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Pathogenic Anti-Desmoglein MAbs Show Variable ELISA Activity because of Preferential Binding of Mature versus Proprotein Isoforms of Desmoglein 3

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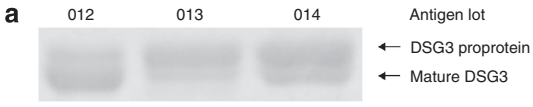
TO THE EDITOR

The desmosomal cadherins desmoglein (DSG) 3 and DSG1 are targets of autoantibodies in the potentially fatal blistering disease, pemphigus vulgaris (PV) (Stanley and Amagai, 2006). DSGs are synthesized as preproproteins, which are processed first in the endoplasmic reticulum to remove the signal sequence and subsequently by the Golgi proprotein convertases to remove the propeptide before transport to the cell surface. The cadherin propeptide is thought to modulate the conformation of the extracellular domains to prevent intracellular aggregation because of interaction with other cadherins within the secretory pathway. Propeptide cleavage occurs upstream of the conserved tryptophan residue at position 2, which is responsible for cadherin strand dimer formation, suggesting that propeptide removal may unmask residues important in intermolecular adhesion. The proprotein convertase furin processes recombinant DSGs in baculoviral overexpression systems (Posthaus *et al.*, 2003), which are widely used for pemphigus research and clinical diagnostic purposes. Commercial DSG ELISA kits use baculovirally produced recombinant DSG antigen (Ag) and have been shown to be a sensitive and specific diagnostic tool for pemphigus (Ishii *et al.*, 1997).

Abbreviations: Ag, antigen; DSG3, desmoglein 3; PV, pemphigus vulgaris



b

Species	PV mAb	Pathogenic	DSG3 deduced a.a. epitope	Conformational epitope
Human	P1	Yes	1–161	Yes
	P2	Yes	1–88	No
	P3	Yes	1–161	Yes
	NP1	No	ND	No
	NP2	No	1–161	No
Mouse	AK23	Yes	1–60	Yes
	AK15	No	195–565	No
	AK18	No	195–565	No

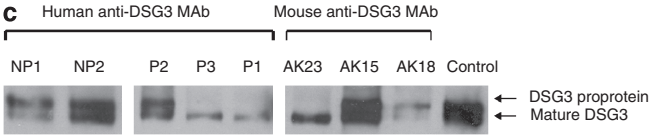


Figure 1. Pathogenic anti-DSG MAbs recognizing conformational epitopes selectively immunoprecipitate mature DSG3. (**a**) SDS-PAGE and coomassie staining of DSG3 antigen for three sequential lots of baculovirally produced recombinant DSG3 (data provided by MBL). (**b**) Immunochemical properties of human and mouse anti-DSG MAbs (Amagai *et al.*, 2000; Payne *et al.*, 2005). ND, not determined. (**c**) Immunoprecipitation of recombinant DSG3 baculoviral supernatants by pathogenic and non-pathogenic human and mouse anti-DSG3 MAbs. Control shows total recombinant DSG3 input, harvested by metal affinity chromatography. Immunoprecipitates were separated by SDS-PAGE, followed by immunoblotting using a horseradish peroxidase-coupled anti-E tag secondary antibody.

Earlier, pathogenic anti-DSG3 MAbs were isolated from human patients and PV model mice (Amagai *et al.*, 2000; Payne *et al.*, 2005). We have recently observed decreased ELISA binding by some pathogenic PV MAbs, despite consistent pathogenicity against endogenously expressed DSG3 in human keratinocytes. We hypothesized that the variability in pathogenic PV MAb ELISA was because of differential binding of mature DSG3 versus DSG3 proprotein, as the proprotein is commonly observed in recombinant Ags purified from baculoviral overexpression systems. We requested the Ag data from Medical and Biological Laboratories Co., Ltd (MBL International, Woburn, MA), the commercial distributor for DSG ELISA. Interestingly, an increase in purified DSG3 proprotein was observed when Ag production methods switched from stationary plate culture (Figure 1a, lot 012) to roller bottle culture (Figure 1a, lots 013 and 014). Baculoviral roller bottle or spinner culture often results in a higher yield of recombinant proteins than stationary plate systems. However, as suggested by Figure 1a, increased cell lysis associated with these cultures can cause the release of immature DSG3 proprotein into culture supernatants.

We evaluated a panel of pathogenic and non-pathogenic human and mouse anti-DSG MAbs (summarized in Figure 1b) for their ability to immunoprecipitate proprotein and mature DSG3 isoforms from recombinant baculoviral culture supernatants. Human pathogenic PV MAbs, P1 and P3, and mouse pathogenic MAb, AK23, selectively immunoprecipitate mature DSG3 (Figure 1c). In contrast, human non-pathogenic MAbs, NP1 and NP2, mouse non-pathogenic MAbs, AK15 and AK18, and one human pathogenic MAb, P2 (which recognizes a non-conformational epitope), immunoprecipitate both mature and proprotein isoforms.

To confirm that furin proprotein convertase cleaves the DSG3 propeptide, recombinant DSG3 was purified from baculoviral supernatants by metal affinity chromatography and incubated with furin (20 U/mg) for 16 hours at room temperature in the manufacturer's recommended buffer (New England BioLabs, Ipswich, MA). Figure 2a shows that furin effectively processes DSG3 proprotein into the mature DSG3 isoform.

To evaluate whether altered ratios of DSG3 isoforms affect ELISA binding by anti-DSG3 MAbs, we treated current commercial DSG3 ELISA wells with furin enzyme (2 U/well in Tris-buffered saline plus 1 mM $CaCl_2$ for 1 hour at room temperature) before incubation with anti-DSG3 MAbs. Furin treatment increases the ELISA binding of all human pathogenic MAbs (P1, P2, and P3), as well as the mouse pathogenic MAb, AK23. Furin treatment also modestly increases the binding of human non-pathogenic NP2 MAb, which recognizes a non-conformational epitope in the amino-terminal domain of DSG3 (Figure 2b). Furin treatment shows no significant effect on the binding of other non-pathogenic human and mouse MAbs.

As increases in proprotein Ag levels seem to disproportionately decrease the binding of pathogenic versus non-pathogenic PV MAbs, we sought to determine whether the clinical performance of the DSG3 ELISA would be affected by the variability of Ag isoforms. MBL produced custom mature DSG3 ELISA plates by furin treatment of DSG3 before Ag adsorption (as shown in Figure 2a). A pilot study of 85 independent PV patient sera indicates that use of the mature DSG3 Ag does not change the diagnostic result compared with the current DSG3 ELISA. However, in 30 of the 85 samples, use of mature DSG3 ELISA increases the serum index value by 15% or more compared with the current DSG3 ELISA (range 15–33%), whereas only 1 of 85 samples shows a decrease in the index value of $\geqslant$15% (value = 15%) (demarcated by the 45 degree dashed line in Figure 2c). The mean serum index value increased from 116 to 129 with use of the mature DSG3 ELISA, which was statistically significant by the paired *t*-test analysis ($P = 1 \times 10^{-14}$). Similar binding of DSG3 isoforms between the two kits was confirmed by anti-E tag ELISA (unpublished data).

In summary, our results indicate that pathogenic PV MAbs preferentially bind epitopes in mature DSG3 that are masked in the proprotein isoform. In contrast, non-pathogenic anti-DSG3 MAbs recognize both mature and proprotein isoforms, correlating with the

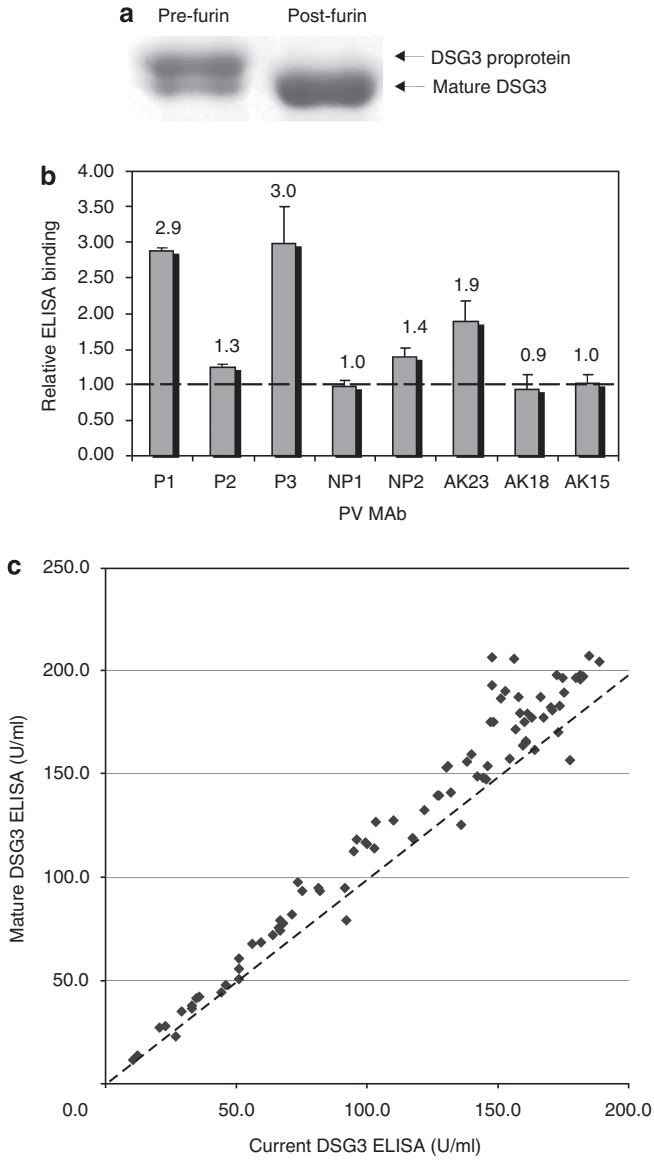


Figure 2. Furin treatment increases the ratio of mature DSG3 versus proprotein, resulting in increased ELISA binding by pathogenic anti-DSG MAbs and PV patient serum. (**a**) Purified recombinant DSG3 was treated with furin enzyme as described in the text, resulting in effective processing of DSG3 proprotein into its mature isoform. (**b**) Furin treatment of commercial DSG3 ELISA increases binding of pathogenic PV Mabs, P1, P2, P3, and AK23, as well as one non-pathogenic PV MAb (NP2) that binds an amino-terminal epitope. (**c**) 85 independent PV sera were tested by ELISA using current kits (containing a mixture of mature and proprotein DSG3) and custom kits produced with mature DSG3 antigen. Dashed line, 45-degree concordance.

binding of non-conformational DSG epitopes. Earlier studies have shown that pathogenic pemphigus antibodies more often bind conformational epitopes in the amino-terminal domain of DSGs, whereas non-pathogenic antibodies bind non-conformational epitopes (Sekiguchi *et al.*, 2001; Li *et al.*, 2003; Payne *et al.*, 2005; Ishii *et al.*, 2008). Therefore, a predominance of proprotein in the DSG3 ELISA might bias the test to the detection of non-pathogenic antibodies. Although the clinical diagnostic value of the ELISA is unaffected by variability in the DSG3 isoform (Figure 2c), we would predict that the mature DSG3 ELISA would correlate better with disease activity. Our study does not directly evaluate this hypothesis, although a concurrent study supports this conclusion (Yokouchi *et al.*, 2009). Commercial DSG ELISA plates will use mature DSG3 Ag, cleaved with furin before adsorption, beginning in December 2008 (lots 101 and up). Ongoing research studies may note changes in optical density values using the new ELISA kits. These findings are relevant for physicians and scientists using baculovirally produced recombinant DSG3 for clinical and basic research studies, including the use of ELISA to track disease activity and for the evaluation of human and mouse anti-DSG MAbs.

CONFLICT OF INTEREST

Keiko Kuroda and Takahisa Hachiya are employees of the Medical and Biological Laboratories Co., Ltd, the commercial distributor of the desmoglein ELISA. Preety M. Sharma, Eun Jung Choi, Ken Ishii, and Aimee S. Payne declare no conflict of interest.

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See related commentary on pg 2096

Non-Neuronal Expression of Transient Receptor Potential Type A1 (TRPA1) in Human Skin

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TO THE EDITOR

The temperature-sensitive channels, which belong to the transient receptor potential (TRP) superfamily, play an important role in skin biology. In addition to being expressed in sensory neurons, several members of this family, TRPV1, TRPV3, and TRPV4, which are activated by warm to hot temperatures (>42, >34–38, and >27–34 °C, respectively), are broadly expressed in non-neuronal cells of the skin and are involved in the control of keratinocyte differentiation, inflammatory skin responses, and hair growth (reviewed in Bíró et al., 2007).

TRPA1 is a distant family member of the TRP superfamily channels, which is localized in a subset of nociceptive sensory neurons, and showed a response to cold temperature starting nearly at 17 °C, the threshold of noxious cold for humans (Story *et al.*, 2003). TRPA1 can also be activated by the number of pungent natural compounds, environmental irritants, and formalin, as well as by endogenous proalgesic agents (Bandell *et al.*, 2004; McNamara *et al.*, 2007; Trevisani *et al.*, 2007). In addition, TRPA1 is capable of mediating acute and inflammatory pain, at least in part, through crosstalk with the signaling pathway induced by the proinflammatory peptide, bradykinin (Bautista *et al.*, 2006). However, the expression and functions of TRPA1 in non-neuronal cells in skin remain as yet unknown.

The aim of this study was to explore a role of TRPA1 in skin biology by studying its expression in distinct cutaneous cell populations (keratinocytes, fibroblasts, and melanocytes) as well as by assessing whether pharmacological activation of TRPA1 would have effects on gene expression programs in epidermal keratinocytes. Human scalp skin samples were obtained from five patients after face-lift surgery, with written consents approved by the Institutional Review Board to ensure subject protection and adherence according to the Declaration of Helsinki Principles. By real-time PCR analysis (see Supplementary Material), the TRPA1 mRNA expression was observed in primary cultures of human epidermal keratinocytes, melanocytes, and fibroblasts (Figure 1a). Relative quantification revealed that TRPA1 mRNA levels were higher in melanocytes than that in fibroblasts and keratinocytes. By western blot analysis, the TRPA1 protein expression was also seen in all cell types examined, and its expression levels in fibroblasts were relatively higher than those in melanocytes and keratinocytes (Figure 1b). To determine localization of TRPA1 in skin, we performed immunofluorescence analysis. TRPA1 immunoreactivity was detected in the basal layer of the epidermis, in the dermis, and in the epithelium of the hair follicle. By double immunofluorescence, we observed colocalization of TRPA1 with the melanocyte marker, pMel-17, in the distinct cells of the basal layer of the epidermis, suggesting that TRPA1 is expressed in the keratinocytes as well as in the melanocytes (Figure 1c and d; for details, see also Supplementary Text).

Epidermal keratinocytes serve as first line of defense that protects organism from environmental stressors, including cold temperature and chemical irritants, which are capable of activating TRPA1. To explore a possible functional role for TRPA1 in the epidermis, primary normal human epidermal keratinocytes were treated with the pharmacological TRPA1 agonist, icilin (10 μM; 24 hours) (Werkheiser *et al.*, 2006; Doerner *et al.*, 2007). Comparative analysis of global gene expression profiles in keratinocytes treated with icilin and vehicle control was performed using Agilent microarray technology (Santa Clara, CA) and real-time PCR (Supplementary Text).

Microarray analysis of the icilin-treated and control keratinocytes showed two-fold or higher changes in the expression of 241 genes encoding the adhesion/extracellular matrix molecules, in cell cycle/apoptosis and cytoskeleton/cell motility markers, and in molecules involved in the control of cell differentiation, metabolism, signaling, and transcription (Figure 2a and b,

Abbreviations: GDF15, growth differentiation factor 15; HSP, heat shock protein; TRP, transient receptor potential