton X-100-insoluble DJM-1 cells treated with PV-IgG, AK23 mAb, and normal mouse IgG, but not from 30-min Triton X-100-insoluble fractions, the same Dsg3-depleting activity profiles were observed; i.e. ~90, 45, and 0% reduction, respectively. In NHEK treated with AK23 mAb and PV-IgG for 24 h, both samples of Triton X-100-soluble and -insoluble fractions also revealed similar Dsg3 depletion profiles to those seen in DJM-1 cells. Bars represent S.D. Statistical significances of differences were indicated with *p* (t test) in the figures.

**FIGURE 6. Differences in effects of different AK single antibody treatments.** Cells were treated with each AK mAb (AK18, AK19, AK20, AK23) at a concentration of 0.5 mg/ml for 30 min and 24 h. Triton X-100-soluble pool, i.e. cytosol/membrane fraction (*left panel*), and Triton X-100-insoluble pellets, i.e. cytoskeleton fraction (*right panel*), were obtained as described under “Experimental Procedures.” The Dsg3-depleting activity is correlated with the pathogenic activity in the Triton X-100-soluble fraction, whereas only AK23 treatment shows significant Dsg3-depleting activity in the Triton X-100-insoluble fraction after a 24-h treatment. Nonpathogenic AK18 and AK20 mAbs did not decrease Dsg3 content of samples from the Triton X-100-soluble pool after 24 h and the Triton X-100-insoluble pools after both 30 min and 24 h. The % content of Dsg3 was obtained by taking a ratio of Dsg3/PG as 100%, when treated with mouse normal IgG. Bars represent S.D. Statistical significances of differences between control mouse IgG and AK23 mAb or its combination treatments were indicated with asterisks; *t* test, *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

**FIGURE 7B.** Equivalent results were obtained in Triton X-100-insoluble samples at 24-h treatments. Together, these results suggest that the combined activity of different anti-Dsg3 monoclonal antibodies, i.e. addition of four AK mAbs at 0.125 mg/ml for individual antibodies (final concentration of 0.5 mg/ml), similar results were obtained: single AK23 mAb treatment and a combination of four different AK mAbs yielded an approximate 34 and 70% reduction of Dsg3 content after a 30-min treatment, equivalent to the results obtained by using 0.125 mg/ml for individual AK mAbs. Furthermore, the combination of four AK mAbs caused the most prominent loss of Dsg3; i.e. 85% reduction at 24 h in the Triton X-100-soluble fractions (Fig. 7B). Equivalent results were obtained in Triton X-100-insoluble samples at 24-h treatments. Together, these results suggest that the combined activity of different anti-Dsg3 monoclonal antibodies, i.e. addition of non-AK mAbs to AK23 mAb, further enhances the Dsg3-depleting activity from desmosomes.

**Dsg3 Depletion from Cells Causes a Reduction of Mechanical Strength of Cell-Cell Adhesion.** To determine whether Dsg3 depletion, in particular from desmosomes, alters the mechanical strength of cell-cell adhesion, we performed a cell dissociation assay (see “Experimental Procedures”). Cells were cultured for 50 h, a point at which many small colonies are formed, but no fused larger colonies are present. This cell stage is used because the most prominent Dsg3 reduction by anti-Dsg3 antibody treatment is observed at this point.

The cells were stimulated with PV-IgG and AK23 mAb for 6 or 24 h and cell colonies were detached by dispase and washed, and then mechanical stress was given by pipetting 5 times with P1000 pipettman. The resultant smaller colonies (consisting of less than 30 cells) were counted, and the ratios of the number of total cells in these smaller colonies to that in the larger colonies were determined. The ratio obtained when treated with normal control IgG was used as the 100% strength index of cell-cell adhesion.

To facilitate cell counting, we used an adapted method with nuclear staining. The intensity of 4',6-diamidino-2-phenylindole fluorescence of cells spread on the glass slide after pipetting was measured. The fluorescence intensity of encircled colonies ranging from 1 to 300 cells was plotted versus the number of cells in the colony (Fig. 8A). Fluorescence intensity is a function of the cell number, with fluorescence intensity of 30,000 being equivalent to a cell number of 30. The cell number of individual colonies was thus determined.
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

The mechanical strength index of cell-cell adhesion was obtained using total cell number in colonies consisting of over 30 cells divided by the total cell number in colonies consisting of below 30 cells. Thus, if the number of cells in the smaller colonies consisting of less than 30 cells increases, the index value decreases.

After 6-h treatments with IgGs, the mechanical strength index of cell-cell adhesion for cells, which show a 27% reduction of Dsg3 content (Fig. 8C, 6 h), treated with AK23 was reduced ~36% (Fig. 8C, 6 h). For cells that show a 47% reduction of Dsg3 content (Fig. 8C, 6 h), treatment with either the four AK mAb mixture or PV-IgG alone reduced Dsg3 content ~50% compared with that of normal IgG-treated cells (Fig. 8B, 6 h). These results reveal that the mechanical strength index is roughly related to the Dsg3 content only when the residual Dsg3 content remains over 50%; however, this relationship is lost when the Dsg3 content is below this level. This conclusion is also supported by the observation that nonpathogenic AK20 mAb caused neither significant reduction of the mechanical strength index nor of Dsg3 content (Fig. 8, B and C, 6 and 24 h).

Interestingly, however, the decrease in the mechanical strength index of cell-cell adhesion was not exactly in proportion to the reduction of Dsg3, when cells were treated with IgGs for 24 h. Although both the mechanical strength indices of cells treated with the four AK mAb mixture or PV-IgG yielded a 51% reduction (Fig. 8B, 24 h), their Dsg3 reduction rates were significantly different, as such 63 and 85% reductions, respectively (Fig. 8C, 24 h). This difference may reflect the partial role of Dsg3 for determining mechanical strength; i.e., being ~50%, with the remaining 50% being compensated by other desmosomal and classical cadherins. Alternatively, this difference may reflect a limiting sensitivity for this method.

**DISCUSSION**

We provide here a novel finding that, similar to PV-IgG (18), a monoclonal antibody to a single extracellular domain of Dsg3 can deplete desmosomes of Dsg3, leaving Dsg2 and DPKs at cell-cell adhesion. This result indicates for the first time that no other antibodies besides the anti-Dsg3 antibody included in PV-IgG are required to deplete desmosomes of Dsg3. In addition, these results suggest that anti-Dsg3 antibody itself cannot completely eliminate desmosomal adhesive function(s), as cell-cell contacts remain in cultured DJM-1 cells, even with the depletion of Dsg3 from desmosomes. However, loss of Dsg3 from the desmosome does weaken the adhesive strength of this structure, as discussed below.

Regarding a distinctive difference in the Dsg3-depleting activity between the most pathogenic AK23 mAb and PV-IgG, which reduces cellular Dsg3 around 45 and 90%, respectively, we examined whether AK23 mAb can deplete cells of Dsg3 as much as PV-IgG, with increased dose and incubation times. Although AK23 mAb treatment dose-dependently increased Dsg3 activity at low AK23 concentrations (i.e., between 0.0 and 0.16 μg/ml), no more than 40–50% of the cellular Dsg3 could be depleted even at the highest mAb concentration (500 μg/ml), revealing a plateau beyond 0.16 μg/ml. This maximum Dsg3 depleting value was almost identical to those obtained in the cells treated with 0.5, 1.0, and 2.0 mg/ml AK23 mAb for 48 h (not shown), and never approached the higher depletion obtained by PV-IgG. Thus, the quantity of free (Triton X-100-soluble) Dsg3 is possibly determined by a balance of expression and degradation of Dsg3 in cells, which is in turn determined by binding activities to Dsg3, but not by quantity, of anti-Dsg3 monoclonal antibody present. This also suggests that AK23 mAb may have a specific association/binding constant with Dsg3 molecules, such that equilibrium between expression and degradation is established.

This result prompted us to further study whether the combination of AK23 mAb with other different monoclonal anti-
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

Dsg3 antibodies could increase the maximum level of Dsg3 depletion. Addition of three other AK monoclonal antibodies with AK23 mAb increased the Dsg3-depleting activity sufficient to reduce ∼70% of the cellular Dsg3. Because each monoclonal antibody is thought to possess a different association constant for Dsg3 binding depending on its distinct epitope, the combination of monoclonal antibodies may increase in the Dsg3-depleting activity either additively or synergistically. Therefore, it is likely that PV-IgG exerts its marked Dsg3-depleting activity due to its accumulative (additive or synergistic) effects of individual monoclonal antibodies contained in polyclonal PV-IgG.

The Dsg3-depleting activity of the four tested monoclonal antibodies also is correlated with their pathogenic activity(ies), suggesting that the depletion of Dsg3 from desmosomes plays a crucial role in the generation of blisters. It is worth noting that the most pathogenic and most Dsg3 depleting active AK23 mAb reacts with the NH2-terminal domain, 1–162 amino acids of mouse Dsg3, and that PV-patient IgG, which reacts with the equivalent NH2-terminal domain (1–161 amino acids) of human Dsg3, is associated with active PV, as studied by enzyme-linked immunosorbent assay utilizing recombinants (38).

Interestingly, additive/synergistic effects of non-AK23-mAb combination to AK23 mAb were exerted when cells were treated with combinations of AK23 plus AK18 or AK19, and of AK23 plus AK18/ AK19 for 24 h, whereas addition of AK20 mAb to any of these combinations does not cause any significant differences in Dsg3-depleting activity (Fig. 7A). This result suggests that binding of distinct monoclonal antibodies, but not AK20 mAb, to different Dsg3 epitopes can generate an additive/synergistic Dsg3-depleting activity. In this regard, it is of interest to note that using different mouse monoclonal anti-Dsg3 antibodies (NAK mAbs), synergistic pathogenic effects of combined monoclonal anti-Dsg3 IgG antibodies on pemphigus vulgaris blister formation has been shown in a dissociation assay in cultured mouse keratinocytes and in an antibody passive transfer model mouse (39).

The mechanism(s) by which anti-Dsg3 antibody(ies) induce Dsg3 depletion from desmosomes remain unresolved. However, the fate of Dsg3 after anti-Dsg3 antibody binds to the antigen has been rather well studied in cultured keratinocytes treated with PV-IgG, and in patient skin using immunofluorescence and immunochemistry (19, 20, 40–43). Our previous study (19) demonstrated that PV-IgG binding to Dsg3 causes the internalization of simple Dsg3 clusters, which represent precursors during desmosome assembly, into endosomes as an immune complex of PV-IgG/Dsg3. In addition, desmosomes do not split during the first 60-min chase period (19). It is of great interest to note that PV-IgG binding to Dsg3 induced rapid internalization from the cell surface in a complex with PG, but not with DPK (20). This observation supports our interpretation that anti-Dsg3 antibody initiates a degradation process for Dsg3 exclusive from DPK. Although PG appears to also associate with endosomes, this protein will not be digested by lysosomal enzymes as it is localized to the cytoplasmic side of the endosome, even if it should be detached and degraded. These findings suggest that anti-Dsg3 antibody binds to either Dsg3, which is unbound to keratin intermediate filaments and before integrating into desmosomes, or to Dsg3, in desmosomes and to induce release detachment from DPK and keratin intermediate filaments. In either case, the anti-Dsg3 antibody-
Dsg3 immune complex is internalized, resulting in the shortage of Dsg3, which in turn forms Dsg3-depleted desmosomes.

Recently, an interesting finding was reported on the fate of Dsg3 in HaCaT keratinocytes treated with PV patient serum, demonstrating that PV serum determines a reduction of Dsg3 half-life in cells, although the total amount of Dsg3 remains unchanged, suggesting that incorporation of PV-IgG-Dsg3 complexes into newly forming desmosomes appears to be perturbed (30). However, our present results clearly show that both PV-IgG and pathogenic AK mAbs deplete cells of Dsg3. This discrepancy may be explained by differences in cell strains and culture conditions, as such that we exposed a smaller number of cells to a larger amount of PV-IgG and AK mAbs at a small colony-forming stage, so that perturbation of Dsg3 incorporation into desmosomes causes a shortage of Dsg3 to form new desmosomes or to supply the desmosomal turnover requirements, resulting in marked depletion of Dsg3 from cells of both DJM-1 and NHEK.

A more recent study on the changes in Dsg1 expression and subcellular localization in cultured keratinocytes subjected to pemphigus foliaceus patient sera and anti-Dsg1 monoclonal antibody demonstrate that anti-Dsg1 antibody induced a transient internalization of Dsg1, and reduced the adhesion strength among keratinocytes. However, binding of IgG to Dsg1 did not correlate with its early depletion from the adhesion complexes, but rather reduced the amount of Dsg1 found in the Triton X-100 soluble pool of protein (44). This observation is consistent with our results for Dsg3 in response to anti-Dsg3 IgGs. In our present study on AK23 mAb-dependent Dsg3 depletion, the maximum reduction (45% reduction) of Dsg3 in Triton X-100-soluble pools was already obtained at the 30-min time point, whereas the levels of Dsg3 in the Triton X-100-insoluble pools was not altered at this same point (30 min); however, by 24 h, the Dsg3 levels in the Triton X-100-insoluble pool were also reduced to 45% of control levels (Fig. 5). These findings suggest that earlier Dsg3 depletion from the Triton X-100-soluble pool could lead secondarily to Dsg3 depletion from desmosomes.

We also present here the first evidence that Dsg3 depletion from desmosomes impairs cell-cell adhesive function, even though cells maintain desmosomal structures. Previously it has been shown that an in vitro keratinocyte dissociation assay, used to evaluate the pathogenicity of anti-Dsg3 IgG autoantibodies in PV, works well when keratinocytes express low Dsg2 levels and in the presence of exfoliative toxin A that specifically digests Dsg1 (36). However, these results in the deletion of most desmosomal cadherins other than Dsg3 and possibly Dsc3. Therefore, it is difficult to evaluate with this method the extent to which the partial loss Dsg3 alone from desmosomes contributes to the impairment of cell-cell adhesion. In the present study, we developed an adapted in vitro dissociation assay that precludes the deletion of other desmosomal cadherins, using an early stage of cell culture, at which a large number of smaller colonies are formed but have not yet fused into larger (confluent) colonies. The Dsg3 depletion from cell-cell contacts induced by treatment with AK23 mAb and PV-IgG was the most prominent, with almost complete disappearance of signal from cells at the colony periphery. Therefore, the formation of numerous small colonies increases the population of cells susceptible to Dsg3 depletion by anti-Dsg3 antibodies. Because the loss of Dsg3 in the central area of individual colonies was not extensive, pipetting cannot readily dissociate colonies, but rather, smaller colony fragments are generated from the periphery of colonies. Thus we counted the total cell number of the resultant smaller colonies consisting of less than 30 cells, and determined the ratio of the number of total cells in these smaller colonies to that of the larger colonies. The ratio obtained with normal control IgG treatment was set as the 100% strength index for cell-cell adhesion. Although this method did not reveal differences in the extent of cell-cell adhesive impairment with 50–90% reduction of Dsg3 in cells, it readily demonstrates that a partial depletion of Dsg3 (around 50%) causes a distinctive decrease in cell-cell adhesion strength. The extreme condition of Dsg3 depletion is observed in the case of Dsg3 knock-out mouse, which shows a Nikolsky sign (i.e. acantholysis due to mechanical stress) that is characteristically seen in the skin of patients with PV (29). Therefore, it is rational to suggest that Dsg3 depletion from cells, including desmosomes, may contribute to blistering due to the reduction of cell-cell adhesive strength. However, to generate blisters (i.e. acantholysis), other factors, such as mechanical stress on the skin and some cell biological responses of keratinocyte, such as outside-in signaling, generated by anti-Dsg3 antibody binding, may also be involved.

In summary, our results that anti-Dsg3 antibody activity alone can generate Dsg3 depletion from desmosomes; without effects due to any other factors, such as antibodies against other peptides on the keratinocyte surface that might be included in PV-IgG from patients. In addition, our results demonstrate that individual monoclonal anti-Dsg3 antibodies have their own characteristic limitation of Dsg3-depleting activity, which correlates with their pathogenic activity. Moreover, the combination of monoclonal antibodies exerts a cumulative or synergistic effect, which may explain the robust Dsg3-depleting capacity of PV-IgG. Finally, although Dsg3-depletion activity is correlated with AK mAb pathogenicity in mouse models, the residual level of Dsg3, when below ~50%, does not correlate with the strength index of cell-cell adhesion as determined by our preset method. This may suggest that the Dsg3 depletion is not indicative for adhesive strength in the presence of other desmosomal cadherins, or that blister formation in patients may require other factors. Thus, it is conceivable that the level of Dsg3 can be used as a read-out of pathogenic changes within the cell. These results suggest that the Dsg3 depletion from desmosomes due to anti-Dsg3 antibody activity contained in PV-IgG plays an important role in the skin fragility or susceptibility to blister formation in PV patients.

Acknowledgment—We greatly thank Dr. Walter M. Holleran (University of California, San Francisco, San Francisco, CA) for review of this manuscript.

REFERENCES
1. Eyre, R. W., and Stanley, J. R. (1988) J. Clin. Investig. 81, 807–812
2. Hashimoto, T., Ogawa, M. M., Konohana, A., and Nishikawa, T. (1990) J. Investig Dermatol. 94, 327–331
Anti-Dsg3 Monoclonal Antibodies Deplete Dsg3

3. Buxton, R. S., Cowin, P., Franke, W. W., Garrod, D. R., Green, K. J., King, I. A., Koch, P. J., Magee, A. I., Rees, D. A., and Stanley, J. R. (1993) J. Cell Biol. 121, 481–483
4. Kitajima, Y. (2003) Arch. Dermatol. Res. 295, S17–S23
5. Koul, L., Kusurni, A., Steinberg, M. S., Klaus-Kovtun, V., and Stanley, J. R. (1984) J. Exp. Med. 160, 1509–1518
6. Andrew, P., Kowalezyk, A. P., Anderson, J. E., Bougwardt, J. E., Hashimoto, T., Stanley, J. R., and Green, K. J. (1995) J. Investig. Dermatol. 105, 147–152
7. Karpati, S., Amagai, M., Prussick, R., Cehrs, K., and Kitajima, Y. (1997) J. Cell Biol. 122, 409–415
8. Amagai, M., Koch, P. J., Nishikawa, T., and Stanley, J. R. (1996) J. Investig. Dermatol. 106, 351–355
9. Mahoney, M. G., Wang, Z., Rothenberger, K., Koch, P. J., Amagai, M., and Stanley, J. R. (1999) J. Clin. Investig. 103, 461–468
10. Stanley, J. R., and Amagai, M. (2006) N. Engl. J. Med. 355, 1800–1810
11. Kitajima, Y., Aoyama, Y., and Seishima, M. (1999) J. Investig. Dermatol. Symp. Proc. 4, 137–144
12. Amagai, M., Karpati, S., Prussick, R., Klaus-Kovtun, V., and Kitajima, Y. (1992) J. Clin. Investig. 90, 919–926
13. Sekiguchi, M., Futai, Y., Fujii, Y., Iwasaki, T., Nishikawa, T., and Amagai, M. (2001) J. Immunol. 167, 5439–5448
14. Futai, Y., Amagai, M., Hashimoto, T., and Nishikawa, T. (2003) J. Am. Acad. Dermatol. 49, 1023–1028
15. Kitajima, Y., Inoue, S., and Yaoita, H. (1987) J. Investig. Dermatol. 89, 167–171
16. Kitajima, Y. (2002) Clin. Exp. Dermatol. 27, 684–690
17. Aoyama, Y., Owada, M. K., and Kitajima, Y. (1999) Eur. J. Immunol. 29, 2233–2240
18. Aoyama, Y., and Kitajima, Y. (1999) J. Investig. Dermatol. 112, 67–71
19. Sato, M., Aoyama, Y., and Kitajima, Y. (2000) Lab. Investig. 80, 1583–1592
20. Calkins, C. C., Setzer, S. V., Jennings, J. M., Summers, S., Tsunoda, K., Amagai, M., and Kowalezyk, A. P. (2006) J. Biol. Chem. 281, 7623–7634
21. Osada, K., Seishima, M., and Kitajima, Y. (1997) J. Investig. Dermatol. 108, 482–487
22. Seishima, M., Satoh, S., Nojiri, M., Osada, K., and Kitajima, Y. (1997) J. Investig. Dermatol. 109, 650–655
23. Esaki, C., Seishima, M., Yamada, T., Osada, K., and Kitajima, Y. (1995) J. Investig. Dermatol. 105, 329–333
24. Pelacho, B., Natal, C., Espana, A., Sanchez-Carpintero, I., Iraburu, M. J., and Lopez-Zabalza, M. J. (2004) FEBS Lett. 566, 6–10
25. Puviani, M., Marconi, A., Cozzani, E., and Pincelli, C. (2003) J. Investig. Dermatol. 120, 164–167
26. Wang, X., Bregegere, F., Frusic-Zlotkin, M., Feinmesser, M., Michel, B., and Milner, Y. (2004) Apoptosis 9, 131–143
27. Caldelari, R., de Bruin, A., Baumann, D., Suter, M. M., Bierkamp, C., Balmer, V., and Muller, E. (2001) J. Cell Biol. 153, 823–834
28. Berkowitz, P., Hu, P., Liu, Z., Diaz, L. A., Enghild, J. J., Chua, M. P., and Rubenstein, D. S. (2005) J. Biol. Chem. 280, 23778–23784
29. Koch, P. J., Mahoney, M. G., Ishikawa, H., Pulkkinen, L., Uitto, J., Shultz, L., Murphy, G. F., Whitaker-Menezes, D., and Stanley, J. R. (1997) J. Cell Biol. 137, 1091–1102
30. Cirillo, N., Gombos, F., and Lanza, A. (2006) FEBS Lett. 580, 3276–3281
31. Amagai, M., Ahmed, A. R., Kitajima, Y., Bystryn, J. C., Milner, Y., Gnia-decki, R., Hertl, M., Pincelli, C., Kurzen, H., Fridkis-Harel, M., Aoyama, Y., Frusic-Zlotkin, M., Muller, E., David, M., Mimouni, D., Vint-Kezunovic, D., Michel, B., Mahoney, M., and Grando, S. (2006) Exp. Dermatol. 15, 815–831
32. Tsunoda, K., Ota, T., Aoki, M., Yamada, T., Nagai, T., Nakagawa, T., Koyasu, S., Nishikawa, T., and Amagai, M. (2005) J. Immunol. 170, 2170–2178
33. Ishii, K., Amagai, M., Hall, R. P., Hashimoto, T., Takayanagi, A., Gamou, S., Shimizu, N., and Nishikawa, T. (1997) J. Immunol. 159, 2010–2017
34. Huen, A. C., Park, J. K., Godiel, L. M., Chen, X., Bannon, L. J., Amargo, E. V., Hudson, T. Y., Mongiu, A. K., Leigh, I. M., Kelsell, D. P., Gumbiner, B. M., and Green, K. J. (2002) J. Cell Biol. 159, 1005–1017
35. Ishii, K., Harada, R., Matsuo, I., Shirakata, Y., Hashimoto, K., and Amagai, M. (2005) J. Investig. Dermatol. 124, 939–946
36. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Biophotonics Intl. 11, 36–42
37. Muller, R., Svoboda, V., Wenzel, E., Gebert, S., Hunzelmann, N., Muller, H. H., and Hertl, M. (2006) Exp. Dermatol. 15, 606–614
38. Kawasaki, H., Tsunoda, K., Hata, T., Ishii, K., Yamada, T., and Amagai, M. (2006) J. Investig. Dermatol. 126, 2621–2630
39. Tsunoda, K., Ota, T., Aoki, M., Yamada, T., Nagai, T., Nakagawa, T., Koyasu, S., Nishikawa, T., and Amagai, M. (2005) J. Investig. Dermatol. 124, 939–946
40. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Biophotonics Intl. 11, 36–42
41. Muller, R., Svoboda, V., Wenzel, E., Gebert, S., Hunzelmann, N., Muller, H. H., and Hertl, M. (2006) Exp. Dermatol. 15, 606–614
42. Kawasaki, H., Tsunoda, K., Hata, T., Ishii, K., Yamada, T., and Amagai, M. (2006) J. Investig. Dermatol. 126, 2621–2630
43. Tsunoda, K., Ota, T., Aoki, M., Yamada, T., Nagai, T., Nakagawa, T., Koyasu, S., Nishikawa, T., and Amagai, M. (2005) J. Investig. Dermatol. 124, 939–946
44. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Biophotonics Intl. 11, 36–42