Clinical Implications

- IL-4 and IL-13, which are Th2 cytokines, downregulate the expression of keratins, filaggrin, and desmosomal cadherins, as well as tight junction components, via an IL-4 receptor-alpha- and STAT6-dependent mechanism.
- The suppression of these proteins leads to fragmentation of cultured keratinocytes through mechanical stress.
- Th2 cytokine–induced instability of the epidermis may have a role in the pathogenesis of atopic dermatitis.

barrier–related proteins at multiple levels. In particular, IL-4 and IL-13, two major Th2 cytokines, suppress the synthesis of proteins that are important at different points in the differentiation process (Figure 1, right panel. IL-4 and IL-13 are highlighted by red-colored letters, because they are studied by Omori-Miyake *et al.* (2014) in this issue of *JID*.).

Thus, keratinocyte differentiation processes are highly regulated by these cytokines. This leads to the concept that abnormal expression of cytokines in the skin, which is evident in AD, may disrupt the skin barrier. Moreover, cytokines were reported to change the synthesis and localization of the tight junction proteins in previous studies (Capaldo and Nusrat, 2009; Figure 1, right panel).

Until recently, AD was thought to develop through immunological mechanisms, particularly through the activities of Th2 cells, which produce a number of Th2 cytokines, including IL-4, IL-5, IL-6, IL-10, IL-13, and GM-CSF (Brandt and Sivaprasad, 2011; Leung, 2013). Among the Th2 cytokines, IL-4 and IL-13 have been studied most extensively (Brandt and Sivaprasad, 2011). In particular, the signal transduction study downstream of IL-4 binding of type I IL-4 receptor and IL-13 binding to type II IL-4 receptor/IL-13 receptor revealed the importance of activated Jak kinase followed by the activation of STAT6.

However, more recently, mutations in the filaggrin gene have been identified in AD patients at significantly high rates, suggesting an important role for the disruption of the skin barrier in AD (Leung, 2013). At about the same time, tight junctions were proven to be present in the epidermis (Tunggal *et al.*, 2005; Kim and Leung, 2012) and to function in the activation of immunological cells, including Langerhans cells. This has suggested that abnormal tight junction function might also be related to AD development.

In contrast, the role of structural components and cell adhesion molecules, which are important for maintaining epidermal stability and integrity, in the development of AD has not been fully examined. In this issue of *JID*, Omori-Miyake *et al.* (2014) report that IL-4 and IL-13, which regulate STAT6

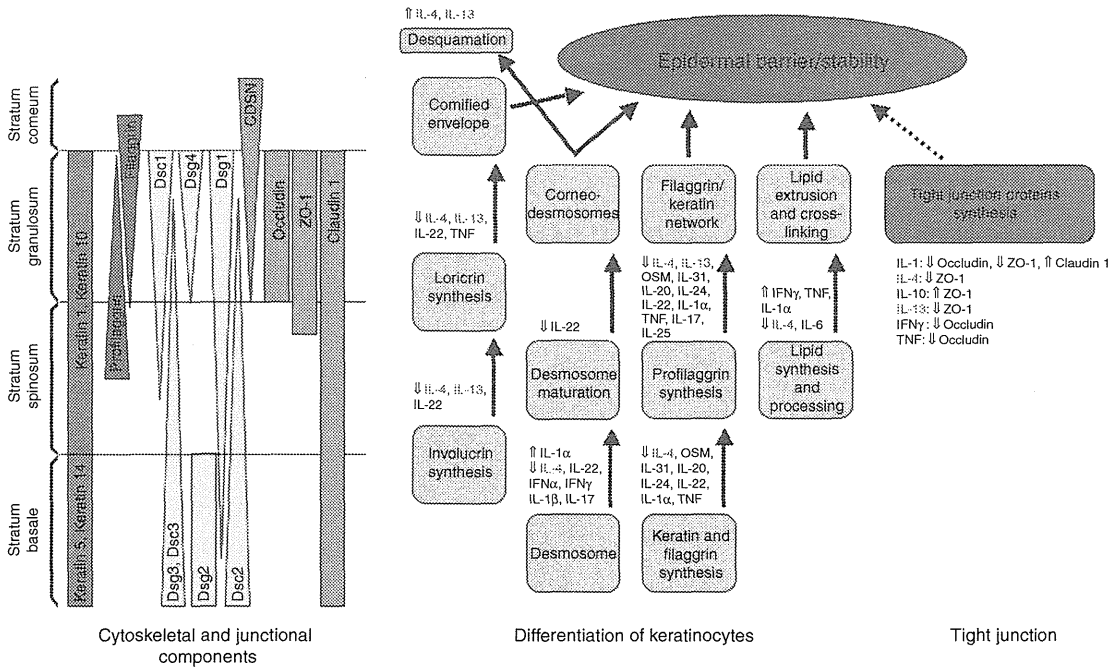

Figure 1. Schematic diagram of four layers of the epidermis, expression profiles of keratins, filaggrin, and desmosomal and tight junction proteins, five terminal differentiation processes, and regulation by cytokines. Left panel shows four distinct epidermal layers and expression patterns of four keratins, filaggrin, four Dsgs, three Dscs, corneodesmosin, and tight junction proteins. Right panel shows detailed information for five distinct epidermal differentiation processes, as well as various cytokines, which upregulate or downregulate the epidermal differentiation. IL-4 and IL-13 are shown in red-colored letters. The left panel was adapted from the figure in the reference of Simpson *et al.* (2011), and the right panel was adapted from figures in the reference of Hänel *et al.* (2013) and Gutowska-Owsiak and Ogg (2013).

and p44/42MAPK signaling, suppress the expression of structural components and cell adhesion molecules in murine and human keratinocyte cultures.

Omori-Miyake *et al.* (2014) first observed that IL-4 would suppress the expression of Dsg1, Dsc1, keratin 1, and keratin 10 at both mRNA and protein levels in murine and human cultured keratinocytes. The authors confirmed that the regulation by IL-4 was dependent on IL-4 receptor-alpha and STAT6.

Moreover, the suppression of expression of Dsg1, keratin 1, and keratin 10 by IL-4 was prevented by the addition of an MEK inhibitor, suggesting that the suppression was regulated via p44/42MAPK signaling. Addition of the p38MAPK inhibitor did not alter the suppressed expression of Dsg1, keratin 1, or keratin 10 by IL-4, suggesting that p38MAPK is not responsible in this suppression. The suppression of Dsc1 expression by IL-4 was not prevented by the addition of the MEK inhibitor, suggesting the important role of STAT6 signaling in Dsc1 expression.

Similar suppressive effects were also found by the addition of IL-13 in parallel experiments. In contrast, the addition of IL-5 did not cause a suppressive effect. These results suggest that IL-4 and IL-13 have important roles in the pathogenesis of AD.

Omori-Miyake *et al.* (2014) also examined mRNA expression levels of keratins and desmosomal components in IL-4 receptor-alpha chain–deficient keratinocytes. No reduction in mRNA expression for these proteins was observed by the addition of IL-4 and IL-13 in these deficient keratinocytes, confirming that IL-4 and IL-13 exerted their effects via the IL-4 receptor-alpha chain.

Finally, the addition of IL-4 and IL-13 to cultured HaCaT cells led to cell fragmentation through downregulation of expression of Dsg1, Dsc1, keratin 1, and keratin 10. The authors speculated that AD may develop or be exacerbated by the disruption of epidermal stability owing to the suppression of structural components and cell adhesion molecules by Th2 cytokines.

This work is the first comprehensive study of the role of Th2 cytokines on the suppression of structural components and cell adhesion molecules, leading to reduced stability and integrity of keratinocytes. It indicates that in addition to immunological and allergic mechanisms, as well as filaggrin mutation and tight junction–related changes in the skin barrier, the stability and integrity of the epidermis itself, which are regulated by cytokines, may also have important roles in AD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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MEETING REPORT

From Epidemiology and Genetics to Diagnostics, Outcome Measures, and Novel Treatments in Autoimmune Bullous Diseases

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The International Pre IID 2013 Satellite Meeting on Autoimmune Bullous Diseases (AIBDs) was held in Lübeck, Germany, from 6th to 7th May 2013, preceding the International Investigative Dermatology (IID) meeting in Edinburgh, UK. This Satellite Meeting followed the tradition of the IID-associated symposia on AIBDs in Kyoto (1993), Salzburg (1998), and Otsu (2008). In total, 180 international researchers and clinicians attended the meeting.

Plenary lectures covered the following topics: (i) epidemiology and genetics of AIBDs; (ii) diagnosis and novel disease entities; (iii) cell biology of desmosomes and disease pathways; (iv) cell biology of hemidesmosomes and disease pathways; and (v) outcome measures and novel treatments. In the exhibit of posters, approximately 60 abstracts were presented. The meeting was flanked by two satellite symposia. Importantly, the meeting provided ample time for interactive discussions, critical analyses of the progress achieved to date, and the identification of questions that still needed to be resolved.

PLENARY PRESENTATIONS

Epidemiology and genetics of AIBDs

Here, two speakers independently indicated a sharp increase in bullous pemphigoid (BP) incidence in various European regions. Moreover, increased mortality rates as well as an association with neurological diseases were noted (Langan *et al.*, 2008). In addition to this epidemiological research, unraveling the genetic predisposition of AIBDs will aid in future clinical decision making and facilitate personalized medical care. The results of the first genome-wide association study (GWAS) in pemphigus vulgaris patients were presented. The study, which was recently published in this journal, led to the identification of ST-18 as a susceptibility gene in pemphigus vulgaris (Sarig *et al.*, 2012). In addition to GWAS, functional and metagenomic genetic studies are expected to provide further insights into AIBD pathogenesis. As an example of a functional study, a strong association was reported between allelic and copy number variations in human Fc gamma receptor genes and an increased susceptibility to AIBD. This study was paralleled by investigations on the impact of these variations on neutrophil function *in vitro*. As an example of a metagenomic study, data on gene interactions, microbiota, and autoimmune disease susceptibility were presented, which subsequently have also been published (Srinivas *et al.*, 2013).

Diagnosis and novel disease entities

A new terminology for AIBD was discussed focusing on both the target antigen and autoantibody isotype. This classification is based on our growing understanding of the molecular biology of AIBD, including the identification of novel autoantigens (Dainichi *et al.*, 2009). In addition, novel methods for serological AIBD diagnosis, such as biochip mosaic-based and bioplex-based techniques, were discussed. Furthermore, the practical importance of differentiating epidermolysis bullosa acquisita (EBA) from other sub-epidermal AIBDs, such as BP, using direct immunofluorescence microscopy of the patient's skin and determining the fluorescence pattern (e.g., either "n-serrated" or "u-serrated) was demonstrated with audience participation (Terra *et al.*, 2013).

Cell biology of desmosomes and disease pathways

Regarding pemphigus, the characterization of anti-desmoglein antibodies generated by phage display from pemphigus patients was presented. This powerful technique facilitates a detailed analysis of the B-cell immune response, exemplified by following the B-cell response in pemphigus patients over

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Meeting report on The International Pre IID 2013 Satellite Meeting on Autoimmune Bullous Diseases, Webpage: http://www.aibd-iid2013.uni-luebeck.de

several years. Using this technique, it was demonstrated that autoreactive B-cell clones persist over a long period of time. Furthermore, generation of an autoimmune B-cell response to Dsg 3 relies on somatic mutations in response to an unrelated antigen (Di Zenzo *et al.*, 2012). Interestingly, various Dsg 3-specific T cells not only contribute to the generation of an anti-Dsg 3 IgG response but also directly cause interface dermatitis *in vivo* (Takahashi *et al.*, 2011). In addition, an environmental, non-infectious agent (sand fly salivary antigen) may drive the generation of autoimmunity in pemphigus in endemic pemphigus foliaceus (Qian *et al.*, 2012). Evidence for the pathogenicity of non-desmoglein-specific autoantibodies (e.g., autoantibodies directed against desmocollin 3) in pemphigus was summarized and discussed in detail (Rafei *et al.*, 2011; Kalantari-Dehaghi *et al.*, 2013).

This session also focused on the involvement of signaling molecules in the pathogenesis of pemphigus vulgaris (e.g., the Dsg3-p38MAPK adhesion receptor complex, which has been identified as a potential drug target in the treatment of pemphigus; Spindler *et al.*, 2013). In addition, the role of apoptosis in pemphigus was widely discussed: on one hand, the evidence for Fas ligand involvement in the pathogenesis of blister formation in pemphigus was provided. On the other hand, data from skin biopsies from pemphigus patients do not support apoptotic involvement. Furthermore, in contrast with Dsg 3 antibodies, Dsg 1 antibodies were shown to induce widening between keratinocytes by diminishing desmosome size and number and altering plakoglobin distribution. These observations support the desmoglein non-assembly depletion hypothesis as a dominant mechanism for acantholysis in pemphigus foliaceus (Oktarina *et al.*, 2011). The actin-binding protein adducin may also control blistering in pemphigus as it was reported to regulate Dsg 3 protein expression.

Cell biology of hemidesmosomes and disease pathways in pemphigoid

The mechanisms involved in blister formation in pemphigoid disease were a focus of this session. Novel data from the BP neonatal mouse model (Liu *et al.*, 1993) indicate that blister induction completely depends on IL-1 signaling, particularly IL-1β and inflammasome activation. In dermatitis herpetiformis, epidermal transglutaminase (TG3) diffuses from human epidermis into the upper dermis. The subsequent binding of anti-TG3 IgA initiates the blistering cascade in dermatitis herpetiformis. Furthermore, the pathogenic relevance of anti-IgE antibodies and cleavage site–specific antibodies in BP were discussed. Convincing evidence was presented for the contribution of anti-BP180 IgE in the pathogenesis of BP, which is mediated by Fc-receptor-independent effects (Messingham *et al.*, 2011) and the regulation of eosinophil functions. In contrast, the pathogenic relevance of cleavage site–specific autoantibodies, as studied in the COL17-humanized mouse model of BP (Nishie *et al.*, 2007), needs to be further evaluated. In addition to targeting blister-inducing mechanisms in pemphigoid, the pathways involved in blister resolution, such as Flightless I, may also be used as novel treatments (Kopecki *et al.*, 2013). After a review of the molecular organization within hemidesmosomes, the biology of type VII collagen (COL7) and its potential use as protein-based treatment in recessive dystrophic epidermolysis bullosa (RDEB) was discussed given that recombinant COL7 forms new anchoring fibrils in RDEB patients after intravenous injection (Wang *et al.*, 2013). Novel insights into the mechanisms leading to loss of tolerance in EBA were also presented: epidemiologic observations, HLA genotyping of EBA patients, and data from mouse models of this disease provide strong evidence for a genetic contribution. In addition, experimental data indicate that specific T-cell subsets and cytokines are involved in the initiation of the autoimmune response (Ludwig *et al.*, 2013).

Outcome measures and novel treatments

In this final session, the attendees discussed the importance of outcome measures for AIBDs (Daniel *et al.*, 2012) to determine disease activity, thereby offering the possibility of comparing therapeutic outcomes among AIBD studies. In an extension of a previous study on the clinical and molecular effects of rituximab treatment in pemphigus vulgaris (Colliou *et al.*, 2013), initial data were presented from a randomized controlled study comparing rituximab and corticosteroids to corticosteroids alone in pemphigus vulgaris patients. Use of veltuzumab, a humanized anti-CD20 mAb, was discussed as an alternative treatment option in rituximab-refractory pemphigus. The evidence of immunoapheresis in bullous diseases was summarized (Schmidt and Zillikens, 2013), and the outline of an ongoing prospective controlled multicenter trial of immunoapheresis in pemphigus patients was discussed. The final results from this study are expected by the end of 2015.

POSTER SESSIONS

Several hours were exclusively reserved for discussions of the posters ($n=58$). Poster prizes were awarded to Volker Spindler (Munich) for the work entitled "Peptide-mediated prevention of pemphigus skin blistering in mice" and to Yvonne Exner (Marburg) for her work entitled "IgG autoantibodies against carboxy-terminal epitopes of desmoglein 3 are pathogenic *in vitro*".

SATELLITE MEETINGS

Meeting of the International Autoimmune Blistering Diseases Genetics Consortium

On 6th May, over 30 conference participants met to discuss the future direction of AIBD genetic studies. The participants agreed that relatively small patient numbers and ethnic differences are the major challenges for AIBD genetic studies. To overcome these challenges, joint efforts have been made to bring together all existing and recruiting cohorts. This effort can be achieved within the framework of the International Autoimmune Blistering Diseases Genetics Consortium. Following the first GWAS in pemphigus vulgaris patients (Sarig *et al.*, 2012), the participants expect results from additional GWAS in BP, mucous membrane pemphigoid, and pemphigus within the next 2–3 years. The participants also agreed that novel technologies, such as next-generation sequencing, will be the driving force behind the progress in this field in

the next years. Collectively, the identification of susceptibility genes will aid in the development of novel compounds targeting the respective pathways.

EBA Consensus Meeting

On 7th May, 35 registrants joined this 4th and final meeting in a series of international EBA consensus meetings cochaired by Dedee Murrell (Australia) and Victoria Werth (USA). Previous meetings were held at the AAD, SID, and EADV over the preceding 2 years.

Definitions agreed upon at the previous meetings and the EBA disease area index (EBADAI) were reviewed and discussed. One challenge in the development of a disease extent tool for both EBA and mucous membrane pemphigoid is scoring ocular involvement. For practical reasons, it would be easier if dermatologists could score ocular involvement themselves. However, a simple scoring sheet could ideally be used by ophthalmologists to ensure accuracy. Two scoring systems for ocular involvement were presented and widely discussed (Munyangango *et al.*, 2013).

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Presence of autoimmune regulator and absence of desmoglein 1 in thymoma associated with a pemphigus foliaceus patient

Running head: AIRE and Dsg1 expression in PF thymoma

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Dear Editor, Autoantigens for pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are desmoglein 1 (Dsg1) and Dsg3, respectively.[1,2] Thymomas are frequently associated with various autoimmune diseases, particularly myasthenia gravis (MG).[3] Thymomas histopathologically divided into types A, AB, B1, B2, B3 and C (renamed as thymic carcinoma).[4]

Autoimmune regulator (AIRE) is a causative molecule in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, and is expressed in thymic medulla in fetal thymus.[5,6] AIRE regulates negative selection of self-reactive T-cells by expressing tissue specific antigen. Thus, AIRE is involved in central immune tolerance and development of autoimmune diseases.[7] AIRE is also expressed in non-thymus tissues, including lymphnodes and spleen, but is absent in all thymomas, except for a few type B1 thymomas.[8,9]

β5t, a subunit of thymus-specific proteasome S28, involves in antigen processing for positive selection in thymus, and is expressed in type B, but not type A, thymomas.[10,11]

In this study, we examined expression of various skin autoantigens and AIRE in thymoma in a PF patient, as well as other thymic tumors. PF thymoma showed unique antigen expression, indicating different AIRE function from that in fetal thymus.

Materials and methods are described in Supporting Information. All specific antibodies are summarized in Table S1. Seven thymomas, one thymic carcinoma and 2 persistent thymi were

obtained from non-pemphigus individuals (Table S2), and were histopathologically classified (Fig.S1).

An 82-year-old Japanese female developed wide-spread blistering erythematous skin lesions without mucous membrane involvement (Fig.1a), and histopathologically showed acantholysis at uppermost epidermis (Fig.1b). ELISAs detected anti-Dsg1 antibodies (index >3000), but not anti-Dsg3 antibodies (index <5), confirming the diagnosis of PF. Computed tomography demonstrated a low-density mass in anterior mediastinum (Fig.1c). Although anti-AChR antibodies were positive, MG was ruled out by neurological examination.

Various treatments could not suppress skin lesions, and the patient died 2 months later. At autopsy, the tumor mass (Fig.1d) and persistent thymus were excised, and pieces of them were either fixed or frozen. Histopathology for the tumor showed short spindle epithelial cells with few lymphocytes (Fig.1e,f), and epithelial cells and lymphocytes were positive for AE1/AE3 and CD45, respectively (Fig.1g,h), suggesting the diagnosis of type A thymoma.

In immunoblotting (IB), PF thymoma expressed strongly Dsg2 and Dsg3, weakly desmocollin 2 (Dsc2) and Dsc3, but not Dsg1, while persistent thymus showed no positive reactivity (Fig.1i). Immunofluorescence (IF) confirmed the reactivity in PF thymoma, while persistent thymus expressed these proteins to less extent (Fig.1j).

In IB, Dsg2/Dsg3, Dsc2/Dsc3, E-cadherin, epiplakin and laminin γ1 were detected in PF thymoma and several non-PF thymomas (Fig.2a, and Table S3). PF thymoma showed much stronger reactivity with these proteins, particularly Dsg2, Dsg3, Dsc2 and laminin γ1. Plectin, periplakin and α6 integrin were detected only in PF thymoma. Dsg1, Dsc2 and Dsc3 were

weakly detected in thymi. Thymic carcinoma showed expression pattern similar to those in PF thymoma. In contrast, BP230, desmoplakin, envoplakin, β4 integrin, laminin α3 and type VII collagen were not expressed in any thymomas.

In addition, Dsg1 and laminin γ1 were weakly detected in commercially available lysates of both fetal and adult thymi, and E-cadherin, plectin and epiplakin were detected in adult thymus (Fig.2b).

Positive reactivity with AE1/AE3 in immunohistochemistry (IHC) for selected samples with favorable condition (Fig.S2) confirmed the presence of epithelial cells in all samples (Fig.2c). Similar to IB results, Dsg2, Dsg3 and Dsc2 were detected.

We next analyzed expression of AIRE in all tumors by IB, IHC and RT-PCR. In IB, the 58-kDa AIRE was clearly detected in extracts of DJM-1 cells and PF thymoma, but not in other thymic tumors and persistent thymi (Fig.2d). AIRE was also expressed in various keratinocyte cell lines, including KU-8, DJM-1 and HaCaT cells, and in commercially available fetal and adult thymi (Fig.S3a). IHC showed positive staining of AIRE in epithelial cell nuclei in PF thymoma, but not in other tumors (Fig.2f). In addition, RT-PCR detected AIRE messages strongly in fetal and adult thymi, weakly in DJM-1 cells and PF thymoma, but not in another thymoma (Fig.S3b).

In IB, the 32-kDa β5t protein bands were only in type B or type AB thymomas, but not in PF thymoma, thymus and thymic carcinoma (Fig.2e). IHC confirmed these results (Fig.2g).

In this study, PF thymoma expressed Dsg2, Dsg3, Dsc2 and Dsc3, but not Dsg1 (PF autoantigen) in all IB, IF and IHC. Some thymic tumors also showed similar expression profile, but PF thymoma showed the strongest reactivity. Additionally, several other epidermal antigens were detected in several thymic samples with the most prominent expression in PF thymoma. Two adult persistent thymi also expressed some of these autoantigens in less extent. Thus, this is the first study of the expression of various skin antigens in human thymic tissue, although this study used only thymomas and adult thymi.

All IB, IHC and RT-PCR showed expression of AIRE in PF thymoma, but not in any other thymomas, persistent thymi or thymic carcinoma. The presence of AIRE in PF thymoma was inconsistent to the results in previous studies.[8,9]

Similar to the previous studies,[10,11] β5t expressed in type B and type AB, but not type A thymomas, suggesting that the absence of β5t in PF thymoma was merely tumor type-specific event.

The strongest expression of various skin autoantigens and AIRE in PF thymoma led us to speculate that expression of autoantigens and AIRE in PF thymoma might cause PF-specific autoimmunity. However, the mechanism for induction of autoimmunity to Dsg1 in our PF patient is currently unknown.

While role of AIRE in transcriptional activation of TSAs (tissue-specific antigens) in medullary thymic epithelial cells, thereby ensuring elimination of autoreactive T-cells, is widely accepted,[7] expression of autoantigens was preserved in AIRE-deficient medullary thymic epithelial cells in a mouse study.[13] Furthermore, recent study reported that levels of several TSAs, particularly autoantigens, were paradoxically increased in some thymomas found in MG

patients, which parallels autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy,[14] suggesting some relationship between expression levels of AIRE and/or TSAs (including autoantigens) within thymoma tissues and development of autoantibodies to autoantigens.

Although thymoma in our patient expressed AIRE but not Dsg1 (autoantigen), PF thymoma showed the strongest expression of Dsg3, PV autoantigen without production of autoantibodies to Dsg3. Thus, we speculate that AIRE in PF thymoma might induce strong expression of Dsg3 and other skin autoantigens, which in turn lead to induction of autoimmune response to Dsg1 through so far unidentified pathway in our case, although this speculation is against the dogma of induction of central immune tolerance.

Lastly, this study and a previous study[12] demonstrated that keratinocytes express AIRE. Therefore, AIRE might increase Dsg1 expression in epidermis and lead to production of anti-Dsg1 antibodies.

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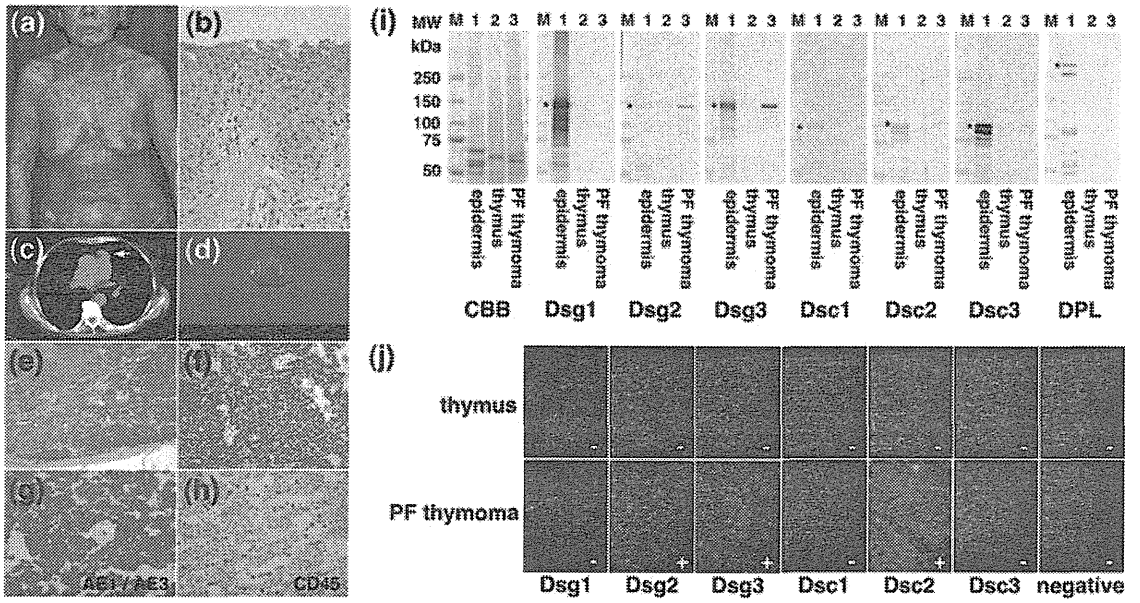
Figure legends

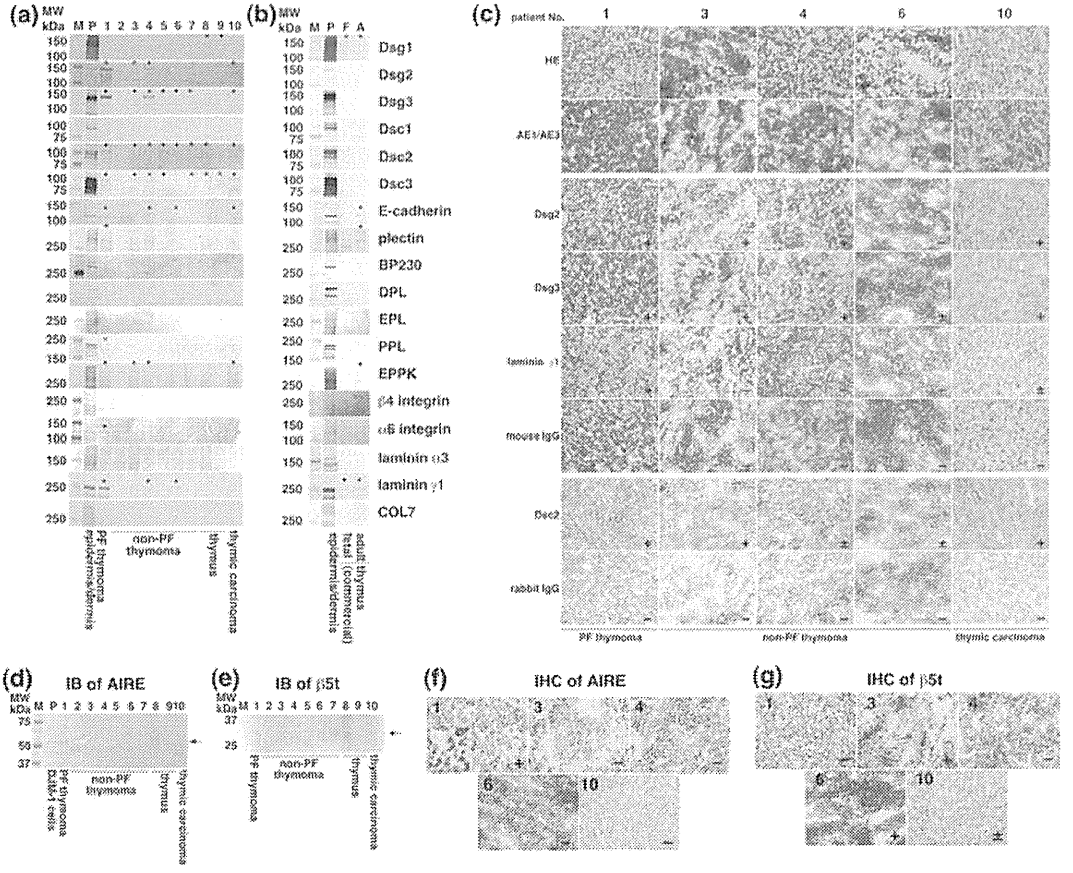
Fig 1. Clinical and histopathological features of PF patient, and IB and IF results of Dsg1-Dsg3, Dsc1-Dsc3 and DPL in PF thymoma. (a) Clinical feature. (b) Histopathological features (hematoxylin and eosin staining, x400). (c) Image of chest computed tomography. Tumor mass is indicated by an arrow. (d) Resected thymoma. (e,f) Histopathological finding of thymoma (hematoxylin and eosin staining; e: x100 and f: x400). (g,h) IHC findings for AE1/AE3 (g) and CD45 (h) (x400). (i) The results of IB studies of epidermal extracts (lanes 1), extract of PF persisting thymus (lanes 2) and extract of PF thymoma (lanes 3). Equal amount (3 ug) of total protein was loaded on each lane, and separated on 5-20% polyacrylamide gel. The positions of MW markers (lanes M) are shown in the left. The asterisks (*) indicates positive reaction. CBB: Coomassie brilliant blue staining. (j) The results of IF of PF persisting thymus (upper panel) and PF thymoma (lower panel) for Dsg1-Dsg3 and Dsc1-Dsc3 (x400). +: Positive reaction. -: Negative reaction.

Fig 2. The results of IB and histopathological studies of various thymic tissues. (a) The results of IB of positive controls (epidermal or dermal extracts) (lane P), thymomas (lanes 1-7), persisting thymi (lanes 8 and 9) and thymic carcinoma (lane 10) for various skin antigens. Each number indicates patient number shown in Table S2. (b) The results of IB of positive controls (epidermal or dermal extracts) (lane P), commercially available protein lysates of fetal (lane F) and adult (lane A) thymi. Equal amount (3 ug) of total protein was loaded on each lane, and separated on 5-20% polyacrylamide gel. The positions of MW markers are shown in the left. The asterisks (*) indicates positive reaction. All specific antibodies showed positive reaction in

positive controls. EPL: envoplakin. PPL: periplakin. EPPK: epiplakin (EPPK). COL7: type VII collagen. (c) Histopathological findings and the results of IHC for various skin autoantigens for 4 representative thymomas and thymic carcinoma (x400). HE: hematoxylin and eosin staining. Each number indicates patient number shown in Table S2. +: Positive reaction. ±: Weakly positive reaction. -: Negative reaction.

Laminin γ1 was detected clearly in PF thymoma (#1), but not in non-PF thymomas (#3, 4, 6) in both IB and IHC. In contrast, laminin γ1was detected by IHC, but not by IB in thymic carcinoma (#10). (d,e) The results of IB studies for expression of AIRE (d) and β5t (e) in extracts of DJM-1 cells as positive control (lane P), thymomas (lanes 1-7), persistent thymi (lanes 8, 9) and thymic carcinoma (lane 10). Arrows indicate positive reaction. The positions of MW markers are shown in the left. Equal amount (3 ug) of total protein was loaded on each lane, and separated on 5-20% polyacrylamide gel. (f,g) IHC for expression of AIRE (f) and β5t (g) in 4 thymomas and one thymic carcinoma (x400, inset figure: x1000). +: Positive reaction. ±: Weakly positive reaction. -: Negative reaction. Each number indicates patient number shown in Supplemental Table S2.





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Letter to the Editor

Epidermal polymeric immunoglobulin receptors: leads from intraepidermal neutrophilic IgA dermatosis-type IgA pemphigus

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Abstract: In this study, we attempted to identify unknown autoantigen for intraepidermal neutrophilic IgA dermatosis-type IgA pemphigus by novel IgA-specific immunoprecipitation. Mass-spectrometry study identified polymeric immunoglobulin receptor (PIGR) as the candidate protein, and we confirmed that PIGR expressed in both epidermis and cultured keratinocytes. Eukaryotic recombinant protein of PIGR expressed in COS7 cells was reacted with both patient and normal sera, indicating that PIGR binds physiologically to IgA. To detect antigen-specific binding by IgA autoantibodies, we performed several experiments using deglycosylated PIGR and $F(ab)_2$ fragments from patient sera. However, these analyses suggested that patient IgA bound physiologically, but not immunologically, to PIGR. Nevertheless, our study provided two important insights. Newly developed IgA-immunoprecipitation system should be a useful tool in the future study of identification of antigens for IgA autoantibodies. Detection of epidermal PIGR in this study confirmed previous results and indicated possible immunological role of PIGR in epidermis.

Key words: IEN-type IgA pemphigus – IgA autoantibody – immunoprecipitation – keratinocyte – polymeric immunoglobulin receptor

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Background

IgA pemphigus is characterized by IgA autoantibodies to keratinocyte cell surfaces and is divided into two subtypes: subcorneal pustular dermatosis (SPD)-type and intraepidermal neutrophilic IgA dermatosis (IEN)-type (1,2). By cDNA transfection method, we previously identified desmocollin 1 as autoantigen in SPD-type (3). In contrast, autoantigen in IEN-type is still unknown. However, our immune-electron microscopic study indicated that antigen resides at extracellular space outside of desmosomal areas, suggesting that IEN-type is a pemphigus disease reactive with non-desmosomal protein (4).

Questions addressed

To identify unknown autoantigen for IEN-type IgA pemphigus by newly developed IgA-specific immunoprecipitation (IP).

Experimental design

Peptide M, which specifically binds to IgA (5), was used in IgA-IP analyses to detect autoantigen for IgA antibodies in IEN-type IgA pemphigus. After identification of polymeric immunoglobulin receptor (PIGR) as the candidate protein for autoantigen, eukaryotic recombinant protein (RP) of PIGR expressed in COS7 cells. To confirm whether PIGR is autoantigen of IEN-type IgA pemphigus, we used deglycosylated PIGR RP and $F(ab)_2$ fragments from patient IgA. All materials and methods were described in Supplementary data.

Results

In IgA-immunofluorescence (IF) of KU-8 cells, IEN-type IgA pemphigus patient serum reacted with cell surfaces of cultured cells (Fig. 1a). However, no specific protein bands were detected by IgA-immunoblotting of KU-8 cell extract (Fig. S1). Therefore, we developed a novel IgA-IP system using peptide M.

In IP of KU-8 cell extract, while the 110-kDa and 90-kDa proteins were detected by all normal and IEN-type IgA pemphigus patient sera, the 80-kDa protein was detected by two of the 10 patient sera (Fig. 1b). At this time, we considered the 110-kDa and 90-kDa proteins as non-specific reactivity and suspected the 80-kDa protein as a candidate of autoantigen. Mass-spectrometry analysis for an identical 80-kDa protein identified PIGR.

In IF of normal human skin, anti-PIGR (C-term) pAb showed cytoplasmic staining in entire epidermis, which was different from staining by IEN-type IgA pemphigus patient serum (Fig. 1c). This staining pattern was also different from that in a previous IF study (6), probably because of different antibodies used.

By immunoblotting for IP products with normal and IEN-type IgA pemphigus patient sera, we compared reactivity of anti-PIGR pAbs to that of streptavidin (Fig. 1d, left panel). Anti-PIGR (C-term) pAb showed the 110-kDa protein in IP products with all sera (Fig. 1d, middle panel). Anti-PIGR pAb showed protein band around the 80-kDa and 90-kDa proteins (Fig. 1d, right panel). However, this reactivity was too weak, indicating that anti-PIGR pAb was not suitable to identify the PIGR epitope by immunoblotting.

To determine whether PIGR is autoantigen, we prepared eukaryotic PIGR RPs using COS7 cells (Fig. S2). In IP of RIGR-I, all normal and IEN-type IgA pemphigus patient sera detected the 110-kDa band (Fig. 2a). PIGR contains several N-glycosylation consensus sites in its extracellular portion, and non-glycosylated molecule is 83.3-kDa (7). Therefore, to examine the nature of the three proteins with different sizes, we performed deglycosylation study using PIGR-I. Deglycosylation of PIGR-I produced two protein bands with different sizes, which were the same as the

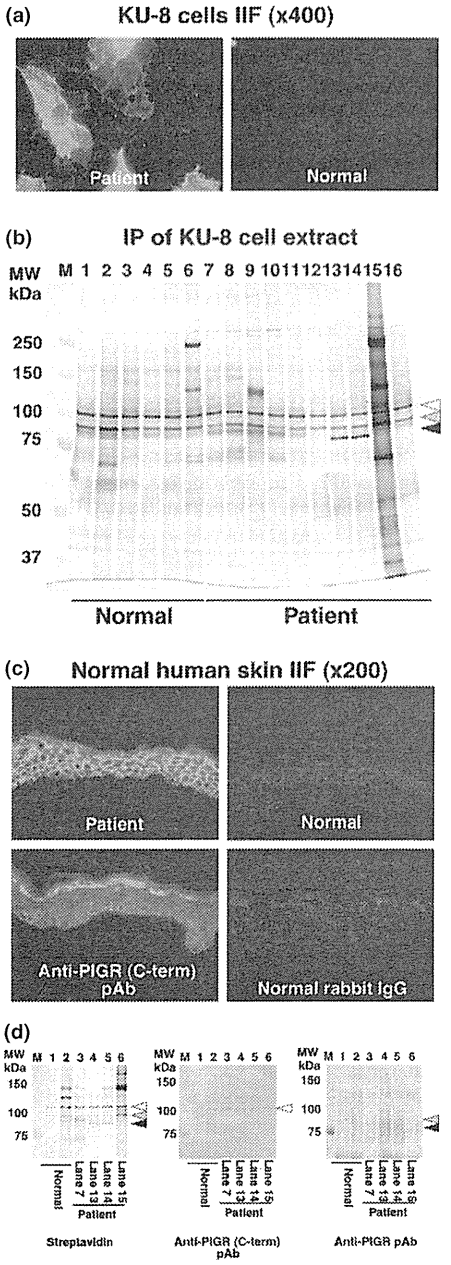

Figure 1. (a) IgA-IF of cultured KU-8 cells. (b) Immunoblotting with HRP-conjugated streptavidin for IP products from biotinylated KU-8 cell extract. Lanes 1–6 are for normal sera, and lanes 7–16 are for IEN-type IgA pemphigus patient sera. White, grey and black arrowheads indicates the 110-kDa, 90-kDa and 80-kDa bands, respectively. (c) IF of normal human skin with IEN-type IgA pemphigus patient and normal sera (upper), or anti-PIGR (C-term) pAb and normal rabbit IgG (lower). (d) Immunoblotting of IP products of KU-8 cell extract with anti-PIGR pAbs. Lanes 1 and 2 are for normal sera, and lanes 3-6 are for IEN-type IgA pemphigus patient sera. Each lane number indicates number in Fig. 1b. Equal volume (10 μl) of IP product was loaded on each lane and separated on 7.5% polyacrylamide gel. The positions of molecular weight markers (MW) are shown in the left.

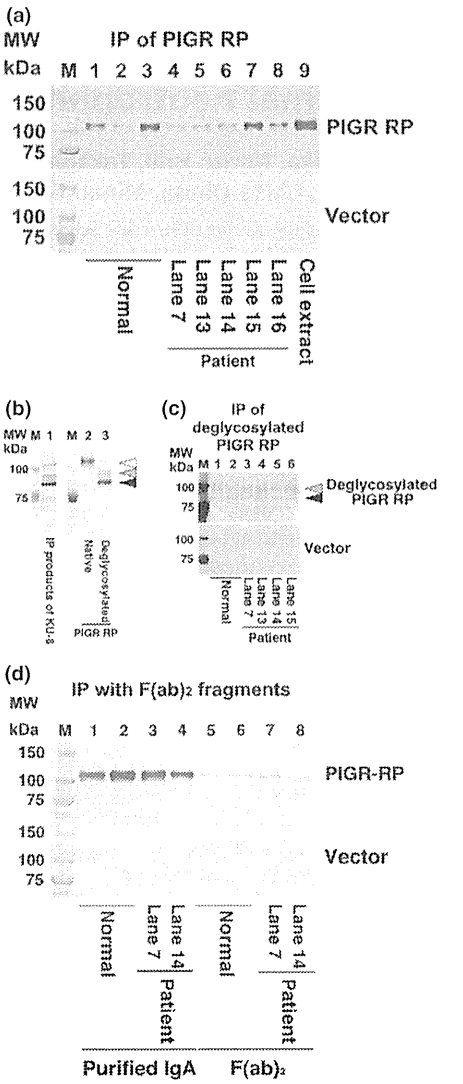

Figure 2. Experiments for PIGR-I RP. (a) Immunoblotting with anti-His mAb for IP products from PIGR-I or empty vector COS7 cells. Lanes 1–3 are for normal sera, and lanes 4–8 are for IEN-type IgA pemphigus patient sera. (b) Immunoblotting with HRP-conjugated streptavidin for IP products of patient serum from biotinylated KU-8 cell extract (lane 1) and anti-His mAb for the cell extract from PIGR-I transfected COS7 cells without (lane 2) or with deglycosylation (lane 3). The 110-kDa, 90-kDa and 80-kDa bands are indicated by white, grey and black arrowheads, respectively. (c) Immunoblotting with anti-His mAb for IP products from deglycosylated PIGR-I or empty vector COS7 cells. Lanes 1 and 2 are for normal sera, and lanes 3-6 are for IEN-type IgA pemphigus patient sera. (d) Immunoblotting with anti-His mAb for IP products of PIGR-I or empty vector COS7 cell extract with purified IgA (lanes 1–4) or $F(ab)_2$ fragments (lane 5–8). Each lane number indicates number in Fig. 1b. Equal volume (10 μl) of IP products was loaded on each lane and separated on 7.5 or 5–20% polyacrylamide gel. The positions of molecular weight markers (MW) are shown in the left of the two panels.

90-kDa and 80-kDa bands in IP products of KU-8 extract (Fig. 2b). Then, we performed IP of deglycosylated PIGR-I. All normal and IEN-type IgA pemphigus patient sera reacted weakly with the deglycosylated PIGR-I (Fig. 2c), which confirmed the results of a previous study that glycosylation was not necessary for immunoglobulin binding (8).

Furthermore, we prepared $F(ab)_2$ fragments from purified IgA of sera by pepsin digestion. Then, IP of PIGR-I was performed with purified IgA and prepared $F(ab)_2$ fragments using protein L.