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Expression of filaggrin-2 protein in the epidermis of human skin diseases: A comparative analysis with filaggrin

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ABSTRACT

Filaggrin-2 is a member of the S100 fused-type protein family, and the structural features and expression of filaggrin-2 are similar to those of profilaggrin, a protein essential for keratinization. In the present study, we investigated the expression profile of filaggrin-2 in patients with skin diseases using antibodies against the repetitive region of filaggrin-2. In tissue samples from patients with skin diseases which are associated with a decrease in filaggrin, including ichthyosis vulgaris, atopic dermatitis and psoriasis vulgaris, the expression level of filaggrin-2 was markedly decreased compared to that in normal skin samples. In contrast, the expression of filaggrin-2 increased in parallel with that of filaggrin in samples of tissue from patients with skin diseases associated with hyperkeratosis, such as lichen planus and epidermolytic ichthyosis. Interestingly, filaggrin-2 signals were observed in slightly higher layers of the epidermis in comparison to those of filaggrin. Similarly, the expression of filaggrin-2 proteins was induced slightly later than filaggrin in the cultured keratinocytes. These findings suggest that filaggrin-2 may play an overlapping role with filaggrin in epithelial cornification; however, it may also have a partially distinct role in the molecular processes of cornification.

1. Introduction

Cornification is an essential process to confer a barrier function upon the skin in terrestrial mammals, which help resist against noxious biological and physicochemical insults from the environments and prevent the loss of body fluid [1]. A family of proteins described as "S100 fused-type proteins" are found within a 2 Mb region at chromosome band 1q21.3 [2]. The S100-fused type proteins include profilaggrin, trichohyalin, hornerin, trichohyalin-like 1, repetin, cornulin and filaggrin-2 [3–10]. Among these proteins, filaggrin plays an important role in the cornification process. Filaggrin is produced by the post-translational proteolysis of a precursor protein, profilaggrin and it promotes the aggregation of keratin filaments [2,11]. Filaggrin-2 is another member of the S100 fused-type protein family and was recently identified [5]. The deduced amino acid sequence of 2391 residues shows typical structural features of the "fused-type" S100 protein family members, which consists of an EF-hand domain at the N-terminus followed by a large repetitive domain. This repetitive domain of filaggrin-2 contains two types of tandem repeats, each 75–77 amino acids in length. The A-type repeats (A1–A9) are similar to the repeats of hornerin (50–77% identity) and the B-type repeats (B1–B14) are similar to the repeats of filaggrin (28–39% identity). Filaggrin-2 protein was detected in the granular and horny layers of normal stratified epithelium, which is the same pattern of distribution as filaggrin. Recently, the expression of filaggrin-2 was shown to be decreased in patients with atopic dermatitis, as has been observed for filaggrin [12]. Furthermore, genetic variation of filaggrin-2 was reported to be associated with more persistent atopic dermatitis in African American subjects [13]. However, the precise biological role of filaggrin-2 is still unknown. In the present study, to clarify the differences in the functions between filaggrin-2 and filaggrin, the expression profile of filaggrin-2 was examined in both skin samples from patients with diseases associated with decreased filaggrin and from patients with hyperkeratotic skin diseases, as well as healthy subjects.

Abbreviations: EDC, the epidermal differentiation complex; RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GST, anti-glutathione S-transferase; DAPI, 6-diamidine-2′-phenylindole dihydrochloride; FLG, filaggrin.

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2. Materials and methods

2.1. Clinical materials

Normal human skin tissue samples and tissue samples from patients with skin diseases were obtained from Toyama University Hospital. The skin tissue samples from subjects with diseases included samples from subjects with ichthyosis vulgaris, atopic dermatitis, psoriasis vulgaris, epidermolytic ichthyosis, lichen planus and actinic keratosis. The diagnosis of atopic dermatitis was performed using the AD diagnostic criteria proposed by Hannifin and Rajka [14]. Other diseases were diagnosed based on the clinical and histological findings by experienced dermatologists and pathologists. All patients gave their written informed consent and the study protocol complied with all of the Principles of the Declaration of Helsinki. This study was approved by the Medical Ethics Committees of the University of Toyama, Toyama.

2.2. Cell culture

Normal human epidermal keratinocytes (Kurabo Industries Ltd., Osaka, Japan) were cultured in Humedia-KG2 (Kurabo Industries Ltd., Osaka, Japan) in a humidified atmosphere with 5% CO_2. To induce the differentiation of keratinocytes, 1.5 mM Ca^{2+} was added to the medium of semi-confluent cultures, and the cells were harvested two, five and seven days after the addition of Ca^{2+}.

2.3. Preparation of specific antibodies against filaggrin-2 proteins

Antibodies against filaggrin-2 were prepared as reported previously [7,10]. Briefly, to prepare antibodies, an oligopeptide TQTGSRSSRASHFQSH corresponding to a part of the type-B repetitive units of filaggrin-2 was synthesized, conjugated with keyhole limpet hemocyanine and injected with adjuvant (TyterMax Gold, CytRx) into rabbits. The resulting antibodies were affinity-purified using a Hitrap NHS-activated column (GE healthcare UK Ltd., Buckinghamshire, England) conjugated with the peptide.

2.4. Preparation of recombinant filaggrin-2 and filaggrin proteins

To prepare recombinant proteins covering a part of repetitive domains of filaggrin-2 and filaggrin, cDNA fragments were amplified using reverse transcription-polymerase chain reaction (RT-PCR), and were subcloned into the pDEST15 gateway vectors (glutathione S-transferase gene fusion vector; Invitrogen, Carlsbad, CA). The primers used for PCR were: filaggrin-2-sense 5′-CACCA CAACTGGAAGAAGGGGATCTAGACT-3′, filaggrin-2-antisense 5′-TC AATGTCTAGACAGTTGCTTGTTTGC-3′, filaggrin-sense 5′-CACCCAGG AGTCCAGGACAAGAAAGCGT-3′ and, filaggrin-antisense 5′-CTAGTC

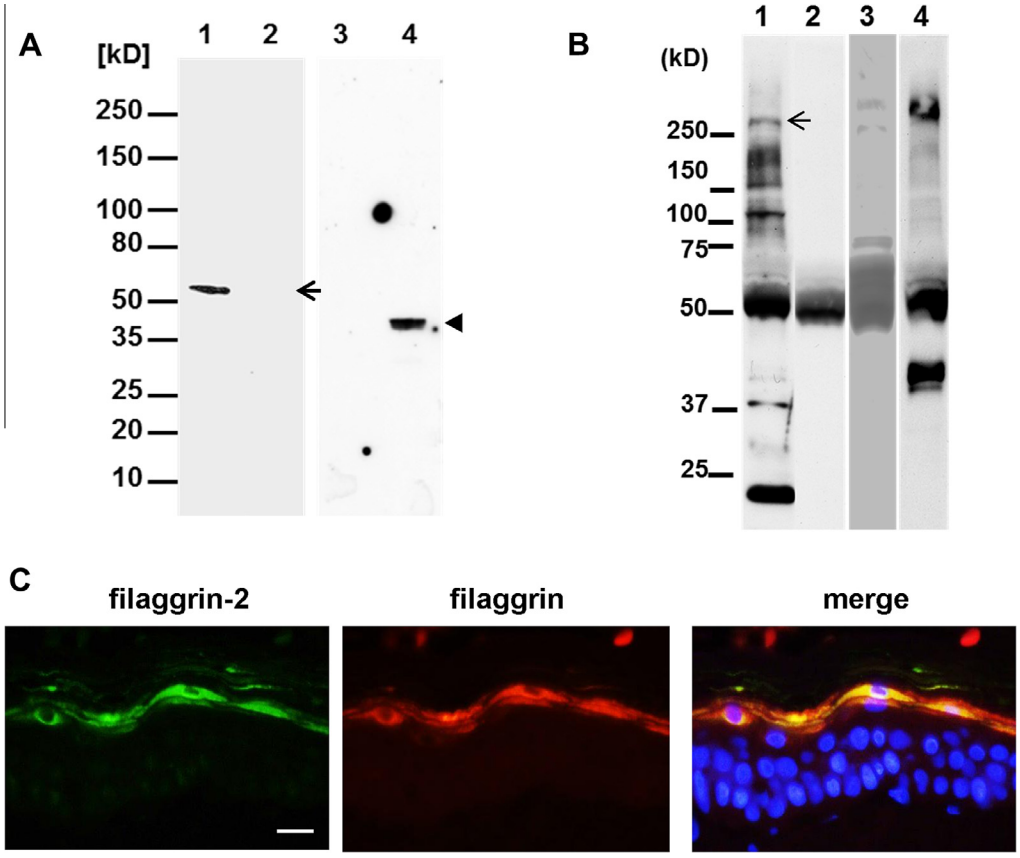

Fig. 1. (A) The specific recognition of the recombinant filaggrin-2 proteins by the antibodies. Purified recombinant filaggrin proteins were applied onto a 10% SDS gel and blotted with anti-filaggrin antibodies (lane 1) and anti-filaggrin-2 antibodies (lane 2). Purified recombinant filaggrin-2 was applied and blotted with anti-filaggrin antibodies (lane 3) and anti-filaggrin-2 antibodies (lane 4). The arrow indicates the size of the recombinant filaggrin protein. The arrowhead indicates the size of the recombinant filaggrin-2 protein. (B) The results of a Western blot analysis of normal human skin. Twenty micrograms of the protein preparation was blotted with an anti-filaggrin-2 antibody (lane 1), preimmune serum from the respective rabbits (lane 2), antiserum pre-absorbed with the peptide of the immunogen (lane 3), or an anti-filaggrin antibody (lane 4). The arrow indicates the expected size of the intact filaggrin-2 protein. (C) Immunostaining for filaggrin-2 proteins in normal human skin tissue samples. The tissue sections of normal human skin were doubly immunostained for filaggrin-2 and filaggrin. The tissue sections were also stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) to visualize the nuclei. The scale bar, 50 μm, is the same for all panels.

TCCTGATTGTTCCTCATTTCGTGT-3′. After inducing protein expression in BL21 cells (Invitrogen, Carlsbad, CA) with 1 mM isopropyl thiogalactopyranoside, the proteins were purified using glutathione-sepharose 4B (GE healthcare UK Ltd., Buckinghamshire, England).

2.5. Western blot analysis

Protein extracts were prepared by homogenizing normal human skin tissues in 0.1% Tris–HCl (pH 7.5), 5 mM EDTA and protease inhibitor mixture diluted according to the manufacturer's instructions (Sigma–Aldrich. Co., St. Louis, MO), 0.2 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich. Co., St. Louis, MO), and 2 mM pepstatin A (Peptide Institute, Osaka, Japan). A trichloroacetic acid solution was added to the homogenates to yield final concentration of 10%, and the same samples were kept on ice for 15 min. After centrifugation, the precipitates were dissolved in 9 M urea, 2% Triton X-100, and 5% 2-mercaptoethanol. Lithium dodecyl sulfate was added to 2%, and the pH of the solution was adjusted to 7.0 with 1 M Tris–HCl pH 8.0. To the prepared protein extracts from cultured keratinocytes, 10% cold trichloroacetic acid was added to the monolayer culture pre-washed with phosphate-buffered saline. The cells were collected with a scraper and centrifuged. The precipitates were processed as described above. Ten or 20 μg equivalents of protein were applied onto a 5–20% gradient SDS/polyacrylamide gel (PAGEL, Atto, Tokyo, Japan), electrophoresed and transferred onto Poly-Screen Transfer Membranes (NEN, Boston, MA). The membranes were treated with the antibodies against filaggrin-2 or a monoclonal anti-human filaggrin antibody (Abcam, Stoughton, MA) and positive signals were visualized by the ECL-plus Western Blotting Detection Reagents (GE healthcare UK Ltd., Buckinghamshire, England). Preimmune serum or the anti-filaggrin-2 antibody pre-absorbed with the immunogen peptide gave consistently negative results.

2.6. Immunohistochemistry

The human skin specimens were obtained from five patients with actinic keratosis, five patients with atopic dermatitis, two patients with Darier's disease, one patient with epidermolytic ichthyosis, two patients with ichthyosis vulgaris, five patients with lichen planus, one patient with pityriasis rubra pilaris, five patients with psoriasis vulgaris and five patients with seborrheic keratosis. The specimens were directly dipped into OCT compound (Ted Pella, Redding, CA) and rapidly frozen in liquid nitrogen. In contrast, cultured keratinocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline for immunostaining. Skin tissue sections and cultured keratinocytes were blocked with Protein Block Serum-Free (DAKO, Carpinteria, CA) for 30 min, incubated with the primary antibodies, which were the anti-human filaggrin-2 antibodies or anti-human filaggrin antibody (Abcam, Stoughton, MA), followed by incubation with an anti-rabbit IgG (H + L) Alexa Fluor 488, or an anti-mouse IgG Alexa Fluor 555 (H + L) (Molecular Probes, Eugene, OR) secondary antibody. Preimmune serum or the anti-filaggrin-2 antibody pre-absorbed with the immunogen peptide gave consistently negative results. The tissue sections were observed by a fluorescence microscopy (Olympus).

2.7. Quantitative reverse transcription-polymerase chain reaction

Total RNA was prepared from five normal skin tissue samples and the skin tissues samples from five patients with atopic dermatitis, two patients with ichthyosis vulgaris and three patients with

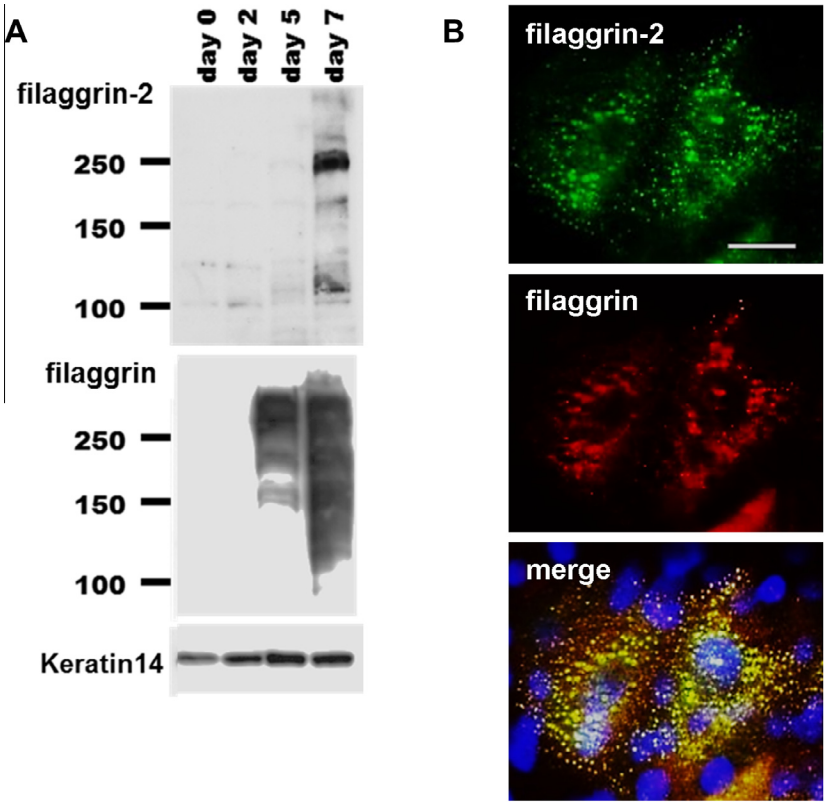

Fig. 2. The differentiation of epidermal keratinocytes induced the expression of filaggrin-2. Primary cultures of normal human keratinocytes were induced to differentiate with 1.5 mM Ca^{2+}. (A) The protein extracts were prepared before or on days 2, 5 and 7 after the addition of Ca^{2+}. The membranes were blotted with an anti-filaggrin-2, anti-filaggrin or anti-keratin 14 antibody. (B) Filaggrin-2 immunostaining of the keratinocytes induced to undergo differentiation with 1.5 mM Ca^{2+} for seven days. Filaggrin-2 was present in association with keratohyalin granules in the differentiating cells, and was co-localized with filaggrin. The scale bar, 10 μm, is the same for all panels.

psoriasis vulgaris by a method described previously [3]. All the RNA samples were pretreated with DNase I (GE healthcare UK Ltd., Buckinghamshire, England) and confirmed to give no positive signals without reverse transcription. Reverse transcription was performed with random hexamers and Superscript III (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using a Quant-Tect SYBR RT-PCR kit according to the of the manufacturer's protocol (Qiagen, Bothell, WA). The primers used were as follows: filaggrin-2; sense 5′-GGAGAAAGAGCTTCATCCAGTT-3′, antisense 5′-TTTCACTTTCTTCTTCTTGGTGT-3′: profilaggrin; sense 5′-ATGTC-TACTCTCCTGGAAAA-3′, antisense 5′-GTTGTGGTCTATATCCAAGT-3′: GAPDH; sense 5′-CTTCACCACCATGGAGAAGGC-3′ and antisense 5′-GGCATGGACTGTGGTCATGAG-3′.

The cDNAs were amplified with SYBR Green under thermocycling conditions of 50 degree for 2 min and 95 degree for 15 min, followed by 40 cycles of 95 degree for 30 s, 55 degree for 30 s, and 72 degree for 30 s. The copy numbers of filaggrin-2 and filaggrin mRNA were estimated by comparison with standard curves and are expressed as the copy ratios to glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

3. Results

3.1. Generation of antibodies against the filaggrin-2

To examine the expression of filaggrin-2 proteins, polyclonal antibodies against the filaggrin-2 were generated in rabbits according to a previous report [7]. The generated antibodies recognized the recombinant glutathione S-transferase (GST)-fused protein of the filaggrin-2 type-B repetitive unit comprising amino acids 2271–2415 of filaggrin-2, but not the recombinant GST-fused protein of the filaggrin repetitive unit comprising amino acids 304–579 of profilaggrin (Fig. 1A). In contrast, the anti-filaggrin antibody (Abcam, Stoughton, MA) did not recognize the recombinant filaggrin-2 protein. In normal adult human skin, the

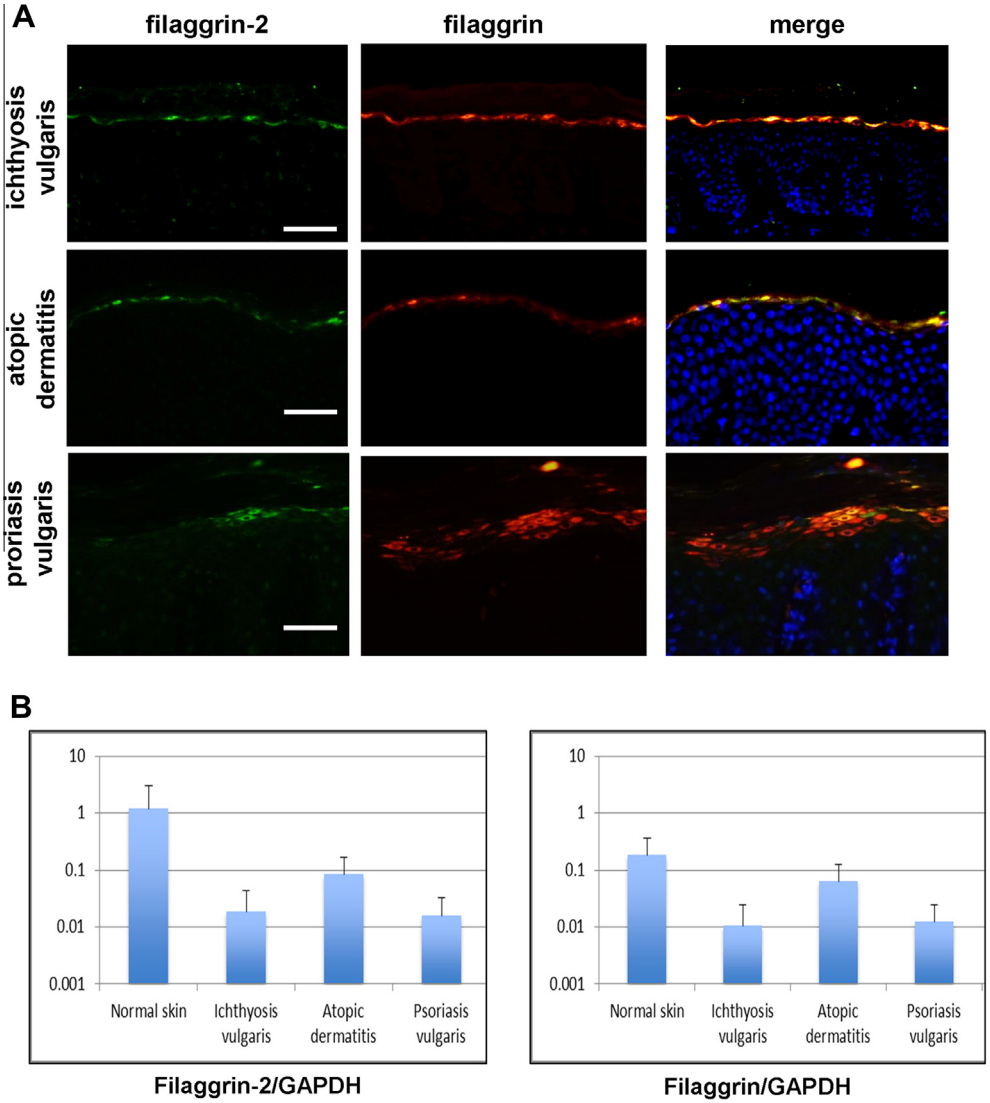

Fig. 3. (A) Immunostaining for the filaggrin-2 proteins in pathological skin tissue samples from patients with diseases associated with decreased filaggrin expression. Ichthyosis vulgaris samples ($n = 2$), atopic dermatitis samples ($n = 5$), and psoriasis vulgaris samples ($n = 5$) were doubly immunostained for filaggrin-2 and filaggrin. Similar results were obtained for the three diseases. The tissue sections were also stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) to visualize the nuclei. The scale bar, 50 μm, is the same for all panels. (B) The expression of filaggrin-2 mRNA in these pathological skin tissue samples. The total RNA was prepared from five normal skin tissue samples and the skin tissue samples from two patients with ichthyosis vulgaris, five patients with atopic dermatitis and three patients with psoriasis vulgaris. The copy numbers of filaggrin-2 and filaggrin mRNA were estimated by comparison with standard curves and are expressed as the copy ratios normalized to GAPDH. Each value represents the mean ± SD.

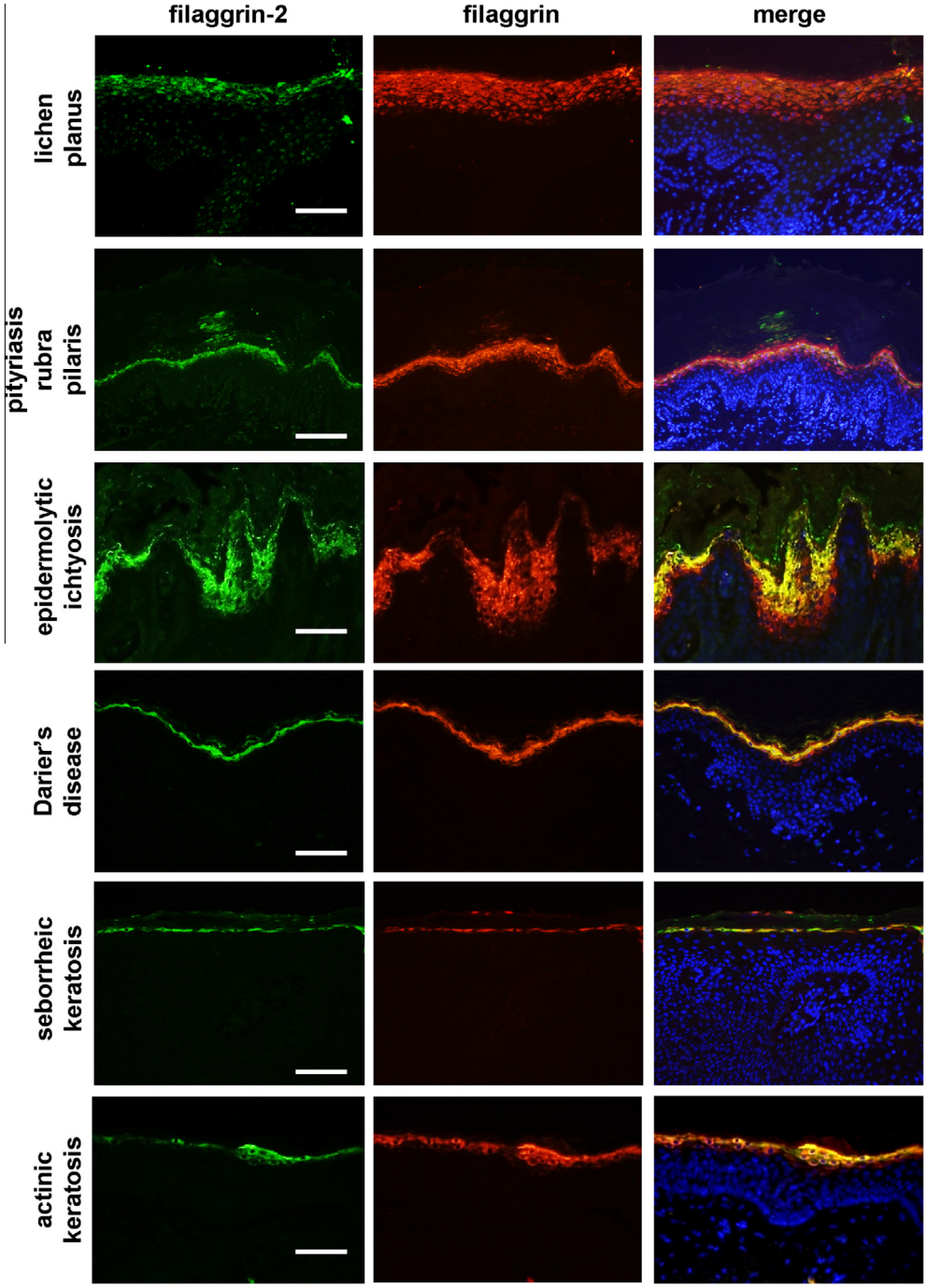

Fig. 4. Immunostaining for filaggrin-2 proteins in pathological skin tissue samples from patients with diseases associated with hyperkeratosis. The tissue samples from patients with lichen planus ($n = 5$), pityriasis rubra pilaris ($n = 1$), epidermolytic ichthyosis ($n = 1$), Darier's disease ($n = 2$), seborrheic keratosis ($n = 5$), and actinic keratosis ($n = 5$) were doubly immunostained for filaggrin-2 and filaggrin. Similar results were obtained for the six diseases. The tissue sections were also stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) to visualize the nuclei. The scale bar, 50 μm, is the same for all panels.

filaggrin-2 antibodies detected a band corresponding to the expected size of the filaggrin-2 protein (~248 kDa) by a Western blot analysis (arrow in Fig. 1B, lane 1). The filaggrin-2 antibodies also detected ladder-like bands, which are usually observed in profilaggrin. No corresponding bands were detected using the pre-immune serum or the antibodies pre-absorbed by the peptides used for immunization (Fig. 1B, lanes 2 and 3). An immunohistochemical study showed that filaggrin-2 protein was localized in granular cells in normal skin (Fig. 1C). The signals for the filaggrin-2 protein were mostly co-localized with those of filaggrin.

3.2. Induction of filaggrin-2 protein in cultured normal human keratinocytes

We examined the expression of filaggrin-2 protein in differentiating epidermal human keratinocytes in culture. Differentiation was induced by the addition of 1.5 mM Ca^{2+} into the medium of semi-confluent cultured cells. A band corresponding to filaggrin-2 was detected in the differentiating cells on day 7. In contrast, the filaggrin expression was observed from day 5 after the addition of 1.5 mM Ca^{2+} (Fig. 2A). The induced filaggrin-2 protein was

distributed on presumptive keratohyalin granules, and the signals for filaggrin-2 were mostly co-localized with those of filaggrin (Fig. 2B).

3.3. Expression of filaggrin-2 protein in pathologic skin tissues which are associated with a decrease in filaggrin

To clarify the role of filaggrin-2, we first examined the expression of filaggrin-2 proteins in tissue samples from patients with skin diseases which are known to be associated with a decrease in filaggrin, including ichthyosis vulgaris, atopic dermatitis and psoriasis vulgaris. In lesional skin samples from patients with ichthyosis vulgaris and atopic dermatitis, the signals of filaggrin were markedly decreased compared to those of normal skin as described previously [15,16]. In these tissue samples, only a few signals for filaggrin-2 were observed in the granular layer of the epidermis (Fig. 3A). Accordingly, the expression levels of filaggrin-2 and filaggrin mRNAs were also markedly decreased compared to those in normal skin samples by a quantitative RT-PCR analysis (Fig. 3B). In the lesional skin of samples of psoriasis vulgaris, the expression levels of both filaggrin and filaggrin-2 seemed to be decreased irregularly as determined by an immunohistochemical study; however, the quantitative RT-PCR analysis showed marked reductions of filaggrin and filaggrin-2 mRNAs in psoriatic skin samples (Fig. 3B).

3.4. Expression of the filaggrin-2 protein in pathologic skin tissues with hyperkeratosis

We next examined the expression levels of the filaggrin-2 and filaggrin proteins in samples of tissue from patients with hyperkeratotic skin diseases, including lichen planus, pityriasis rubra pilaris, epidermolytic ichthyosis, Darier's disease, seborrheic keratosis and actinic keratosis. In the lesional skins of the patients with lichen planus and epidermolytic ichthyosis, the filaggrin expression was markedly increased, and the signals corresponded to keratohyalin granules. The filaggrin-2 expression also increased in the granular layers of the tissue samples of patients with these diseases (Fig. 4). Although the signals of filaggrin-2 were almost co-localized with those of filaggrin, filaggrin-2 signals were observed in slightly higher layers of the epidermis in comparison to filaggrin, In the samples from patients with other diseases, the expressions levels of both filaggrin-2 and filaggrin were slightly increased compared to those of normal skin.

4. Discussion

Filaggrin-2 is a novel S-100 fused protein identified in 2009, and it is considered to be associated with the formation of the skin barriers, similar to filaggrin [5]. The present study clearly described the expression profile of filaggrin-2 in patients with skin diseases in comparison with that of filaggrin.

In the lesional skin of patients with ichthyosis vulgaris and atpic dermatitis, the expression level of filaggrin-2 was markedly decreased compared to that in normal skin samples. The expression level of filaggrin-2 was also decreased in psoriatic lesions. Ichthyosis vulgaris is caused by a mutation of the filaggrin (*FLG*) gene, resulting in a lack of filaggrin protein [15]. A loss-of-function of filaggrin is also reported in patients with atopic dermatitis [16]. Furthermore, not only the genetic risk factors, but also the level of proinflammatory cytokines, including IL-4, IL-13 and IL-25, have been suggested to modulate the expression of filaggrin-2 and filaggrin in atopic skin lesions [12]. Previous studies also reported the down-regulation of filaggrin-2 in the lesional skin of patients with atopic dermatitis [5,12]. In psoriatic lesions, the filaggrin expression has been reported to be reduced because of the altered differentiation of keratinocytes [17]. Based on these results, filaggrin-2 appeared not to compensate for the function of filaggrin in forming the skin barrier.

On the other hand, in the tissue samples of patients with skin diseases associated with both hyperkeratosis and hypergranulosis, the expression level of filaggrin-2 was markedly increased in parallel with that of filaggrin. Therefore, we consider that the induction of filaggrin-2 and filaggrin expressions may be coordinated. A recent study showed a genomic mechanism that coordinates the developmental expression of the epidermal differentiation complex genes via *cis*-regulatory elements that could play a role in human skin diseases. [18]. This mechanism might be associated with the regulation of filaggrin-2 and filaggrin expression during cornification.

In the present study, the signals of filaggrin-2 seemed to correspond to keratohyalin granules, and were generally co-localized with those of filaggrin. However, the filaggrin-2 signals were detected in slightly higher layers of the epidermis in comparison to filaggrin and both proteins were not completely co-localized. This finding seems to be in agreement with the observation that the expression of filaggrin-2 protein was induced slightly later than filaggrin in the cultured keratinocytes, as shown in Fig. 2A. These results suggest that the timing of filaggrin-2 induction in the epidermis may be different from that of filaggrin. A previous report also mentioned that filaggrin-2 and filaggrin did not show complete co-localization; thus, these proteins might have not any direct intermolecular cross-linking between them [5]. Accordingly, we speculate that filaggrin-2 may play an overlapping role with filaggrin in epithelial cornification; however, it may also have a distinct role in the molecular processes of cornification.

Further studies are needed to understand the biological functions of filaggrin-2 in the differentiation of keratinocytes and the pathogenesis of skin diseases; however, our findings of the expression profile of filaggrin-2 in skin diseases will contribute to extending the knowledge about filaggrin-2.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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