CXCL8 in the NOD1 shRNA-expressing cells is mediated by such receptors.

In summary, to our knowledge this is the first report demonstrating that the intracellular receptor NOD1 is functional expressed in human keratinocytes, suggesting that NOD1 may be involved in cutaneous innate immunity. Further studies are needed to understand the contribution of intracellular innate immune receptors to cutaneous host defense.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
We thank Joel Whitfield for help with the ELISAs and Peter Kuffa for lab assistance. This study was supported by NIH grant R01 DK61707 and a Heisenberg fellowship of the Deutsche Forschungsgemeinschaft (DFG) given to J Harder.

***Jürgen Harder*[1,2] *and Gabriel Núñez*[1,2]**

[1]Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan, USA and [2]Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Michigan, USA
E-mail: jharder@dermatology.uni-kiel.de

REFERENCES

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124:783–801

Baker BS, Ovigne JM, Powles AV, Corcoran S, Fry L (2003) Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. *Br J Dermatol* 148:670–9

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L *et al.* (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 4: 702–707

Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411:826–33

Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA *et al.* (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* 20:1436–47

Franchi L, Park JH, Shaw MH, Marina-Garcia N, Chen G, Kim YG *et al.* (2008) Intracellular NOD-like receptors in innate immunity, infection and disease. *Cell Microbiol* 10:1–8

Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J *et al.* (2003a) Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* 300:1584–7

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G *et al.* (2003b) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278:8869–72

Gutierrez O, Pipaon C, Inohara N, Fontalba A, Ogura Y, Prosper F *et al.* (2002) Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J Biol Chem* 277:41701–5

Hisamatsu T, Suzuki M, Podolsky DK (2003) Interferon-gamma augments CARD4/NOD1 gene and protein expression through interferon regulatory factor-1 in intestinal epithelial cells. *J Biol Chem* 278: 32962–32968

Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S *et al.* (2003) Crohn's disease and the NOD2 gene: a role for Paneth cells. *Gastroenterology* 125:47–57

Masumoto J, Yang K, Varambally S, Hasegawa M, Tomlins SA, Qiu S *et al.* (2006) Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment *in vivo*. *J Exp Med* 203:203–13

Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 276:4812–8

Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF *et al.* (2003) Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* 52:1591–7

Pivarcsi A, Bodai L, Rethi B, Kenderessy-Szabo A, Koreck A, Szell M *et al.* (2003) Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immunol* 15:721–30

Rosenstiel P, Fantini M, Brautigam K, Kuhbacher T, Waetzig GH, Seegert D *et al.* (2003) TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 124:1001–9

Travassos LH, Carneiro LA, Girardin SE, Boneca IG, Lemos R, Bozza MT *et al.* (2005) Nod1 participates in the innate immune response to *Pseudomonas aeruginosa*. *J Biol Chem* 280:36714–8

Underhill DM, Ozinsky A (2002) Toll-like receptors: key mediators of microbe detection. *Curr Opin Immunol* 14:103–10

Voss E, Wehkamp J, Wehkamp K, Stange EF, Schroder JM, Harder J (2006) NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. *J Biol Chem* 281:2005–11

Prevalent and Rare Mutations in the Gene Encoding Filaggrin in Japanese Patients with Ichthyosis Vulgaris and Atopic Dermatitis

Journal of Investigative Dermatology (2009) **129,** 1302–1305; doi:10.1038/jid.2008.372; published online 27 November 2008

TO THE EDITOR

Mutations in the gene encoding filaggrin (*FLG*) were identified as the underlying cause of ichthyosis vulgaris (IV; OMIM #146700) and also shown to predispose to atopic dermatitis (AD; Palmer *et al.*, 2006; Smith *et al.*, 2006). Although *FLG* is considerably difficult to analyze because of its large size (>12 kb) and highly repetitive nature, PCR strategy that permits routine and comprehensive sequencing of the entire *FLG* has been developed recently (Sandilands *et al.*, 2007).

Using this methodology, we have identified four prevalent *FLG* mutations in Japanese patients with IV (Nomura *et al.*, 2008). We also demonstrated that *FLG* mutations were significantly associated with AD and the frequency of these *FLG* mutations observed in our Japanese AD cohort was about 20%. However, the frequency in our cohort

Abbreviations: AD, atopic dermatitis; FLG, filaggrin; IV, ichthyosis vulgaris

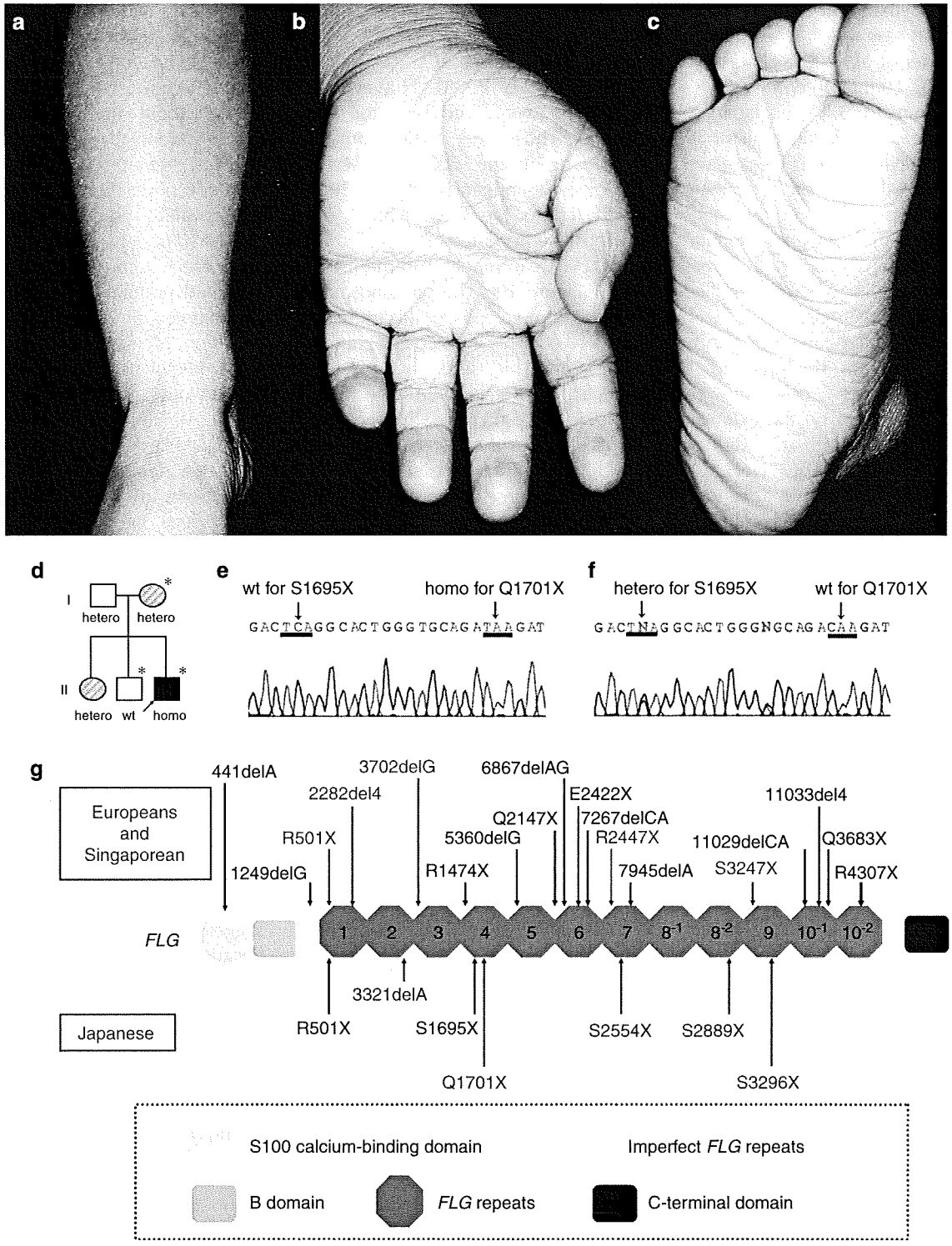

Figure 1. Clinical features and results of mutation analysis. (**a**) Fine scaling clearly visible on the proband's leg. (**b**, **c**) He also showed marked palmoplantar hyperlinearity. (**d**) A family tree of the ichthyosis vulgaris family shows the semidominant inheritance pattern. Solid symbols refer to the marked ichthyosis vulgaris presentation; cross-hatched symbols refer to the milder ichthyosis vulgaris presentation. In addition, the proband, his mother, and sister had concomitant dermatologist-diagnosed atopic dermatitis (*). wt, wild type for Q1701X; hetero, heterozygous; homo, homozygous. (**e**, **f**) A homozygous transition mutation c.5101C>T was identified in the proband, resulting in Q1701X. A heterozygous transition mutation c.5084C>G was identified in one Japanese individual in the control population, resulting in S1695X. Mutation S1695X is located only six amino acids upstream from Q1701X. (**g**) Loss-of-function *FLG* mutations are shown in a schematic of profilaggrin. Mutations shown in red are prevalent; those in black are rare. Some individuals have duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8-1, 8-2, 10-1, and 10-2.

was still lower than that seen in analogous European case series, where it is up to 48% (Barker *et al.*, 2007; Sandilands *et al.*, 2007). Furthermore, it was reported that up to 37% of Japanese patients with AD had concomitant IV (Uehara and Hayashi, 1981; Uehara and Miyauchi, 1984). Taken together, there might be further prevalent *FLG* mutations to be discovered in the Japanese population. Here we have studied a further Japanese family with IV and identified two further *FLG* mutations.

A newly recruited Japanese family with IV was studied. The proband, a one-year-old Japanese boy, showed marked scaly dry skin on the extensor limbs and trunk (Figure 1a). Marked palmoplantar hyperlinearity was also evident (Figure 1b and c). A diagnosis of IV was made from these clinical observations. His mother and sister also showed scaly dry skin and palmoplantar hyperlinearity, but the clinical severity was mild compared to the proband (Figure 1d). Therefore, the inheritance pattern seemed semidominant. The proband, his mother, and his brother had concomitant AD.

The medical ethical committee at Hokkaido University Graduate School of Medicine approved all the studies. The study was conducted according to the Declaration of Helsinki Principles. Participants or their legal guardians gave their written informed consent. Following informed consent, genomic DNA from all family members was extracted from peripheral blood according to standard procedures. Initially, all family members were screened for five *FLG* mutations identified in Japanese population so far, R501X, 3321delA, S2554X, S2889X and S3296X, by restriction enzyme digestion, fluorescent PCR, and direct DNA sequencing as described previously (Nomura *et al.*, 2007, 2008; Hamada *et al.*, 2008). However, all individuals were wild type for these variants. Thus, we carried out full sequencing of the *FLG* as described previously (Sandilands *et al.*, 2007), which led to the identification of a previously unreported nonsense mutation Q1701X in repeat 4 in the present family (Figure 1e). The proband turned out to be homozygous for this truncation mutation and his non-consanguineous parents and his sister heterozygous, whereas his brother wild type (Figure 1d). It was also confirmed that they carry no pathogenic mutations in the other *FLG* repeats. Then, we screened 118 unrelated Japanese patients with AD and 134 unrelated Japanese control individuals for Q1701X by direct DNA sequencing. The diagnosis of AD in our case series was made by experienced dermatologists, according to the AD diagnostic criteria by Hannifin and Rajka (1980). Notably, mutation Q1701X was also identified in two Japanese patients with AD (1.7%), which brings the total number of recurrent *FLG* mutations so far identified in Japanese population to five.

During the screening for Q1701X, we identified another previously unreported *FLG* mutation, S1695X, which is located only six amino acids upstream from Q1701X, in the general Japanese control population (Figure 1f). We screened 33 Japanese patients with IV and 118 with AD for S1695X, but all patients were wild type for this mutation. Only one heterozygote was identified in the control population. Therefore, S1695X seems to be an extremely rare *FLG* mutation in Japanese individuals. The control individuals had not been examined in relation to AD or IV status, that is, they were population controls rather than "hypernormal" controls, so no clinical details about the individual carrying S1695X are available. In total, there are at least seven *FLG* variants in the Japanese population, including five that are prevalent and two that are quite rare.

The *FLG* genotype data in the Japanese AD case series and ethnically matched population control series are summarized in Table 1. In this study, case–control association analyses were performed by using Pearson's r^2 statistics, as previously described (Palmer *et al.*, 2006). All alleles were observed to be in normal Hardy–Weinberg equilibrium. Here we demonstrate that about 25% of patients in our Japanese AD case series carry one or more of these seven *FLG* mutations (combined minor allele frequency = 0.127, $n = 236$) and these variants are also carried by 4% of general Japanese control individuals (combined minor allele frequency = 0.019, $n = 268$). There is significant statistical association between the seven *FLG* mutations and AD (r^2 $P = 1.75 \times 10^{-6}$). Moreover, AD was manifested in heterozygous carriers of these *FLG* mutations with a Fisher's exact test odds ratio for AD of 6.8 (95% CI 2.5–18.5, $P = 3.7 \times 10^{-5}$), implying a causal relationship between *FLG* mutations and AD. Taken together, these data strongly suggest that skin barrier impairment because of reduced filaggrin expression is important in the pathogenesis in AD.

To date, 24 *FLG* mutations, including the two identified in this study, have been reported in the European, Japanese, and Singaporean populations (Sandilands *et al.*, 2007; Chen *et al.*, 2008; Nomura *et al.*, 2008). Interestingly,

Table 1. Atopic dermatitis case–control association analysis for *FLG* null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		Combined	
Genotypes	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
AA	134	118	133	113	133	118	134	116	133	112	132	105	134	114	129	91
Aa	0	0	1	5	1	0	0	2	1	6	2	13	0	4	5	24
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Total	134	118	134	118	134	118	134	118	134	118	134	118	134	118	134	118

For combined genotype, r^2 $P = 1.75 \times 10^{-6}$; Fisher's exact test odds ratio=6.8 (95% CI 2.5–18.5).

mutations found in Japanese are different from those found in Europeans and Singaporean (Figure 1g), except in one case of the common European R501X mutation occurring as a very rare mutation on a different haplotype in the Japanese population (Hamada *et al.*, 2008). These observations imply that every population is highly likely to have a unique set of *FLG* mutations.

In conclusion, we have identified two further *FLG* mutations in the Japanese population. We also showed that at least about 25% of Japanese patients with AD carried one or more of *FLG* mutations. As we have sequenced more than 30 Japanese patients with IV, there is now little possibility that further highly prevalent mutations underlie the Japanese population. Taking the high frequency (up to 37%) of concomitant IV in patients with AD into account, however, it is still possible that there might be further multiple low-frequency *FLG* mutations to be discovered in the Japanese population. Further *FLG* mutation analysis will be necessary to understand the more precise genetic architecture of filaggrin-related AD in Japan.

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

ACKNOWLEDGMENTS

We thank the patients and their families for their participation. We also thank Ai Hayakawa for the fine technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M Akiyama (Kiban B 20390304). Filaggrin research in the McLean laboratory is supported by grants from The British Skin Foundation; The National Eczema Society; The Medical Research Council (reference number G0700314); A*STAR, Singapore; and donations from anonymous families affected by eczema in the Tayside region of Scotland.

Toshifumi Nomura[1,2], Masashi Akiyama[1], Aileen Sandilands[2], Ikue Nemoto-Hasebe[1], Kaori Sakai[1], Akari Nagasaki[1], Colin N.A. Palmer[3], Frances J. D. Smith[2], W.H. Irwin McLean[2] and Hiroshi Shimizu[1]

[1]Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [2]Epithelial Genetics Group, Division of Molecular Medicine, Colleges of Life Sciences and Medicine, Dentistry and Nursing, University of Dundee, Dundee, UK and [3]Population Pharmacogenetics Group, Biomedical Research Center, Ninewells Hospital and Medical School, University of Dundee, Dundee, UK
E-mails: akiyama@med.hokudai.ac.jp, w.h.i.mclean@dundee.ac.uk

REFERENCES

Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP *et al.* (2007) Null mutations in the filaggrin gene (*FLG*) determine major susceptibility to early-onset atopic dermatitis persists into adulthood. *J Invest Dermatol* 127:564–7

Chen H, Ho JCC, Sandilands A, Chan YC, Giam YC, Evans AT *et al.* (2008) Unique and recurrent mutations in the filaggrin gene in Singaporean Chinese patients with ichthyosis vulgaris. *J Invest Dermatol* 128:1669–75

Hamada T, Sandilands A, Fukuda S, Sakaguchi S, Ohyama B, Yasumoto S *et al.* (2008) *De novo* occurrence of the filaggrin mutation p.R501X with prevalent mutation c.3321delA in a Japanese family with ichthyosis vulgaris complicated by atopic dermatitis. *J Invest Dermatol* 128:1323–5

Hannifin JM, Rajka G (1980) Diagnostic features of atopic dermatitis. *Acta Derm Venereol* 92:44–7

Nomura T, Akiyama M, Sandilands A, Nemoto-Hasebe I, Sakai K, Nagasaki A *et al.* (2008) Specific filaggrin mutations cause ichthyosis vulgaris and are significantly associated with atopic dermatitis in Japan. *J Invest Dermatol* 128:1436–41

Nomura T, Sandilands A, Akiyama M, Liao H, Evans AT, Sakai K *et al.* (2007) Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Allergy Clin Immunol* 119:434–40

Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP *et al.* (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 38:441–6

Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson RM *et al.* (2007) Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 39:650–4

Smith FJD, Irvine AD, Terron-Kwiatkowski A, Sandilands A, Campbell LE, Zhao Y *et al.* (2006) Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat Genet* 38:337–42

Uehara M, Hayashi S (1981) Hyperlinear palms: association with ichthyosis and atopic dermatitis. *Arch Dermatol* 117:490–491

Uehara M, Miyauchi H (1984) The morphologic characteristics of dry skin in atopic dermatitis. *Arch Dermatol* 120:1186–90

"White" Nevi and "Red" Melanomas: Association with the RHC Phenotype of the *MC1R* Gene

Journal of Investigative Dermatology (2009) **129,** 1305–1307; doi:10.1038/jid.2008.378; published online 4 December 2008

TO THE EDITOR

In 2002, we reported on three patients presenting with melanocytic nevi lacking pigmentation, which we named "white" dysplastic melanocytic nevi (DMN) due to their peculiar clinical appearance of white to pale red macules with accentuated skin markings and a silvery "shining" when observed with tangential light (Zalaudek *et al.*, 2002). Notably, all three patients had melanoma, and in one patient white DMN were associated with two primary amelanotic melanomas (AMMs).

We present herein a 25-year-old woman (skin type I, red hair, and blue eyes), who sought consultation for a mole check. Clinical examination revealed, besides approximately 30 slightly atypical light brown nevi on

Abbreviations: AMM, amelanotic melanoma; DMN, dysplastic melanocytic nevi; RHC, red hair color

A Novel Humanized Neonatal Autoimmune Blistering Skin Disease Model Induced by Maternally Transferred Antibodies[1]

Wataru Nishie,[2]* Daisuke Sawamura,*† Ken Natsuga,* Satoru Shinkuma,* Maki Goto,* Akihiko Shibaki,* Hideyuki Ujiie,* Edit Olasz,‡ Kim B. Yancey,§ and Hiroshi Shimizu*

All mammal neonates receive maternal Abs for protection against pathogenic organisms in the postnatal environment. However, neonates can experience serious adverse reactions if the Abs transferred from the mother recognize self-molecules as autoAgs. In this study, we describe a novel model for autoimmune disease induced by transferred maternal Abs in genetically transformed Ag-humanized mice progeny. Bullous pemphigoid is the most common life-threatening autoimmune blistering skin disease that affects the elderly, in which circulating IgG autoAbs are directed against epidermal type XVII collagen (COL17). We have established a genetically manipulated experimental mouse model in which maternal Abs against human COL17 are transferred to pups whose skin expresses only human and not mouse COL17, resulting in blistering similar to that seen in patients with bullous pemphigoid. Maternal transfer of pathogenic Abs to humanized neonatal mice is a unique and potential experimental system to establish a novel autoimmune disease model. *The Journal of Immunology,* **2009, 183: 4088–4093.**

During pregnancy and after birth, all mammal neonates receive various factors from their mothers to adapt to the new environment, including Abs for protection against pathogenic organisms (1, 2). However, this can result in serious adverse reactions in neonates if the transferred Abs recognize self-molecules as autoAgs. For example, neonatal lupus, which is clinically characterized by skin eruptions and fatal congenital heart block, is induced by autoAbs against Ro/SSA, Ro/SSB, or U1 ribonuclear protein transferred from mothers affected with Sjögren syndrome or systemic lupus erythematosus (3, 4). In addition, maternally transferred autoAbs against acetylcholine receptors can induce the characteristic features of myasthenia gravis in human neonates (5). This suggests that mothers, in experimental animal models, might be able to induce autoimmunity in their offspring.

One possible approach to using maternal Abs to produce disease models for autoimmune diseases is the use of gene-targeted mice (6). Immunizing Ag-knockout female mice with a targeted Ag can induce Abs against the antigenic molecule. Mating these immunized females with wild-type males could mimic autoimmune diseases in the neonates expressing antigenic peptides transcribed by paternal genes in the presence of circulating maternally transferred Ag-specific IgG (6). However, this approach has not achieved practical application, probably because gene-targeted mice often die soon after birth, especially when the targeted genes encode functionally important proteins (7–11). Consequently, another method that does not use lethal gene-deleted maternal mice is desirable. The difference in immune systems between humans and mice is another important problem underlying most of the current experimental autoimmune disease models. In fact, the autoAgs in existing autoimmune disease models have been the mouse's own proteins, which are expected to differ from those in the human autoimmune disease condition (12–14). Therefore, autoimmune disease models with human autoAg expression would be ideal.

In this study, we tried to produce a novel neonatal autoimmune disease model induced by passage of maternal IgG. We aimed at the most common and life-threatening autoimmune blistering skin disease, bullous pemphigoid (BP).[3] In BP, circulating IgG autoAbs are directed against type XVII collagen (COL17, formerly known as BP180 or BPAG2) in the skin (15, 16). COL17 is a type-II-oriented, 180kD hemidesmosomal transmembrane protein that anchors basal keratinocytes to the underlying epidermal basement membrane. The pathogenic epitope in COL17 is tightly clustered within the noncollagenous (NC) 16A stretch of its ectodomain (17, 18). Interestingly, due to significant differences between humans and rodents in the amino acids sequence in the NC16A region, mice that have received human IgG from BP patients fail to show any clinical, histological, or immunological findings consistent with BP (13, 14). We recently generated *Col17a1* gene-targeted ($mCol17^{-/-}$) mice as well as COL17-humanized mice by introducing human *COL17A1*cDNA ($hCOL17^{+/+}$) transgene driven under keratin 14 promoter into $mCol17^{-/-}$ mice (12, 19). Importantly, the $mCol17^{-/-}$ mice were too fragile to mate with male mice, but reproductive ability was restored in COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) mice (12). In this study, we used these genetically manipulated COL17-humanized mice to produce a novel neonatal autoimmune disease model induced by passage of maternal IgG.

*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; †Department of Dermatology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan; ‡Department of Dermatology, Medical College of Wisconsin, Milwaukee, WI 53226; and §Department of Dermatology, University of Texas Southwestern Medical Center, Dallas, TX 75390

Received for publication February 5, 2008. Accepted for publication July 16, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Grant-in-Aid for Young Scientists Start-up and Young Scientists A (19890005 and 20689021 to W.N.); by Grant-in-Aid for Exploratory Research (19659279 to H.S.); and by Health and Labour Science Research Grants for Research on Measures for Intractable Diseases, from the Ministry of Health, Labour, and Welfare of Japan (to H.S.) and by Program for Promotion of Fundemental Studies in Health Science of the National Institute of Biomedical Innovation (NIBIO; to H.S.).

[2] Address correspondence and reprint requests to Dr. Wataru Nishie, Department of Dermatology, Hokkaido University Graduate School of Medicine, N15 W7, Sapporo, Japan. E-mail address: nishie@med.hokudai.ac.jp

[3] Abbreviations used in this paper: BP; boullous pemphigoid; COL17; type XVII collagen; NC; noncollagenous; Tg, transgenic; IIF, indirect immunofluorescence; DIF; direct immunofluorescence, GST; gulutathione S-transferase.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0800389

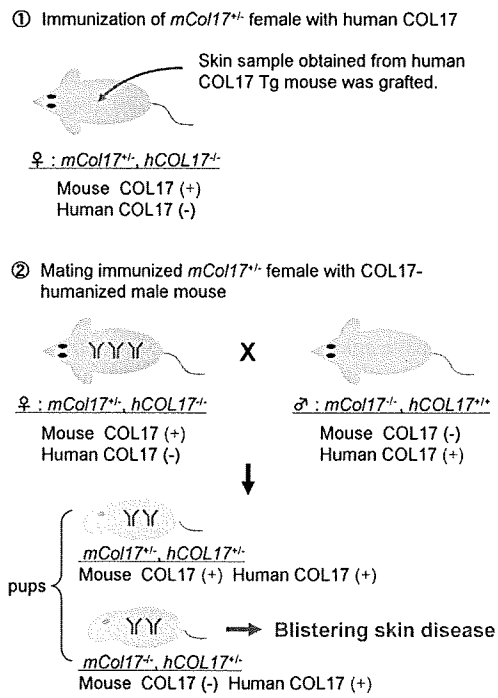

FIGURE 1. Schematic of the method for generating the neonatal BP model. Four-to 6-wk-old heterozygous $mCol17^{+/-}$ female mice (C57BL/6 background) were immunized against human COL17 by grafting skin obtained from gender-matched, syngeneic human COL17 cDNA Tg mouse driven under keratin 14 promoter (1). Two weeks after grafting, the immunized female mice were crossed with 6- to 8-wk-old COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) males. Theoretically, half of the newborns should express only human COL17 in the skin ($mCol17^{-/-}$, $hCOL17^{+/-}$), and these are expected to be a neonatal BP model (2).

Materials and Methods

Gross summary of strategy

We selected a breeding pair consisting of heterozygote *Col17a1*-deficient ($mCol17^{+/-}$) female mice and COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) male mice (Fig. 1). Theoretically, half of the pups from this pair should express only human COL17 in the skin while the other half should express both mouse and human COL17 (Fig. 1). Wild-type mice can develop quite high titers of circulating anti-human COL17 IgG when grafted with human COL17 transgenic (Tg) mouse skin (19). We first immunized $mCol17^{+/-}$ mother mice with skin grafts obtained from human COL17 Tg mouse, and then we mated the immunized $mCol17^{+/-}$ mother mice with COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) male mice. Neonatal COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) mice retained skin stability against mechanical friction (12); similarly, neonatal COL17-humanized mice heterozygously carrying human COL17 cDNA transgene ($mCol17^{-/-}$, $hCOL17^{+/-}$) showed none of the skin abnormalities seen in $mCol17^{-/-}$ mice, although it was possible to detach the epidermis by moderate mechanical friction (our unpublished data). We hypothesized that immunized $mCol17^{+/-}$ mother mice would produce circulating anti-human COL17 IgG that would be transferred into their neonates including those whose skin expressed only human and not mouse COL17 ($mCol17^{-/-}$, $hCOL17^{+/-}$), resulting in natural blistering that replicates human BP disease (Fig. 1).

Immunization of the heterozygote $mCol17^{+/-}$ female mice

Four- to 6-wk-old heterozygote-null $mCol17^{+/-}$ female mice (F_1 mouse was 129/SvEv × C57BL/6 background, back-crossed with C57BL/6 over 10 generations) were immunized against human COL17 as previously described (19), with minor modifications. In brief, 1 × 1 cm of back skin obtained from gender-matched, syngeneic human COL17 cDNA Tg mice was grafted onto the back of the recipient $mCol17^{+/-}$ female mice. As a control, back skin obtained from wild-type C57BL/6 was grafted onto recipient $mCol17^{+/-}$ female mice ($n = 5$). The grafted skin was sutured, and bandages were removed 7 days after skin grafting.

Generation of neonatal BP mice

Two weeks after skin grafting, the immunized and the control $mCol17^{+/-}$ female mice were crossed with 6- to 8-wk-old COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) male mice (12). Half of their newborns ($mCol17^{-/-}$, $hCOL17^{+/-}$) were predicted to express only human COL17 and not mouse COL17 in the skin and the other half of the newborns ($mCol17^{+/-}$, $hCOL17^{+/-}$) to express both mouse and human COL17 in the skin (Fig. 1).

Evaluation of serum anti-human COL17 IgG in the immunized mother mice and their neonates

Sera from immunized $mCol17^{+/-}$ females (before immunization and 1 to 4 wk after immunization) and their neonates (at birth and 1 to 4 wk after birth, respectively) were sampled, followed by ELISA and indirect immunofluorescence (IIF) to evaluate the circulating mouse IgG Abs directed against human COL17 (12, 19). The ELISA index value against the human COL17 NC16A domain peptide was measured using BP180 ELISA kit (MBL) with minor modifications. In brief, this kit is designed to detect human IgG against human COL17; therefore, HRP-conjugated goat polyclonal anti-mouse IgG (1/20,000 dilution, Jackson ImmunoResearch Laboratories) was used as a secondary Ab substitute for prepared HRP-conjugated anti-human IgG. The absorbance was measured at 450 nm by microtiter plate readers (Bio-Rad). For IIF studies, serum from the mice was serially diluted in PBS. Normal or 1M NaCl split human skin samples were obtained from a healthy volunteer and incubated with the sera for 30 min at 37°C, followed by staining with FITC-conjugated polyclonal goat anti-mouse IgG (1/100 dilution, Jackson ImmunoResearch Laboratories) as described previously (12, 19).

Immunopathological analysis of neonatal BP

For histological investigations, back skin of the mice was obtained at birth and 1 to 4 wk after birth, and processed for H&E staining and direct immunofluorescence (DIF) microscopy. For DIF study, FITC-conjugated goat polyclonal anti-mouse IgG (1/100 dilation, Jackson ImmunoResearch Laboratories), rat monoclonal anti-mouse IgG1, IgG2a, IgG2b (1/100 dilution, BD Pharmingen), goat polyclonal anti-mouse IgG2c (1/400 dilation, Bethyl Laboratories), and FITC-conjugated goat anti-mouse C3 (1/200 dilation, Cappel) were used (12, 14, 20).

Passive transfer of maternal IgG with or without immunoadsorption against human COL17 NC16A protein into neonatal COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) mice

Total IgG was purified from pooled sera obtained from 5 immunized $mCol17^{+/-}$ females (10 wks after skin grafting) using HiTrap Protein G HP (GE Healthcare) according to the manufactur's instructions. Recombinant human COL17 NC16A (amino acid: 490–566) protein was generated as a gulutathione S-transferase (GST) fusion protein as previously described (12), and 6 mg of the purified protein was coupled with 1 ml of GSTrap FF (GE Healthcare). Half of the purified total IgG was coupled with the human COL17 NC16A-GST protein in the column to eliminate Abs directing to human COL17 NC16A protein, and flow-through samples were collected. Total IgG with or without immunoadsorption using human COL17 NC16A protein were concentrated by Amicon Ultra-50 ultracentrifuge (Millipore), and each was adjusted to be 2.1 $\mu g/\mu l$. Fifty μl of Abs was i.p. injected into neonatal COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) mice as previously described (12).

All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hokkaido University, and fully informed consent from all patients was obtained for the use of their materials.

Results

High titers of IgG Abs against human COL17 were induced in recipient mother mice and they were efficiently transferred to their neonates

Consistent with the previous report in which high titer of IgG against human COL17 were successfully induced when human COL17 Tg mouse skin was grafted onto the wild-type mice (19), the heterozygote $mCol17^{+/-}$ female mice also developed high titers of circulating anti-human COL17 IgG after skin grafting of human COL17 cDNA Tg mice skin (Fig. 2, *a–c*). ELISA studies clearly showed the presence of circulating anti-human COL17 IgG at 3 wk after skin grafting, and a maximum titer was reached at

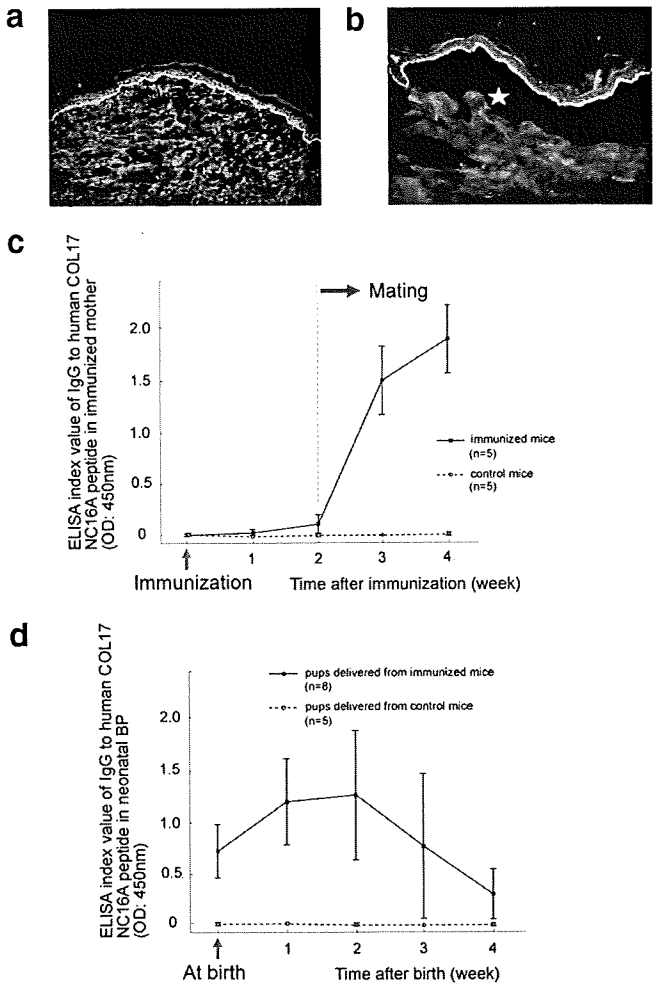

FIGURE 2. Profile of IgG Abs against human COL17 in immunized heterozygote *mCol17*$^{+/-}$ female and neonatal mice. *a*, IIF study using normal human skin as a substrate demonstrated the presence of IgG Abs against the dermal-epidermal junction in the sera of immunized mothers. *b*, These Abs reacted with the epidermal side of the basement membrane zone in skin incubated with 1M-NaCl (star shows cleft between epidermis and dermis). *c*, Heterozygous recipient *mCol17*$^{+/-}$ female mice developed high titers of circulating anti-human COL17 IgG around 3 wk after immunization. *d*, Maternal IgG was efficiently transferred into neonates, rapidly decreasing 2 to 3 wk after birth. The majority of maternal IgG had disappeared by 4 wk after birth.

4 wk (Fig. 2*c*). IIF study using 1M NaCl split normal human skin as a substrate demonstrated that this anti-human COL17 IgG reacted with the epidermal side of the basement membrane (Fig. 2*b*), consistent with the reactivity of human BP autoAbs (15, 16). We then crossed immunized heterozygous *Col17*-deficient (*mCol17*$^{+/-}$) female mice and COL17-humanized (*mCol17*$^{-/-}$, *hCOL17*$^{+/+}$) male mice to give birth to their neonates (Fig. 1). In the neonates delivered from these immunized female mice, maternally transferred IgG against human COL17 was retained at high titers for at least 2 wk after birth, after which it decreased, disappearing by 4 wk after birth (Fig. 2*d*). In the control, *mCol17*$^{+/-}$ female mice, which had been grafted with wild-type mice skin, no anti-human COL17 IgG Abs could be observed nor in their pus delivered after mating them with COL17-humanized (*mCol17*$^{-/-}$, *hCOL17*$^{+/+}$) male (Fig. 2, *c* and *d*).

Neonatal BP mice developed severe blistering

All (n = 12) of the neonatal BP mice that expressed only human COL17 and not the mouse ortholog in the skin with maternally transferred IgG against human COL17 showed severe skin fragility and the epidermis easily detached with minor mechanical friction (Nikolsky phenomenon, Fig. 3*a*). Notably some mice developed spontaneous small blisters and pustules (Fig. 3, *a* and *c*). These skin lesions gradually disappeared in the first week after birth, leaving small, round, crusted lesions similar to those seen in BP patients (Fig. 3*b*). Although epidermal detachment could be induced by moderate (but not minor) friction in humanized mice heterozygously carrying the human COL17 cDNA transgene (*mCol17*$^{-/-}$, *hCOL17*$^{+/-}$), the skin fragility observed in neonatal BP mice was obviously more severe, and minor friction easily produced extensive epidermal detachment. In contrast, none of the other neonates (n = 13) that expressed both human and mouse COL17 in skin (*mCol17*$^{+/-}$, *hCOL17*$^{+/-}$) demonstrated any distinct skin abnormalities following exposure to maternal IgG, including spontaneous blister formation or Nikolsky phenomenon (data not shown).

Neonatal BP mice showed histological and immunological features identical with those seen in patients with BP

This system is characterized by complete humanization of the Ag in neonatal mice with ensuing inflammatory cascades that are completely mouse-derived. Therefore, the system is able to induce specific IgG-Ag reactions and lead to skin inflammation consistent with BP in humans. Notably, histological examinations demonstrated distinctive subepidermal blister formation with numerous inflammatory cell infiltrates predominately consisting of neutrophils (Fig. 3*c*). DIF studies of BP model mice skin revealed deposition of mouse IgG and of mouse complement (C3) in epidermal basement membrane until the third and the first to second weeks after birth, respectively (Fig. 3*d*). Subclass analysis of in vivo deposition of IgG showed that IgG1 and IgG2c predominated at the dermal-epidermal junction (Fig. 3*e*). This characteristic of IgG subclass deposition was the same for immunized *mCol17*$^{+/-}$ females as for their neonates, as shown by IIF on the normal human skin as a substrate (data not shown).

IgG Abs to the NC16A domain of human COL17 play a major role in inducing blistering skin disease

We previously demonstrated that IgG Abs to the NC16A domain of human COL17 play a major role in induce blistering disease; this was demonstrated by passive-transfer experiments using IgG autoAbs from BP patients in neonatal COL17-humanized (*mCol17*$^{-/-}$, *hCOL17*$^{+/+}$) mice (12). To assess and characterize the role of IgG Abs in immunized *mCol17*$^{+/-}$ female mice in the current model, we performed passive-transfer experiment using IgG Abs obtained from immunized female mice with or without immunoadsorption against human COL17 NC16A protein (n = 2, respectively). By IIF study using normal human skin as a substrate, both immune-adsorbed and without immunoadsorption purified IgG reacted to the dermal-epidermal junction until 5120 and 20480 times dilution respectively (data not shown). The passive-transfer experiment showed that purified total IgG without immunoadsorption with human COL17 NC16A protein resulted in skin fragility associated with IgG deposition along the dermal-epidermal junction (Fig. 4, *a* and *b*). In contrast, treatment of IgG with COL17 NC16A protein resulted in no blistering phenotype, although slight deposition of IgG could be observed along the dermal-epidermal junction (Fig. 4, *a* and *b*). These results clearly suggest that IgG Abs to NC16A domain of human COL17 played the major role to induce blistering skin disease in vivo.

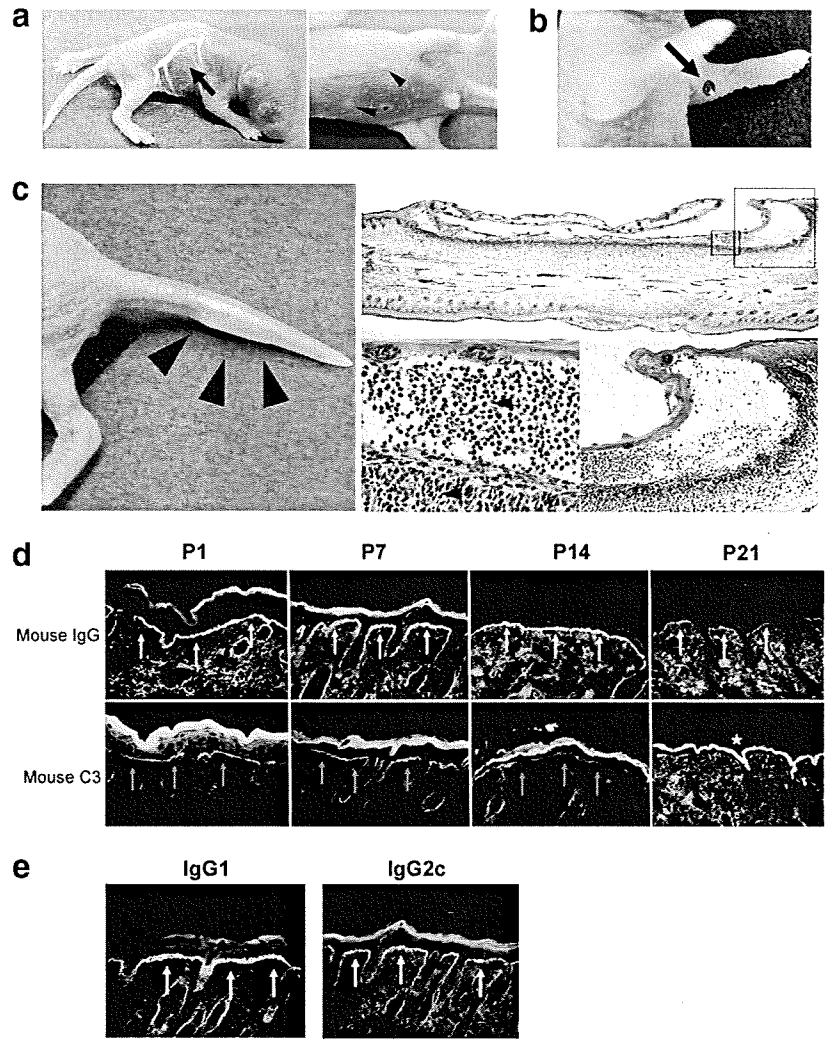

FIGURE 3. *a*, Neonatal BP mice showed severe skin fragility, with the epidermis easily detaching from mechanical friction (Nikolsky phenomenon, arrow). Spontaneous small blisters and pustules were scattered over the entire body (arrowheads). *b*, Small, round, crusted lesion developed around the arm in 4-day-old neonatal BP mice. *c*, Histological finding of blistering lesion on the tail. Subepidermal blister formation associated with numerous infiltrations of neutrophils (arrowheads) was observed. *d*, DIF study revealed in vivo skin deposition of mouse IgG (yellow arrows) until 3 wk after birth, and activated mouse C3 (red arrows) was detected within 1 to 2 wk after birth. Note the Abs to mouse C3 strongly cross-reacted to the corneal layer of the epidemis (star). *e*, In vivo deposition of IgG1 and IgG2c was detected at the dermal-epidermal junction of a neonatal BP mouse soon after birth (arrows).

Maternal IgG to human COL17 was transmissible into neonatal circulation via milk even after birth

Interestingly, some of the mice showed elevated IgG Ab titers to human COL17 by ELISA around 1 to 2 wk after birth (Fig. 2*d*). It has been reported that mouse IgG can be transferred from milk via neonatal FcR expressed in gut, which is different from humans (2, 21, 22). To investigate this possibility, COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) neonatal mice delivered from unrelated pairs were moved soon after birth to a lactating preimmunized $mCol17^{+/-}$ female mouse (ELISA index value to human COL17 of 1.33). As a result, it was found that, at 1 wk of breast-feeding from the immunized female mouse, serum IgG in these pups to human COL17 was markedly elevated (ELISA index titer: 0.73 ± 0.20, $n = 4$), and mouse IgG reacted positively to the dermal-epidermal junction in the skin until 1/1280 dilution (Fig. 5*a*). In contrast, IgG Abs to human COL17 of the pups breast-fed from the nonimmunized female mouse were not increased (ELISA index titer: 0.02 ± 0.03, $n = 4$), and mouse IgG did not react to the normal human skin (Fig. 5*a*). These results clearly indicate that maternally anti-human COL17 IgG Abs were transmitted from milk. However, these COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) neonatal mice with maternal milk-derived Abs to human COL17 in their circulation showed no skin fragility (data not shown). In Neonatal BP mice, active blistering skin disease could be observed for several days after birth, therefore, we assessed in vivo deposition of mouse IgG in the neonatal COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) mice skin at 2 days after being breast-fed by the lactating preimmunized $mCol17^{+/-}$ female to find how maternal IgG from milk could contribute to the blistering skin disease soon after birth. As a result, very low amount of mouse IgG could be detected at the dermal-epidermal junction

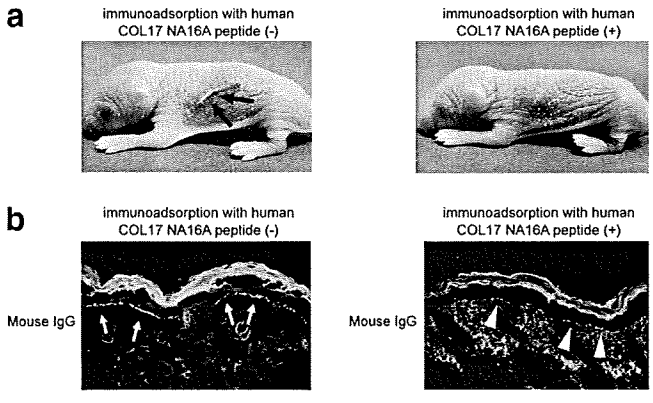

FIGURE 4. IgG Abs to the NC16A domain of human COL17 play a major role in inducing blistering skin disease. *a*, Neonatal COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) mice that received IgG Abs without immunoadsorption with human COL17 NC16A protein from immunized $mCol17^{+/-}$ females resulted in skin fragility (positive Nikolsky sign, arrows), whereas no epidermal detachment could be observed in mice that received immune-adsorbed IgG (50 μl of 2.1 μg/μl IgG Abs, respectively). *b*, In vivo deposition of mouse IgG was more intense in the skin obtained from mice that received IgG Abs without adsorption with human COL17 NC16A protein (arrows) compared with that being adsorbed with the protein (arrowheads).

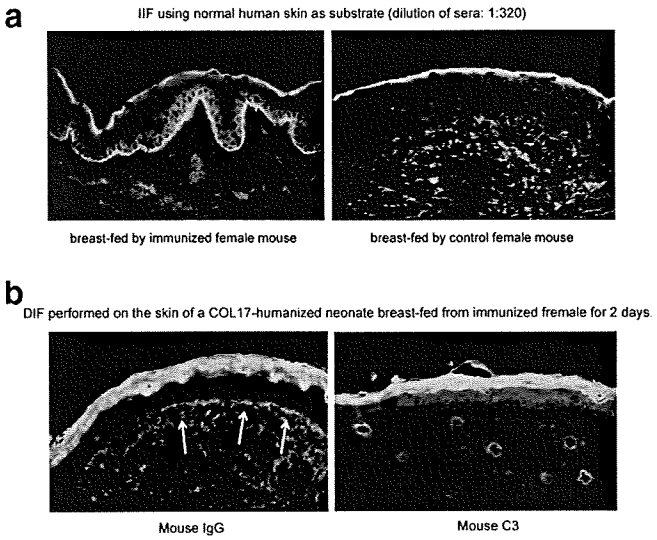

FIGURE 5. Maternal anti-human COL17 Abs transferred from milk. *a*, Serum IgG in these pups to human COL17 reacted to the dermal-epidermal junction at 1 wk of breast-feeding from an immunized female mouse (1/320 dilution). *b*, COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) neonatal mice skin at 2 days of breast-feeding from an immunized mother mouse showed mild in vivo deposition of IgG Abs along the dermal-epidermal junction.

(Fig. 5*b*), suggesting that IgG from milk would play a minor role in the pathogenesis of this Neonatal BP mice model.

Discussion

This study showed successful production of a novel autoimmune disease model in which efficiently transferred maternal IgG induced distinctive Ab-mediated blistering skin disease in COL17-humanized neonates. The COL17 is crucial for maintaining the structural stability of skin. Indeed, loss of COL17 in skin as a result of null mutations results in a novel form of epidermolysis bullosa (OMIM: 226650) (23, 24). Similarly, $mCol17^{-/-}$ mice skin is so fragile that these mice could not be intercrossed (12). Therefore, we used heterozygote-null, but phenotypically normal $mCol17^{+/-}$ female mice to develop Abs against human COL17. Furthermore, by mating immunized female mice with COL17-humanized male mice, human COL17 could be introduced into neonates as an Ag. This simple method enabled us to develop distinctive neonatal BP mice that demonstrate a more severe blistering phenotype characterized by spontaneous blister formation with numerous inflammatory cell infiltrates, a phenotype that is very similar to that seen in humans with BP (15, 16).

Our neonatal BP mice has several advantages over previous mouse models of BP (12, 14). First, unlike other BP models that have relied on the injection of pathogenic IgG Abs into neonatal mice, our model does not require the technically difficult injection procedure. Second, the pathogenic IgG remains in circulation longer in the new model than in conventional models that use injected IgG. Third, the immune reaction is totally dependent on the mouse immune system, while the Ag remains human COL17. The mouse complement system does not work as efficiently during the neonatal period as during adulthood (25). Accordingly, the present system using Abs from the same species is suitable for promoting the subsequent inflammation cascade, including activation of the mouse complement. Finally, immunized heterozygous $mCol17^{+/-}$ female mice induced both IgG1 and IgG2c autoAbs which were transferred to neonates. Mouse IgG1 Abs do not fix complement (20), whereas IgG2c does fix mouse complement (26); therefore, activation of complement in the present system would be induced predominately by IgG2c. Activation of complement has been reported to play a pivotal role in BP blistering (27, 28). In light of this, the current model can be regarded as accurately reproducing human BP disease.

Maternal IgG Abs to human COL17, especially to the NC16A domain, via placenta plays a major role in the current neonatal BP mice model to induce blistering skin disease, in which the most severe disease could be observed soon after birth. However, although it is a rare possibility, maternal transferred lymphocytes as well as pathogenic IgG from milk might contribute to the blistering skin disease. In particular, we were able to clearly demonstrate that maternal IgG Abs transferred into pups via milk 1 wk after birth, although no active blistering skin disease could be observed in the recipient unrelated COL17-humanized ($mCol17^{-/-}$, $hCOL17^{+/+}$) neonatal mice. The reason we could not observe blistering skin disease is probably due to the lower amount of IgG to human COL17 than in neonatal BP mice, in which maternal IgG could be transferred from not only milk but also via placenta, especially soon after birth. IgG transferred from milk might work in part, but it will not be a major player in inducing blistering skin disease, because blistering skin disease was the most severe soon after birth and no active blistering skin disease could be observed at 1 wk, when a lot of fur had grown.

Using maternally transferred pathogenic Abs and introducing human Ags in neonates, we succeeded in inducing autoimmune disease model in neonates whose Ags are functionally important. However, this system does not truly represent autoimmunity in human patients, because Abs to human COL17 in diseased neonates are transferred Abs. In addition, for immunized heterozygote *Col17*-deficient ($mCol17^{+/-}$) female mice, human COL17 is not an autoAg but alloantigen therefore, pathogenic Abs to human COL17 in this system is not strictly an autoAbs. Nevertheless, maternally transferred Abs in genetically transformed Ag-humanized neonates will be useful in the study of autoimmune diseases as a novel method for generating diseases in neonates.

Acknowledgments

We thank Ai Hayakawa, Yuko Hayakawa, and Akari Nagasaki for their technical assistance, and Dr. James R. McMillan and Dr. Heather Ann Long for their language editing and proofreading.

Disclosures

The authors have no financial conflict of interest.

References

1. Kohler, P. F., and R. S. Farr. 1966. Elevation of cord over maternal IgG immunoglobulin: evidence for an active placental IgG transport. *Nature* 210: 1070–1071.
2. Van de Perre, P. 2003. Transfer of antibody via mother's milk. *Vaccine* 21: 3374–3376.
3. Lee, L. A. 1993. Neonatal lupus erythematosus. *J. Invest. Dermatol.* 100: 9S–13S.
4. Lee, L. A., M. B. Frank, V. R. McCubbin, and M. Reichlin. 1994. Autoantibodies of neonatal lupus erythematosus. *J. Invest. Dermatol.* 102: 963–966.
5. Vernet-der Garabedian, B., M. Lacokova, B. Eymard, E. Morel, M. Feltin, J. Zajac, O. Sadovsky, M. Dommergues, and J. F. Bach. 1994. Association of neonatal myasthenia gravis with antibodies against the fetal acetylcholine receptor. *J. Clin. Invest.* 94: 555–559.
6. Ni, H., P. Chen, C. M. Spring, E. Sayeh, J. W. Semple, A. H. Lazarus, R. O. Hynes, and J. Freedman. 2006. A novel murine model of fetal and neonatal alloimmune thrombocytopenia: response to intravenous IgG therapy. *Blood* 107: 2976–2983.
7. van der Neut, R., P. Krimpenfort, J. Calafat, C. M. Niessen, and A. Sonnenberg. 1996. Epithelial detachment due to absence of hemidesmosomes in integrin β 4 null mice. *Nat. Genet.* 13: 366–369.
8. Georges-Labouesse, E., N. Messaddeq, G. Yehia, L. Cadalbert, A. Dierich, and M. Le Meur. 1996. Absence of integrin α 6 leads to epidermolysis bullosa and neonatal death in mice. *Nat. Genet.* 13: 370–373.
9. Ryan, M. C., K. Lee, Y. Miyashita, and W. G. Carter. 1999. Targeted disruption of the LAMA3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. *J. Cell Biol.* 145: 1309–1323.

10. Heinonen, S., M. Männikkö, J. F. Klement, D. Whitaker-Menezes, G. F. Murphy, and J. Uitto. 1999. Targeted inactivation of the type VII collagen gene (Col7a1) in mice results in severe blistering phenotype: a model for recessive dystrophic epidermolysis bullosa. *J. Cell. Sci.* 112: 3641–3648.
11. Meng, X., J. F. Klement, D. A. Leperi, D. E. Birk, T. Sasaki, R. Timpl, J. Uitto, and L. Pulkkinen. 2003. Targeted inactivation of murine laminin γ2-chain gene recapitulates human junctional epidermolysis bullosa. *J. Invest. Dermatol.* 121: 720–731.
12. Nishie, W., D. Sawamura, M. Goto, K. Ito, A. Shibaki, J. R. McMillan, K. Sakai, H. Nakamura, E. Olasz, K. B. Yancey, et al. 2007. Humanization of autoantigen. *Nat. Med.* 14: 378–383.
13. Anhalt, G. J., C. F. Bahn, R. S. Labib, J. J. Voorhees, A. Sugar, and L. A. Diaz. 1981. Pathogenic effects of bullous pemphigoid autoantibodies on rabbit corneal epithelium. *J. Clin. Invest.* 68: 1097–1101.
14. Liu, Z., L. A. Diaz, J. L. Troy, A. F. Taylor, D. J. Emery, J. A. Fairley, and G. J. Giudice. 1993. A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen. *BP180. J. Clin. Invest.* 92: 2480–2488.
15. Korman, N. 1987. Bullous pemphigoid. *J. Am. Acad. Dermatol.* 16: 907–924.
16. Stanley, J. R., P. Hawley-Nelson, S. H. Yuspa, E. M. Shevach, and S. I. Katz. 1982. Characterization of bullous pemphigoid antigen: a unique basement membrane protein of stratified squamous epithelia. *Cell* 24: 897–903.
17. Gatalica, B., L. Pulkkinen, K. Li, K. Kuokkanen, M. Ryynänen, J. A. McGrath, and J. Uitto. 1997. Cloning of the human type XVII collagen gene (COL17A1), and detection of novel mutations in generalized atrophic benign epidermolysis bullosa. *Am. J. Hum. Genet.* 60: 352–365.
18. Zillikens, D., P. A. Rose, S. D. Balding, Z. Liu, M. Olague-Marchan, L. A. Diaz, and G. J. Giudice. 1997. Tight clustering of extracellular BP180 epitopes recognized by bullous pemphigoid autoantibodies. *J. Invest. Dermatol.* 109: 573–579.
19. Olasz, E. B., J. Roh, C. L. Yee, K. Arita, M. Akiyama, H. Shimizu, J. C. Vogel, and K. B. Yancey. 2007. Human bullous pemphigoid antigen 2 transgenic skin elicits specific IgG in wild-type mice. *J. Invest. Dermatol.* 127: 2807–2817.
20. Sitaru, C., M. T. Chiriac, S. Mihai, J. Büning, A. Gebert, A. Ishiko, and D. Zillikens. 2006. Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. *J. Immunol.* 177: 3461–3468.
21. Appleby, P., and D. Catty. 1983. Transmission of immunoglobulin to foetal and neonatal mice. *J. Reprod. Immunol.* 5: 203–213.
22. Israel, E. J., V. K. Patel, S. F. Taylor, A. Marshak-Rothstein, and N. E. Simister. 1995. Requirement for a β_2-microglobulin-associated Fc receptor for acquisition of maternal IgG by fetal and neonatal mice. *J. Immunol.* 154: 6246–6251.
23. McGrath, J. A., B. Gatalica, A. M. Christiano, K. Li, K. Owaribe, J. R. McMillan, R. A. Eady, and J. Uitto. 1995. Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa. *Nat. Genet.* 11: 83–86.
24. Shimizu, H., Y. Takizawa, L. Pulkkinen, J. J. Zone, K. Matsumoto, T. Saida, J. Uitto, and T. Nishikawa. 1998. The 97 kDa linear IgA bullous dermatosis antigen is not expressed in a patient with generalized atrophic benign epidermolysis bullosa with a novel homozygous G258X mutation in COL17A1. *J. Invest. Dermatol.* 111: 887–892.
25. Pihlgren, M., A. Fulurija, M. B. Villiers, C. Tougne, P. H. Lambert, C. L. Villiers, and C. A. Siegrist. 2004. Influence of complement C3 amount on IgG responses in early life: immunization with C3b-conjugated antigen increases murine neonatal antibody responses. *Vaccine.* 23: 329–335.
26. Baudino, L., S. Azeredo da Silveria, M. Nakata, and S. Izui. 2006. Molecular and cellular basis for pathogenicity of autoantibodies: lessons from murine monoclonal autoantibodies. *Springer. Semin. Immunopathol.* 28: 175–184.
27. Liu, Z., G. J. Giudice, S. J. Swartz, J. A. Fairley, G. O. Till, J. L. Troy, and L. A. Diaz. 1995. The role of complement in experimental bullous pemphigoid. *J. Clin. Invest.* 95: 1539–1544.
28. Nelson, K. C., M. Zhao, P. R. Schroeder, N. Li, R. A. Wetsel, L. A. Diaz, and Z. Liu. 2006. Role of different pathways of the complement cascade in experimental bullous pemphigoid. *J. Clin. Invest.* 116: 2892–2900.

FLG mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema

I. Nemoto-Hasebe,* M. Akiyama,* T. Nomura,*† A. Sandilands,† W.H.I. McLean† and H. Shimizu*

*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
†Epithelial Genetics Group, Division of Molecular Medicine, University of Dundee, Colleges of Life Sciences and Medicine, Dentistry & Nursing, Dundee, U.K.

Correspondence
Masashi Akiyama.
E-mail: akiyama@med.hokudai.ac.jp

Accepted for publication
4 June 2009

Key words
atopic dermatitis, atopy, ichthyosis, phenotype, profilaggrin

Conflicts of interest
W.H.I.M. has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

DOI 10.1111/j.1365-2133.2009.09406.x

Summary

Background Mutations in the gene encoding filaggrin (*FLG*) have been shown to predispose to atopic eczema (AE).

Objectives Further to establish population genetics of *FLG* mutations in the Japanese population and to elucidate effects of *FLG* mutations to filaggrin biosynthesis in skin of patients with AE.

Methods We searched for *FLG* mutations in 19 newly recruited Japanese patients with AE. We then screened 137 Japanese patients with AE and 134 Japanese control individuals for a novel mutation identified in the present study. In addition, we evaluated *FLG* mRNA expression by real-time reverse transcription–polymerase chain reaction and profilaggrin/filaggrin protein expression by immunohistochemical staining in the epidermis of the patients carrying the novel mutation.

Results We identified a novel *FLG* nonsense mutation c.12069A>T (p.Lys4021X) in one patient with AE. Upon further screening, p.Lys4021X was identified in four patients with AE (2·9% of all the patients with AE). In total, there are at least eight *FLG* variants in the Japanese population. Here we show that about 27% of patients in our Japanese AE case series carry one or more of these eight *FLG* mutations and these variants are also carried by 3·7% of Japanese general control individuals. There is a significant statistical association between the eight *FLG* mutations and AE (χ^2 $P = 6{\cdot}50 \times 10^{-8}$). Interestingly, the present nonsense mutation is in the C-terminal incomplete filaggrin repeat and is the mutation nearest the C-terminal among previously reported *FLG* mutations. Immunohistochemical staining for filaggrin revealed that this nonsense mutation leads to remarkable reduction of filaggrin protein expression in the patients' epidermis.

Conclusions We clearly demonstrated that *FLG* mutations are significantly associated with AE in the Japanese population. The present results further support the hypothesis that the C-terminal region is essential for proper processing of profilaggrin to filaggrin.

Filaggrin is a protein essential to skin barrier function. Mutations in *FLG*, the gene encoding profilaggrin/filaggrin, have been demonstrated as the underlying cause of ichthyosis vulgaris (IV; OMIM 146700) and have been shown to be an important predisposing factor for atopic eczema (AE).[1–4] The presence of population-specific *FLG* mutations in Europeans, Chinese-Singaporeans, Japanese and Taiwanese has been reported.[3,5–9] Recently, it was clarified that *FLG* mutations were found in approximately 25% of Japanese patients with AE.[6,8]

Materials and methods

We searched for *FLG* mutations in 19 newly recruited Japanese patients with AE. All these patients had been diagnosed with AE based on widely recognized diagnostic criteria.[10] Initially, using genomic DNA, patients with AE were screened for seven *FLG* mutations previously identified in the Japanese population by restriction enzyme digestion, fluorescent polymerase chain reaction (PCR) and/or direct DNA sequencing as described previously.[8] Subsequently, for the patients with AE without

any known *FLG* mutation, we sequenced the entire coding region of FLG. The medical ethics committee of Hokkaido University Graduate School of Medicine approved all the studies, which were conducted according to the Declaration of Helsinki Principles. The participants or their legal guardians gave written informed consent.

Results

This sequencing revealed a novel nonsense mutation c.12069A>T (p.Lys4021X) in repeat 11 (imperfect filaggrin repeat) of one patient with AE (Fig. 1a–c). The nucleotide change was not detected in 50 unrelated, healthy Japanese individuals (100 alleles).

Subsequently, we screened for the newly identified FLG mutation p.Lys4021X in all 137 Japanese patients with AE we had collected to date and 134 unrelated Japanese control individuals. The 118 patients with AE and 134 control individuals were identical to those in a previous study,[8] and the data except for those on p.Lys4021X were reported by Nomura *et al.*[8] We identified p.Lys4021X in four patients with AE (2·9% of all the patients with AE) in our Japanese AE cohort.

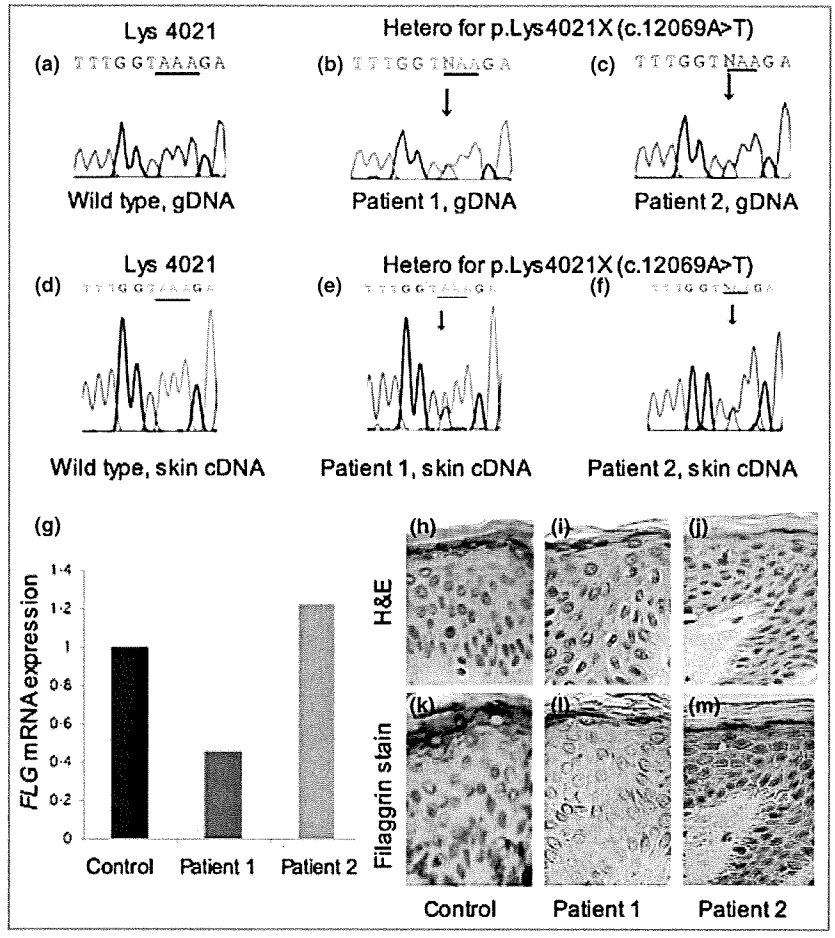

Fig 1. *FLG* mutation analysis and expression of p.Lys4021X mutant alleles. (a–c) Identification of the *FLG* mutation. Direct sequence analysis of *FLG* was performed on genomic DNA from peripheral leucocytes. (a) Normal control sequence from filaggrin repeat 11 in exon 3. (b, c) A heterozygous transition mutation c.12069A>T was identified in patient 1 (b) and patient 2 (c), resulting in p.Lys4021X. (d–f) Direct sequence analysis of *FLG* cDNA from mRNA expressed in skin samples from the back. (d) Normal control cDNA sequence derived from filaggrin repeat 11 in exon 3. (e, f) Expression of mRNA derived from both wild-type alleles and mutant alleles was confirmed in patient 1 (e) and patient 2 (f). The expression level of mRNA derived from the mutant allele was lower than that from the wild-type allele in patient 1 (e), although expression levels of mRNA from both mutant and wild-type alleles were roughly equal in patient 2 (f). (g) Real-time reverse transcription–polymerase chain reaction analysis of *FLG* mRNA expression in the skin. *FLG* expression was reduced in patient 1, but not in patient 2: patient *FLG* mRNA expression/control *FLG* mRNA expression = 0·46 for patient 1 and 1·23 for patient 2. mRNA expression of *FLG* in patients 1 and 2 was not significantly different from that in control skin. (h–m) Histological features of patients 1 and 2: (h–j) haematoxylin and eosin (H&E) staining; (k–m) immunohistochemical staining using antifilaggrin monoclonal antibody against an epitope conserved in all filaggrin repeat peptides. Patient 1 (i) and patient 2 (j) showed a lack of granular layers in the epidermis, where only a small amount of a basophilic substance, which resembled keratohyaline granules, was occasionally present. In contrast, normal control skin (h) had abundant keratohyaline granules in the granular layers. A marked reduction in staining for filaggrin was seen in the epidermis from both patient 1 (l) and patient 2 (m), relative to the strong staining in normal control skin (k).

It was confirmed that these four patients with AE carry no other known *FLG* mutations. None of the control individuals had the p.Lys4021X mutation. The four patients with AE with the newly discovered mutation – three women and one man – were aged 12–31 years, and all four patients had severe AE symptoms. There was no specific clinical feature of AE characteristic to the four patients. None of the four patients had apparent clinical features or a family history of IV.

We investigated *FLG* mRNA expression by real-time reverse transcription (RT)-PCR and sequencing, and studied profilaggrin/filaggrin protein expression in the skin by immunohistochemistry in two of the patients with AE harbouring p.Lys4021X. Real-time RT-PCR analysis revealed that mRNA expression of *FLG* was not reduced significantly (Fig. 1g). The expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles (Fig. 1d–f). However, histopathological examinations of the patients' skin showed reductions in keratohyaline granules in the granular layers (Fig. 1h–j). Immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the patients' epidermis (Fig. 1k–m).

Eight *FLG* mutations including the present mutation p.Lys4021X have been identified in the Japanese population (Fig. 2). Case–control association analyses were performed for *FLG* mutations in Japanese patients with AE and normal controls using Pearson χ^2 statistics, as previously described.[2] The *FLG* genotype data in the Japanese AE case series and ethnically matched population control series are summarized in Table 1. All alleles were observed to be in normal Hardy–Weinberg equilibrium. Here we demonstrate that about 27% of the patients in our Japanese AE case series carry one or more of the eight *FLG* mutations (combined minor allele frequency = 0·150, n = 274) and these variants are also carried by 3·7% of Japanese control individuals (combined minor allele frequency = 0·019, n = 268). There is a statistically significant association between the eight *FLG* mutations and AE

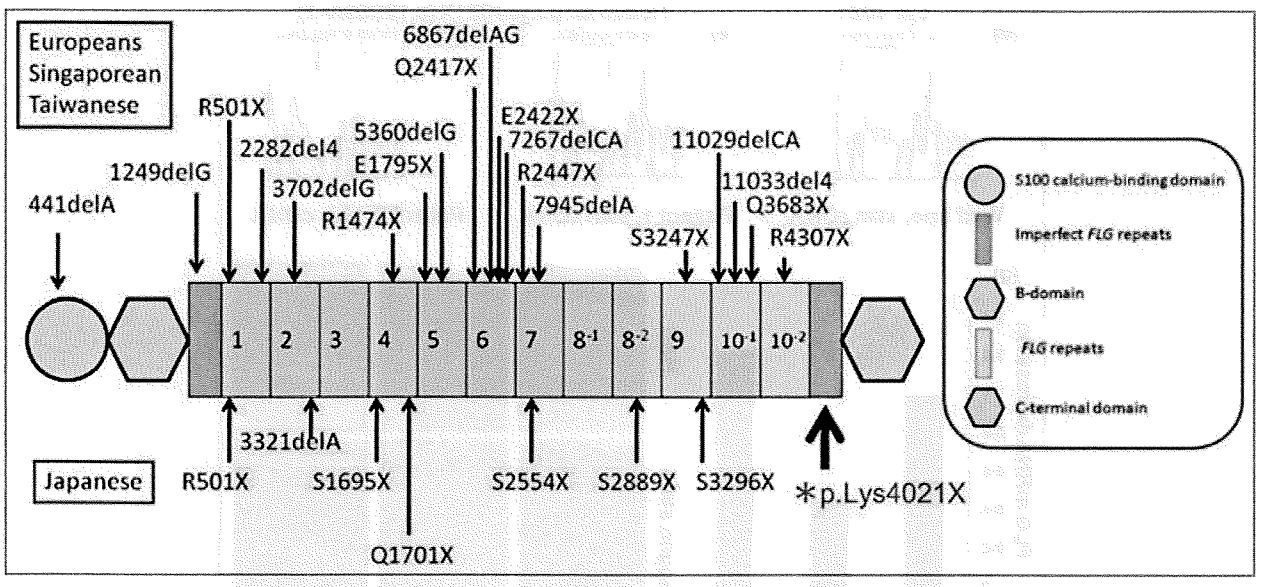

Fig 2. The present and previously reported *FLG* mutations are shown in a scheme of profilaggrin peptide. Some individuals have a duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8^{-1}, 8^{-2}, 10^{-1} and 10^{-2}. *Indicates the present mutation p.Lys4021X. This mutation is the nearest to the C-terminus domain among all the reported mutations and is located in the C-terminal incomplete filaggrin repeat downstream of all the filaggrin repeats.

Table 1 Atopic eczema case–control association analysis for *FLG* null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		K4021X		Combined	
Genotypes	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
AA	134	137	133	131	133	137	134	134	133	129	132	122	134	132	134	133	129	96
Aa	0	0	1	6	1	0	0	3	1	8	2	15	0	5	0	4	5	33
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Total	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137

For combined genotype, $\chi^2 = 29{\cdot}218$, $P = 6{\cdot}50 \times 10^{-8}$; Fisher's exact test odds ratio = 9·94 (95% confidence interval 3·77–26·2, $P = 2{\cdot}35 \times 10^{-8}$).

(χ^2 $P = 6{\cdot}50 \times 10^{-8}$). Moreover, AE was manifested in heterozygous carriers of these *FLG* mutations with a Fisher's exact test odds ratio for AE of 9·94 (95% confidence interval 3·77–26·2, $P = 2{\cdot}35 \times 10^{-8}$), suggesting a causal relationship between *FLG* mutations and AE.

Discussion

Filaggrin is synthesized initially as profilaggrin, an approximately 500-kDa polypeptide that contains two imperfect filaggrin-repeat domains flanking 10–12 essentially identical filaggrin repeats.[11]

Previous studies reported that *FLG* truncation mutations in both filaggrin repeats 1 and 7 lead to a severe filaggrin deficiency, despite the synthesis of a short N-terminal profilaggrin peptide.[1,3,12] The present mutation p.Lys4021X is in the C-terminal incomplete filaggrin repeat, and the truncation site is the nearest to the C-terminal among *FLG* mutations identified to date (Fig. 2). The longest truncated profilaggrin peptide containing all the complete filaggrin repeats may theoretically be produced from the mutant allele. However, our immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the epidermis of the patients with p.Lys4021X, even though *FLG* mRNA expression was not reduced significantly and expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles. These results suggest that the truncated profilaggrin peptides are degenerated and are not processed to filaggrin peptides even when the mutation site is in the C-terminal incomplete filaggrin repeat.

In conclusion, we have identified another prevalent *FLG* mutation in the Japanese population. We have also shown that about 27% of Japanese patients with AD carry one or more *FLG* mutations. The present nonsense mutation p.Lys4021X in the C-terminal incomplete filaggrin repeat leads to filaggrin deficiency and our results further support the hypothesis that the C-terminal region is essential for proper processing of profilaggrin to filaggrin peptides.

Acknowledgments

We thank the patients and their families for their participation. We also thank Ai Hayakawa for fine technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M.A. (Kiban B 20390304). Filaggrin research in the McLean laboratory is supported by grants from the British Skin Foundation, the National Eczema Society, the Medical Research Council (reference number G0700314), A*STAR, Singapore and anonymous donations from families affected by eczema in the Tayside Region of Scotland.

References

1 Smith FJ, Irvine AD, Terron-Kwiatkowski A *et al.* Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat Genet* 2006; **38**:337–42.
2 Palmer CN, Irvine AD, Terron-Kwiatkowski A *et al.* Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006; **38**:441–6.
3 Sandilands A, Terron-Kwiatkowski A, Hull PR *et al.* Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 2007; **39**:650–4.
4 O'Regan GM, Sandilands A, McLean WH, Irvine AD. Filaggrin in atopic dermatitis. *J Allergy Clin Immunol* 2008; **122**:689–93.
5 Nomura T, Sandilands A, Akiyama M *et al.* Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Allergy Clin Immunol* 2007; **119**:434–40.
6 Nomura T, Akiyama M, Sandilands A *et al.* Specific filaggrin mutations cause ichthyosis vulgaris and are significantly associated with atopic dermatitis in Japan. *J Invest Dermatol* 2008; **128**:1436–41.
7 Chen H, Ho JC, Sandilands A *et al.* Unique and recurrent mutations in the filaggrin gene in Singaporean Chinese patients with ichthyosis vulgaris. *J Invest Dermatol* 2008; **128**:1669–75.
8 Nomura T, Akiyama M, Sandilands A *et al.* Prevalent and rare mutations in the gene encoding filaggrin in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Invest Dermatol* 2009; **129**:1302–5.
9 Hsu C-K, Akiyama M, Nemoto-Hasebe I *et al.* Analysis of Taiwanese ichthyosis vulgaris families further demonstrates differences in FLG mutations between European and Asian populations. *Br J Dermatol* 2009; **161**:448–51.
10 Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl (Stockh)* 1980; **92**:44–7.
11 Gan SQ, McBride OW, Idler WW *et al.* Organization, structure, and polymorphisms of the human profilaggrin gene. *Biochemistry* 1991; **29**:9432–40.
12 Sandilands A, O'Regan GM, Liao H *et al.* Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis vulgaris and predispose individuals to atopic dermatitis. *J Invest Dermatol* 2006; **126**:1770–5.

ORIGINAL ARTICLE

Clinical Severity Correlates with Impaired Barrier in Filaggrin-Related Eczema

Ikue Nemoto-Hasebe[1], Masashi Akiyama[1], Toshifumi Nomura[1], Aileen Sandilands[2], WH Irwin McLean[2] and Hiroshi Shimizu[1]

Mutations in the gene-encoding filaggrin (*FLG*), a key molecule involved in skin barrier function, have been shown to be a major predisposing factor for atopic dermatitis (AD; eczema). To elucidate the pathomechanisms underlying filaggrin-related AD, we investigated stratum corneum (SC) hydration and transepidermal water loss (TEWL) as parameters of barrier function in AD patients harboring *FLG* mutations compared to AD patients without any *FLG* mutation. In filaggrin-related AD, SC hydration was both significantly reduced ($P<0.01$–0.05) and thicker ($P<0.01$–0.05) than that in healthy controls. TEWL was demonstrably increased in non-filaggrin AD compared to healthy controls ($P<0.01$–0.05). The objective score of atopic dermatitis (OSCORAD), a disease clinical severity index, significantly correlated with TEWL ($r=0.81$, $P<0.005$), SC hydration ($r=-0.65$, $P<0.05$), and SC thickness ($r=0.59$, $P<0.05$) in filaggrin-related AD. On the contrary, there was no correlation between these parameters and the OSCORAD in non-filaggrin AD. Furthermore, a significant correlation was obtained between the OSCORAD and specific IgE for house dust ($r=0.66$, $P<0.05$), mite allergen ($r=0.53$, $P<0.05$), and cat dander ($r=0.64$, $P<0.05$) in filaggrin-related AD, but not in non-filaggrin AD. All these data suggest that experimentally demonstrable skin barrier defects due to *FLG* mutations may play a crucial role in the pathogenesis of AD.

Journal of Investigative Dermatology (2009) **129,** 682–689; doi:10.1038/jid.2008.280; published online 25 September 2008

INTRODUCTION

Atopic dermatitis (AD; also known as atopic eczema) is a common skin disease that affects 15–20% of children in the developed world (Roll *et al.*, 2004). AD is thought to have a variety of heterogeneous etiologic factors including genetic predisposing factors and environmental factors. Recently, mutations within the gene-coding filaggrin (*FLG*) were reported to cause ichthyosis vulgaris (IV; Smith *et al.*, 2006) and to be a major genetic predisposing factor for AD (Palmer *et al.*, 2006; Sandilands *et al.*, 2007). Filaggrin is essential for the cell compaction process that precedes chemical cross-linking in the biogenesis of the stratum corneum (SC). Therefore, filaggrin is a key molecule in the initiation and maintenance of skin barrier function. Profilaggrin is the main protein component of the keratohyalin granules within the last living cell layers of the epidermis (Irvine and McLean, 2006). In addition, the terminal degradation products of filaggrin may act as a "natural moisturizing substance" (Rawlings and Harding, 2004). The fact that *FLG* mutations have been reported as an important predisposing factor for AD and secondary, less penetrant, atopic phenotypes such as atopic asthma, suggests that the skin barrier defect is a primary key event leading to allergic sensitization and development of AD and related allergic phenotypes (Weidinger *et al.*, 2006).

FLG null mutations are found from 15 to 55% of AD patients in European populations (Palmer *et al.*, 2006; Weidinger *et al.*, 2006; Sandilands *et al.*, 2007). Major differences exist in the spectra of *FLG* mutations observed between different ancestral groups. Specifically, *FLG* ancestral mutations p.R501X and c.2282del4 in the European population were not found in the Japanese population (Nomura *et al.*, 2007, 2008). However, very recently, we identified four unique *FLG* mutations p.Ser2554X, c.3321del, p.Ser2889X, and p.Ser3296X in Japanese IV families and clarified that these four mutations were found more than 24% of the Japanese AD patients (Nomura *et al.*, 2007, 2008).

Transepidermal water loss (TEWL) and SC hydration, which are measurements of skin barrier function, were reported to increase in AD patients due to their skin barrier insufficiency (Aalto-Korte, 1995; Chamlin *et al.*, 2002). Significant correlations were observed between penetration rates of a hydrophilic dye and elevated IgE levels in patients with severe AD (Hata *et al.*, 2002). In addition, percutaneous

[1]*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan and* [2]*Epithelial Genetics Group, Division of Molecular Medicine, Colleges of Life Sciences and Medicine, Dentistry and Nursing, University of Dundee, Dundee, UK*

Correspondence: Dr Masashi Akiyama or Dr Hiroshi Shimizu, Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan.
E-mails: akiyama@med.hokudai.ac.jp or shimizu@med.hokudai.ac.jp

Abbreviations: AD, atopic dermatitis; EOS, eosinophil; FLG, *filaggrin gene; IV, ichthyosis vulgaris; LDH, lactate dehydrogenase; MAST, multiple antigen simultaneous test; SC, stratum corneum; SCORAD, score of atopic dermatitis; TEWL, transepidermal water loss*

Received 26 February 2008; revised 30 June 2008; accepted 30 July 2008; published online 25 September 2008

penetration of sodium lauryl sulphate was reported to be increased in uninvolved skin of patients with AD (Jakasa *et al.*, 2006). Taken together, these findings strongly support the hypothesis that patients with AD have a skin barrier defect.

In this context, we hypothesized that, in filaggrin-related AD, skin barrier defects caused by *FLG* deficiency is a primary abnormality leading to the AD symptoms. In the present study, to confirm this hypothesis, we evaluated skin barrier function in two AD patient groups divided by presence or absence of *FLG* mutations, by measurements of TEWL, SC hydration, and thickness that are useful markers of skin barrier function (Holm *et al.*, 2006).

RESULTS

Significant decrease of hydration and increase of TEWL in AD

We have summarized the details of clinical information from the patients and included *FLG* mutations (Table S1) and data on clinical severity as the objective score of atopic dermatitis (OSCORAD), SC hydration, TEWL, and SC thickness and in three representative regions (both the flexor and extensor aspects of the forearm, as well as the back; Table 1 and Figure 1). Scores for regions in each AD patient are shown in Table S2. SC hydration in AD patients was decreased in all the three regions of the body, as shown in Table 1 and Figure 1.

There were significant differences in SC hydration between filaggrin-related AD and normal controls on the back ($P<0.01$) and on the extensor aspect of the forearm ($P<0.05$), and to a lesser extent between non-filaggrin AD and normal control skin on the extensor aspect of the forearm ($P<0.05$). Average SC hydration values from the three regions were reduced in filaggrin-related AD patients compared with normal controls ($P<0.01$).

Transepidermal water loss values in each group are summarized in Table 1 and Figure 1. TEWL in non-filaggrin AD patients was significantly increased compared with that in normal controls on the extensor aspect of the forearm, on the back and the average of the three regions ($P<0.01$) and slightly reduced on the flexor aspect of the forearm ($P<0.05$). There was a significant TEWL increase in non-filaggrin AD patients compared with filaggrin-related AD individuals on the extensor aspect of the forearms ($P<0.05$), on the back ($P<0.05$), and for the average TEWL in the three regions ($P<0.05$).

It was statistically confirmed that SC hydration was significantly lower and that the TEWL was significantly higher in the filaggrin-related AD compared to those of the non-filaggrin AD by using the Wilcoxon rank sum test and Turkey–Kramer's honestly significant difference test.

SC thickness was significantly increased in filaggrin-related AD compared with that in non-filaggrin AD

Stratum corneum thickness in normal controls, filaggrin-related AD, and non-filaggrin AD is summarized in Table 1 and Figure 1.

Stratum corneum thickness in filaggrin-related AD was significantly increased compared to that of normal controls on the flexor aspect of the forearm ($P<0.05$), on the extensor aspect of the forearm ($P<0.01$), and on the back ($P<0.05$). Interestingly, there was a significant increase in SC thickness from filaggrin-related AD individuals compared with back skin from non-filaggrin AD patients ($P<0.05$). Average SC thickness was remarkably increased in filaggrin-related AD compared with normal controls ($P<0.01$) and compared to non-filaggrin AD patients ($P<0.05$).

Increased SC thickness in filaggrin-related AD was verified by conventional histology, as follows. SC thickness measured

Table 1. Summary of the patients' SC hydration, TEWL, and SC thickness

Group	Flexor aspect of the forearm	Extensor aspect of the forearm	Back	Average of the three regions
SC hydration (μs) (95% confidence interval)				
Filaggrin-related AD	7.67 ± 5.98 (3.87–11.46)	6.28 ± 5.00* (3.10–9.46)	9.46 ± 6.63** (5.25–13.67)	7.80 ± 5.17** (4.51–11.09)
Non-filaggrin AD	9.93 ± 5.61 (6.37–13.49)	7.39 ± 6.30* (3.38–11.39)	14.65 ± 8.88 (9.01–20.29)	10.66 ± 5.11 (7.41–13.91)
Control	11.84 ± 6.40 (7.78–15.91)	12.71 ± 4.48 (9.86–15.56)	20.40 ± 7.43 (15.68–25.12)	14.99 ± 5.06 (11.77–18.20)
TEWL ($g\,m^{-2}$ per hour) (95% confidence interval)				
Filaggrin-related AD	14.20 ± 5.58 (10.65–17.75)	11.05 ± 3.70# (8.70–13.39)	12.68 ± 6.49# (8.55–16.80)	12.64 ± 3.90# (10.17–15.12)
Non-filaggrin AD	20.44 ± 19.29* (8.18–32.69)	19.94 ± 11.98** (12.32–27.55)	20.87 ± 11.57** (13.52–28.22)	20.42 ± 12.51** (12.47–28.37)
Control	7.07 ± 2.45 (5.51–8.62)	6.40 ± 1.77 (5.27–7.52)	6.84 ± 1.47 (5.91–7.78)	6.77 ± 1.74 (5.67–7.87)
SC thickness (μm) (95% confidence interval)				
Filaggrin-related AD	57.74 ± 34.90* (35.56–79.92)	72.87 ± 50.27** (40.93–104.81)	62.24 ± 55.61*,# (26.91–97.57)	64.28 ± 42.28** (37.42–91.15)
Non-filaggrin AD	40.92 ± 32.88 (20.03–61.81)	42.92 ± 19.63 (30.45–55.39)	26.14 ± 15.31 (16.41–35.87)	36.66 ± 18.30 (25.03–48.29)
Control	28.09 ± 12.55 (20.11–36.06)	22.73 ± 10.10 (16.31–29.15)	20.45 ± 7.59 (15.63–25.27)	23.76 ± 8.88 (18.11–29.40)

Abbreviations: AD, atopic dermatitis; SC, stratum corneum; TEWL, transepidermal water loss.
*$P<0.05$, **$P<0.01$ vs Control #$P<0.05$ vs non-filaggrin AD (Tukey–Kramer's honestly significant difference test).

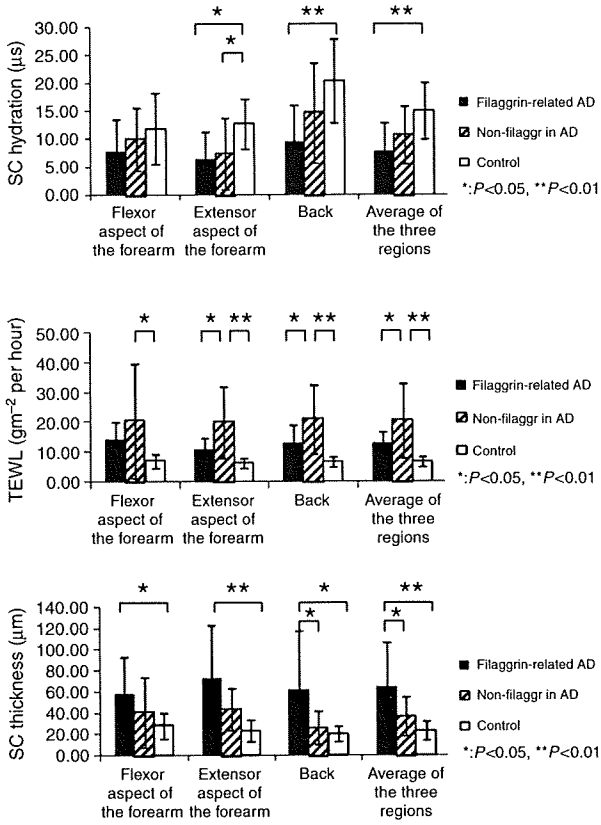

Figure 1. SC hydration, TEWL, and SC thickness on the flexor and extensor aspects of the forearm, on the back and average of the three regions. (Top) Comparison of SC hydration (μs) between filaggrin-related AD, non-filaggrin AD and the control group. (Middle) Comparison of TEWL ($g\,m^{-2}$ per hour) between filaggrin-related AD, non-filaggrin AD and the control group. (Bottom) Comparison of SC thickness (μm) between filaggrin-related AD, non-filaggrin AD and the control group. Data with *P*-values $*P<0.05$ were evaluated as significant and $**P<0.01$ were evaluated as highly significant.

in conventional histological slides was 42.8 μm in patient 3 (filaggrin-related AD), 23.8 μm in patient 21 (non-filaggrin AD), and 14.2 μm in patient 27 (IV without concomitant AD; Figure 2). SC thickness as measured by the corneometer was 47.6 μm in patient 3 (filaggrin-related AD), 25.3 μm in patient 21 (non-filaggrin AD), and 14.7 μm in patient 27 (IV without concomitant AD). These results confirmed that SC thickness data obtained using the corneometer are reliable and reflect the true SC thickness. Using hematoxylin and eosin-stained sections from patient 3 (filaggrin-related AD), additional layers of corneocytes in the SC were seen. Thus, the increase in SC thickness in filaggrin-related AD seems to be due to increased layers of corneocytes.

AD clinical severity was correlated with SC barrier defects indicated by TEWL and SC hydration in filaggrin-related AD, but not in non-filaggrin AD

There was no significant difference in AD severity as indicated by OSCORAD between filaggrin-related AD and those of non-filaggrin AD using the Wilcoxon rank sum test and box-whisker plots; OSCORAD (interquartile range or interquartile interval: filaggrin-related AD, 14.71–31.93; non-filaggrin AD, 26.88–35.75; Figure S1).

In filaggrin-related AD, negative correlation was confirmed by simple regression analysis between the clinical AD severity indicated with OSCORAD and average SC hydration on all the three examined sites (correlation coefficient $r=-0.65$, $P<0.05$; Figure 3). Simple regression analysis revealed a significant, positive correlation between the OSCORAD and average TEWL on all the three examined sites (correlation coefficient $r=0.81$, $P<0.005$) and between the OSCORAD and average SC thickness (correlation coefficient $r=0.59$, $P<0.05$; Figure 3).

In non-filaggrin AD, simple regression analysis revealed that there was no significant correlation between the OSCORAD and average TEWL (correlation coefficient $r=0.01$, $P>0.5$), between the OSCORAD and average SC hydration (correlation coefficient $r=-0.21$, $P>0.5$), or between the OSCORAD and SC thickness (correlation coefficient $r=-0.05$, $P>0.5$; Figure 3).

Significant correlation was obtained between the OSCORAD and specific IgE for house dust, mite allergen, and cat dander in filaggrin-related AD

We have shown the clinical history including the duration of AD, presence, or absence of AD family history, complication of asthma, rhinitis, and seasonal changes of disease activity, and laboratory data including peripheral blood eosinophil count (EOS), lactate dehydrogenase (LDH), total serum IgE, and allergen-specific IgE tests (IgE-multiple antigen simultaneous test (MAST), Table S1).

Atopic dermatitis family history was frequently observed in both AD patient groups (filaggrin-related AD, 10/12; non-filaggrin AD, 7/12). As complications, asthma (filaggrin-related AD, 5/12; non-filaggrin AD, 6/12), and rhinitis (filaggrin-related AD, 7/12; non-filaggrin AD, 9/12) were frequently seen. Patients whose skin lesions tended to get worse in winter were 3/12 in filaggrin-related AD and 2/12 in non-filaggrin AD. Patients whose skin lesions tend to get worse in summer were 0/12 in filaggrin-related AD and 4/12 in non-filaggrin AD. Due to the limited number of patients, it is difficult to draw firm conclusions about the clinical features including complications, family history, and seasonal changes in disease severity.

The IgE-MAST score of both AD groups showed high average, including IgE-MAST against house dust (filaggrin-related AD, 16.01; non-filaggrin AD, 24.00), mite (filaggrin-related AD, 63.41; non-filaggrin AD, 66.00), grass pollen (filaggrin-related AD, 23.46; non-filaggrin AD, 20.35), cedar pollen (filaggrin-related AD, 10.41; non-filaggrin AD, 11.66), fungal allergen (filaggrin-related AD, 6.33; non-filaggrin AD, 9.52), canine dander (filaggrin-related AD, 18.86; non-filaggrin AD, 37.07), feline dander (filaggrin-related AD, 29.64; non-filaggrin AD, 34.38), egg albumen (filaggrin-related AD, 4.21; non-filaggrin AD, 6.80), milk (filaggrin-related AD, 2.92; non-filaggrin AD, 2.32), wheat (filaggrin-related AD, 2.06; non-filaggrin AD, 2.30), and soy beans (filaggrin-related AD, 5.12; non-filaggrin AD, 2.90). No significant difference was seen in IgE-MAST

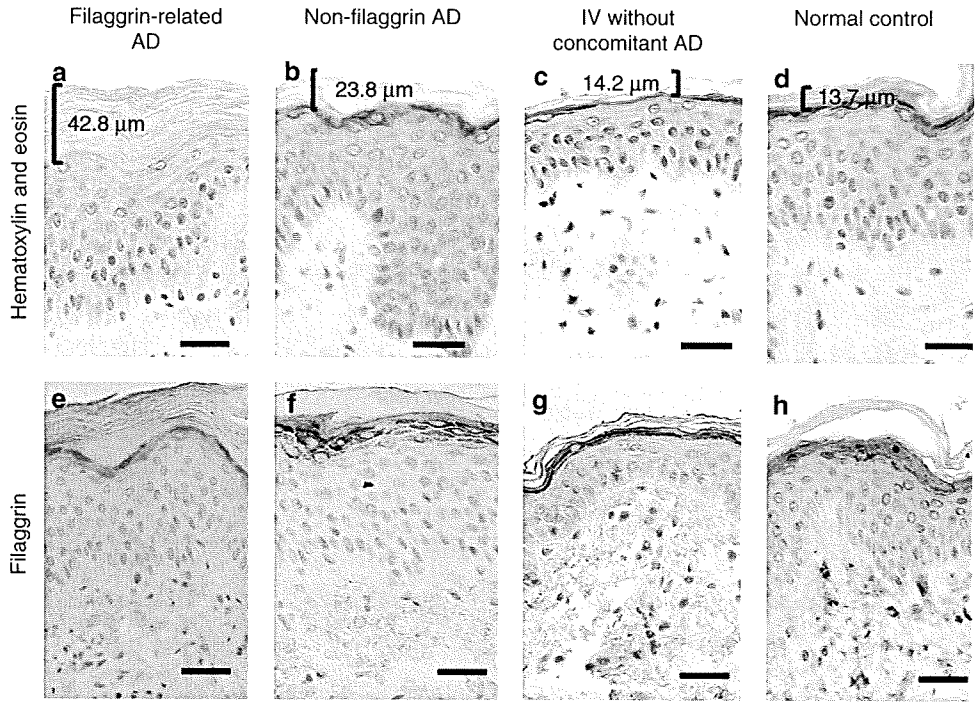

Figure 2. SC thickness measured by conventional histological methods and filaggrin immunostaining. Note remarkably increased SC thickness in filaggrin-related AD (**a**) due to an increased number of cornified cell layers. Immunohistochemical staining with anti-filaggrin monoclonal antibody against an epitope conserved in all filaggrin repeat peptides revealed a marked reduction of filaggrin staining in the epidermis of filaggrin-related AD (**e**) and IV without concomitant AD (**g**). (**a–d**) Hematoxylin and eosin staining; (**e–h**) filaggrin immunostaining. (**a**, **e**) Patient number 3 with compound heterozygous *FLG* mutations, c.3321delA, and p.Ser2554X (filaggrin-related AD); (**b**, **f**) patient number 21 without any *FLG* mutation (non-filaggrin AD); (**c**, **g**) patient number 27, IV without concomitant AD, harboring heterozygous *FLG* mutation p.Ser2554X; (**d**, **h**) normal control. Bars, 25 μm.

scores for any antigen between filaggrin-related AD and non-filaggrin AD.

Neither EOS, total serum IgE nor serum LDH showed any apparent association with the OSCORAD in either filaggrin-related AD or non-filaggrin AD by Wilcoxon rank sum test. Although no significant correlation was obtained between EOS, total serum IgE, LDH, and the OSCORAD in either filaggrin-related AD or in non-filaggrin AD by simple regression test, the OSCORAD and specific IgE for several allergens in filaggrin-related AD revealed a significant correlation coefficient, such as between OSCORAD and IgE for house dust (correlation coefficient $r=0.66$, $P<0.05$) in filaggrin-related AD, between the OSCORAD and IgE for mite allergen (correlation coefficient $r=0.53$, $P<0.05$) in filaggrin-related AD, and between the OSCORAD and IgE for cat dander (correlation coefficient $r=0.64$, $P<0.05$) in filaggrin-related AD. IgE for the other allergens in filaggrin-related AD did not show any correlation with the OSCORAD. IgE MAST scores for none of the allergens exhibited any correlations with the OSCORAD in non-filaggrin AD.

DISCUSSION

Atopic dermatitis is thought to comprise a group of patients with heterogeneous pathogenic factors. For a long time, abnormalities in the immune system have been highlighted as causative factors underlying AD. Recently, *FLG* mutations were found to be an important predisposing factor for AD and epidermal barrier defects have been attracting attention as an important pathomechanisms leading to AD.

The epidermal barrier function, in ichthyosis patients measured by SC hydration, thickness, and TEWL are known to be associated with the severity of the disease (Tomita *et al.*, 2005). Additionally, in AD patients, TEWL was reported to be increased, although controversy still remains as to whether the defective barrier function in AD patients is a primary cause of AD or a secondary consequence following dermatitis (Leung, 2000). Several studies have proven that there is a close correlation between clinical severity assessed using the SCORAD and skin barrier dysfunction in AD patients (Chamlin *et al.*, 2002; Sugarman *et al.*, 2003).

Stratum corneum hydration was marginally lower in filaggrin-related AD compared to non-filaggrin AD, although no statistical significance was obtained between our two AD groups. In contrast, TEWL was higher in non-filaggrin AD compared to in filaggrin-related AD. Low SC hydration in filaggrin-related AD might be indirectly related to barrier defects, but could primarily be related to a deficiency of water-binding filaggrin breakdown products (natural moisturizing factor) within the SC of filaggrin-related AD patients.

From the results of the present study, SC thickness was significantly thicker in filaggrin-related AD than in non-filaggrin AD. In contrast, the TEWL increase observed in

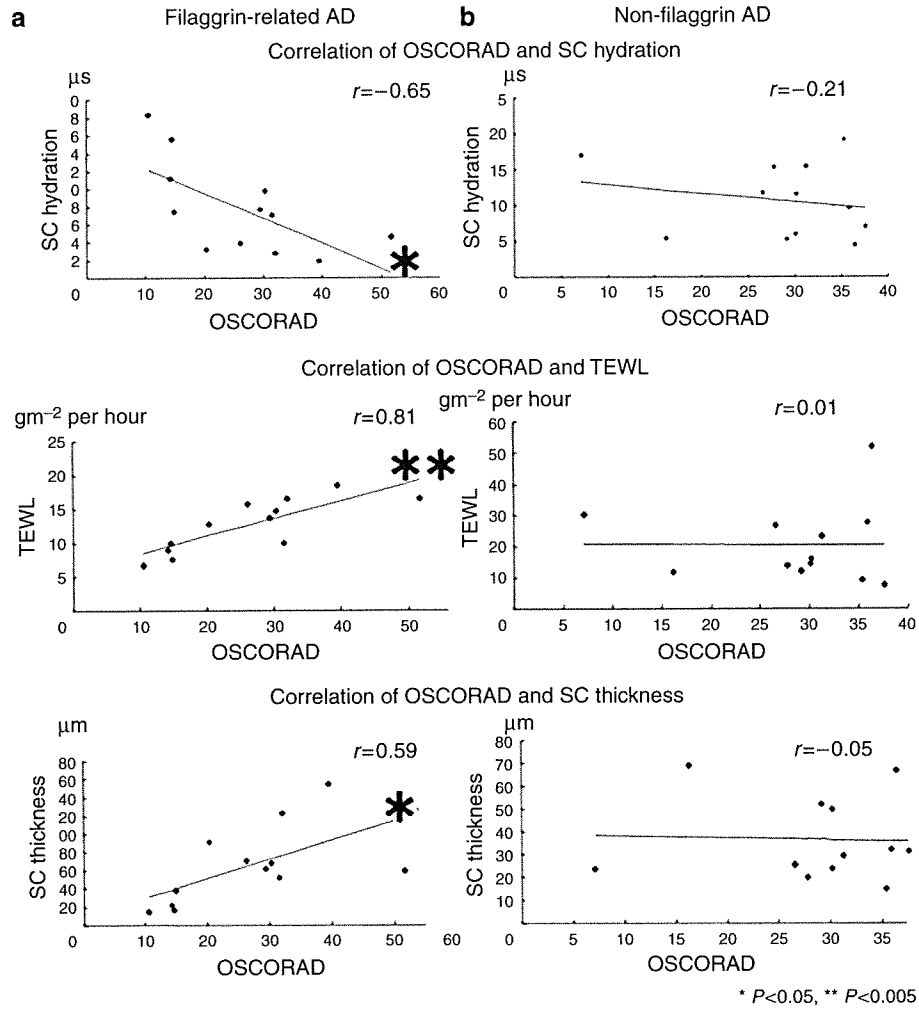

Figure 3. Correlation of clinical severity (OSCORAD) and SC hydration (average values of the three regions, μs), OSCORAD and TEWL (average values of the three regions, g m^{-2} per hour), and OSCORAD and SC thickness (average values of the three regions, μm). (**a**) Filaggrin-related AD. A significant correlation was shown between OSCORAD and each parameter. Data with *P*-values **P*<0.05 were evaluated as significant and ***P*<0.005 were evaluated as highly significant. (**b**) Non-filaggrin AD. No statistically significant correlation was shown between OSCORAD and any parameter.

non-filaggrin AD was significantly greater than that in filaggrin-related AD. It appears interesting that there seems to be a clear difference in barrier formation (SC thickness) and function (TEWL) between the two groups. In total, 4 patients out of 12 in filaggrin-related AD exhibited concomitant IV, although none of non-filaggrin AD patients had concomitant IV. Thus, we cannot exclude the possibility that remarkable SC thickness in filaggrin-related AD is related to the IV characteristics of patients, though SC was generally thinner in IV patients without AD in the present study. In addition, in our study, a negative correlation between the clinical AD severity (OSCORAD) and SC hydration and a positive correlation between the OSCORAD and TEWL were confirmed only in filaggrin-related AD. There was no significant correlation between the OSCORAD and SC hydration and between the OSCORAD and TEWL in non-filaggrin AD. However, according to a previous report studying French AD patients (Hubiche *et al.*, 2007), no significant difference was observed in the level of barrier function defects between filaggrin-related AD and non-filaggrin AD. It should be noted that in the previous study (Hubiche *et al.*, 2007), that the AD cohort was screened only for the two common filaggrin mutations, p.R501X, and c.2282del4 and was not screened for the many newly identified European filaggrin mutations, some of which are also prevalent in European populations (Sandilands *et al.*, 2007).

The OSCORAD scoring in the present study showed slight skewing between the two AD groups. Filaggrin-related AD seemed in general to have a relatively low OSCORAD score. This fact could have affected the interpretation of the other data. The effect of omitting treatments for 24 hours might also make non-filaggrin AD patient's disease worse than that of filaggrin-related AD patients. Thus, we checked this skewing using the Wilcoxon rank sum test and box-whisker plots, whereby the skewing was in fact revealed to be nonsignificant (Figure S1). No clinically relevant differences were seen between filaggrin-related AD and non-filaggrin AD in the present study.

From our results, increased SC thickness, TEWL, and reduced SC hydration are thought to be good indicators to evaluate the severity of filaggrin-related AD. Furthermore, in filaggrin-related AD, increased SC thickness and TEWL and reduced SC hydration might be useful to predict clinical course of the patients. In addition, given the strong correlations between the AD severity score (OSCORAD) and all the three parameters of skin barrier (TEWL, SC hydration, and thickness) obtained only in filaggrin-related AD, we are able to speculate that epidermal barrier defects may be one of the primary abnormalities in filaggrin-related AD.

Only in filaggrin-related AD, significant positive correlations were confirmed between the OSCORAD and IgE for house dust, between the OSCORAD and IgE for mite allergen, and between the OSCORAD and IgE for cat dander. There was no significant correlation between the OSCORAD and allergen-specific IgE in non-filaggrin AD. These results indicated the possibility of percutaneous sensitization for the allergens due to skin barrier defects in filaggrin-related AD. Mechanisms by which barrier defects due to *FLG* mutations contribute to the overall clinical end points of AD have yet to be completely clarified. Profilaggrin/filaggrin is crucial for maintaining the epidermal barrier function (Hudson, 2006). *FLG* mutations result in complete or incomplete loss of profilaggrin/filaggrin peptides and seem to be important in facilitation of allergic sensitization in AD patients. Defective epidermal barrier function provides easy access of allergens, antigens, and irritants through the epidermis, leading to stimulation of active T cells that contribute to more inflammation of the skin (Leung *et al.*, 1995).

We examined the histopathological features of three patients, one filaggrin-related AD, one non-filaggrin AD, and one IV without concomitant AD. Our results demonstrate that SC thickness measured in conventional histological slides correlated well to SC thickness as measured by a corneometer. Concordance of SC thickness measured by a corneometer and that measured by conventional histological methods was previously reported in ichthyosis patients (Tomita *et al.*, 2005). Thus, we feel that SC thickness measured by a corneometer without any further invasive procedures may be a useful and reliable parameter of hyperkeratosis. From histopathological observations, the thickening of SC in filaggrin-related AD (patient 3) appeared to be due to an increased number of cornified cell layers in SC. This observation suggests that hyperkeratosis in filaggrin-related AD might be caused by reduced desquamation of cornified cells, but not by diminished compaction of the corneocytes in the SC. These observations further emphasize the close pathophysiological relationship between ichthyosis vulgaris and filaggrin-related AD.

In our study, AD patients showing aggravation in summer were restricted to the AD group of the patients without *FLG* mutations, although the number of patients included in the present study was limited. Overexposure to sweat antigens was suggested to be an accelerating factor for AD eczema (Tanaka *et al.*, 2006). AD patients with summer aggravation might have other predisposing factors including sweat antigen exposure, but not epidermal barrier defects. Further study with a larger number of patients is needed to verify this hypothesis.

Our results showed remarkable barrier dysfunction (increased TEWL) in AD patients who did not have any *FLG* mutations. Jakasa *et al.* (2007) reported altered penetration of polyethylene glycols into uninvolved AD patient skin. There are likely a variety of mechanisms that modulate barrier integrity, other than the profilaggrin/filaggrin system, although it should be noted that there may be other filaggrin mutations still remain undetected in the Japanese population. Other structural molecules may also contribute to skin barrier formation by as yet unknown mechanisms.

MATERIALS AND METHODS

Patients

We selected 24 patients with AD according to criteria proposed by Hannifin and Rajka (1980), 14 males and 10 females, with a mean age of 21.36 years (range, 6–33 years). Twelve patients harbored *FLG* mutations (filaggrin-related AD) and the other twelve patients had no apparent *FLG* mutation (non-filaggrin AD). They were all treated by topical steroid ointment ranged from moderate to very strong, topical tacrolimus, moisturizer (heparinoid), or oral anti-histamines. We interviewed patients about disease duration of AD, presence or absence of family history of AD, and other atopic disorders including asthma or allergic rhinitis, and seasonal difference in AD severity. Patient numbers 3, 4, 10, and 11 had features of concomitant IV including hyperlinearity in the palms and scales on the lower legs. Patient numbers 1, 2, 5, 6, 7, 8, 9, 12, and 13–24 had dry skin, but no apparent concomitant IV features. In addition, three typical IV patients without concomitant AD harboring *FLG* mutations were included in the present study.

Age-matched healthy volunteers were included in the present study as controls. The control group consisted of 12 healthy individuals aged 6–30 years (eight male and four female) without any past or present skin disease.

The present study was approved by the Institutional Ethical Committee of Hokkaido University Graduate School of Medicine. This study was conducted according to all the Declaration of Helsinki Principles. Participants or their legal guardians gave their written informed consent.

Filaggrin genotyping

FLG mutation analysis was performed in patients and their family members. Briefly, genomic DNA isolated from peripheral blood was subjected to PCR amplification, followed by direct automated sequencing using ABI PRISM 3100 or 3730 genetic analyzers (Applied Biosystems, Foster City, CA). Mutations p.Ser2554X, p.Ser2889X, and p.Ser3296X were screened using restriction enzyme digestion of PCR products (Nomura *et al.*, 2007, 2008). Mutation c.3321del was screened by fluorescent PCR (Nomura *et al.*, 2007). Primers and PCR conditions were as described previously (Nomura *et al.*, 2007, 2008).

Disease severity

The SCORAD (severity scoring of AD, score range 0–103; European Task Force on Atopic Dermatitis, 1993) utilizes the rule of nines with