Sequential Reorganization of Cornified Cell Keratin Filaments Involving Filaggrin-Mediated Compaction and Keratin 1 Deimination

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The final step of keratinocyte differentiation, transition from the granular cells to the cornified cells, involves various post-translational modifications that include deimination of arginine residues. Major deiminated epidermal proteins are derived from K1. Two preferred deimination sites were identified in mouse K1, one in the V1 and the other in the V2 subdomains. An antibody against the deiminated peptide sequence in the V2 subdomain recognized not only deiminated mouse K1 but also deiminated human K1. In this study we analyzed distribution of deiminated K1 in normal human skin and in bullous congenital ichthyosiform erythroderma at light and electron microscopic levels. In normal skin the first few (1–3) cornified cell layers were positive for filaggrin and negative for the antibody against deiminated mouse K1 peptide, whereas the more superficial cells were negative for filaggrin and strongly positive for the antibody against deiminated mouse K1 peptide, indicating slightly delayed onset of K1 deimination at the initial stage of cornification. The clumped keratin in bullous congenital ichthyosiform erythroderma that was not properly compacted with filaggrin was poorly positive to the antibody against deiminated mouse K1 peptide. In addition, K1 derivatives in bullous congenital ichthyosiform erythroderma reacted poorly with the antibody against deiminated mouse K1 peptide compared with the normal control in immunoblot analyses. Our results suggest sequential reorganization of cornified cell keratin filaments involving filaggrin-mediated compaction and K1 deimination. Abnormal keratin aggregation in bullous congenital ichthyosiform erythroderma is likely to disturb the normal deimination of K1. ***Key words: citrulline/epidermolytic hyperkeratosis/filaggrin/peptidylarginine deiminases. J Invest Dermatol 118:282–287, 2002***

The process of epidermal differentiation or keratinization is characterized by a series of morphologic changes as keratinocytes leave the basal compartment and move upwards following the programmed cell death pathway (Ishida-Yamamoto *et al*, 1999). The cells finally differentiate from the granular cells through transitional cells to enucleated, dead cornified cells. This final step involves post-translational modifications of various proteins. Deimination is among such modifications and is catalyzed by a family of calcium-dependent enzyme, peptidylarginine deiminases (PAD) that convert protein arginine residues to citrulline residues (Rothnagel and Rogers, 1984). Three types of PAD (types I, III, and IV) are thought to be expressed in the epidermis (Watanabe *et al*, 1988; Terakawa *et al*, 1991; Nishijyo *et al*, 1997; Ishigami *et al*, 1998; Yamakoshi *et al*, 1998; Rus'd *et al*, 1999; Kanno *et al*, 2000).

It has previously been shown that major deiminated epidermal proteins are derived from keratin, K1, and minor deiminated proteins are from filaggrin and K10, using a monospecific antibody (AMC) that recognizes chemically modified citrulline residues (Senshu *et al*, 1995, 1996). Two preferred deimination sites were identified in mouse K1, one in the V1 and the other in the V2 subdomains (Senshu *et al*, 1999b). These subdomains in K1 are characterized by high glycine and serine contents with a few arginine residues as only charged amino acids (Steinert *et al*, 1985). We have raised an antibody against a deiminated undecapeptide containing the deimination site in the V2 subdomain of mouse K1 (ACP) (Senshu *et al*, 1999a). Similar sequences are present in V subdomains of human K1. ACP reacted with deiminated mouse K1 and also cross-reacted with deiminated human K1 (Ishida-Yamamoto *et al*, 2000), but did not react with unmodified K1; also, it did not react with deiminated filaggrin. Albeit these studies the biologic significance of deimination of V subdomains of K1 is still unknown. To gain some insights into this question, we analyzed ultrastructural localization of the deiminated K1 in normal human skin and in bullous congenital ichthyosiform erythroderma

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Abbreviations: ACP, antibody against deiminated mouse K1 peptide; AMC, antibody against chemically modified citrulline; BCIE, bullous congenital ichthyosiform erythroderma; PAD, peptidylarginine deiminase.

(BCIE), a genetic skin disease of K1/K10 (Fuchs, 1997; Ishida-Yamamoto *et al*, 1998; Irvine and McLean, 1999). Our results suggest that there is a sequential reorganization of cornified cell keratin filaments involving deimination and compaction with filaggrin. In addition, we performed comparative immunoblot analyses of stratum corneum samples from normal and BCIE subjects using ACP and AMC. The results suggested that keratin aggregation in BCIE interferes with the domain specific K1 deimination and causes aberrant deimination of K1 and other epidermal proteins, thus providing insights into the mechanisms and biologic significance of K1 deimination.

MATERIALS AND METHODS

Samples Normal human skin was obtained from the neck and the chest at the time of plastic surgery. Biopsy from lesional skin of two patients with BCIE (cases 1 and 2) was taken after obtaining informed consent. These patients correspond to cases 8 and 6 from previous reports, respectively (Ishida-Yamamoto *et al*, 1992, 1994). Keratin mutations in these patients have not been determined. Scales of three other typical BCIE patients (cases 3–5; mutations in keratins in these patients are to be published elsewhere) were collected by scraping. The outermost cornified cells were collected from four healthy volunteers by tape-stripping.

Immunofluorescence Preparation and affinity purification of the rabbit polyclonal antibody (ACP) to a deiminated undecapeptide corresponding to the identified deimination site in the V2 subdomain of mouse K1 (amino acid residues 545 GSSGGGRGGSS 555) were described previously (Senshu *et al*, 1999a). Immunofluorescence studies on semithin sections of Lowicryl-embedded skin (see below) or cryostat sections of unfixed normal skin were incubated with either a mixture of ACP (4 μg per ml) and a mouse monoclonal antibody, 34βB4, that recognizes suprabasal keratins, including K1 (Gown and Vogel, 1984) (Enzo Diagnostics, New York, NY, 1:20 dilution in phosphate-buffered saline) or a mixture of ACP and a mouse monoclonal anti-filaggrin antibody (BT576, Biomedical Technologies, Stoughton, MA, 1:1000–4000 dilution) for 30 min at 37°C. This was followed by incubation with a mixture of fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins (1:20 dilution, DAKO, Glostrup, Denmark) and Texas red-conjugated sheep anti-mouse immunoglobulins (1:10 dilution, Amersham Life Science, Buckinghamshire, U.K.) or R-phycoerythrin-conjugated F(ab′)2 fragment of goat anti-mouse immunoglobulins (1:10 dilution, DAKO). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Nacalai Tesque, Kyoto, Japan). Fluorescence immunolabeling was observed using an Olympus BX-FLA-1 system (Tokyo, Japan). Digital images were captured using an electric-cooled CCD camera (SenSys, Photometrics, Tucson, AZ). The camera and image processing were controlled using IP Laboratory Spectrum software (Signal Analytics, Vienna, VA).

Immunoelectron microscopy Skin tissue samples were cryofixed, cryosubstituted, and embedded in Lowicryl K11M resin (Chemische Werke Lowi, Waldkraiburg, Germany) according to the methods previously described (Shimizu *et al*, 1989; Ishida-Yamamoto *et al*, 1996). Ultrathin sections were cut, collected on formvar-coated nickel grids, and immunostained as described previously (Ishida-Yamamoto *et al*, 1996). As the primary antibodies ACP (4 μg per ml), 34βB4 (1:20 dilution) and anti-filaggrin antibody (BT576, 1:4000 dilution) were used. As labels, 5 or 10 nm gold-conjugated goat anti-rabbit or mouse IgG (Amersham, 1:10 dilution) were used. In some experiments, the immunogold particles were enhanced using a silver staining kit (Amersham). For all immunohistochemistry, negative controls included incubation in the presence of a secondary antibody alone, and incubation with unrelated primary antibodies. For quantitative evaluations, numbers of gold particles per μm^2 were counted over cytoplasmic areas filled with filamentous keratin and that of amorphous keratin clumps in the stratum corneum of a BCIE patient (case 1). For each compartment 30 areas were randomly chosen on photomicrographs of immunoelectron microscopy. Differences in labeling densities were tested for significance using the Student's t test.

Biochemical analyses of deiminated keratins An epidermal extract enriched with undegraded keratins derived from the viable epidermal cells of normal skin was prepared as described previously (Senshu *et al*, 1996; Ishida-Yamamoto *et al*, 2000). Scale samples of three BCIE patients (cases 3–5) were homogenized with 62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 2% 2-mercaptoethanol (Laemmli, 1970) by sonication. The homogenate was centrifuged at 10,000 × **g** for 5 min The supernatant containing keratins was used for the analyses. Tape-stripped cornified cells were extracted with the same buffer. Protein concentrations were estimated by the method of Lowry *et al* (1951). Proteins in the extract were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for western blotting as described previously (Senshu *et al*, 1996; Ishida-Yamamoto *et al*, 2000). The blot was first incubated with ACP, that reacts preferentially with deiminated peptide sequences in V subdomains of K1, and then horseradish peroxidase-labeled goat anti-rabbit IgG (Bio-Rad, Hercules, CA) for the detection by the enhanced luminol reaction using Renaissance (Dupont NEN, Boston, MA). The chemiluminescence image was recorded using a Bio-Rad Fluor S Max Chemiluminescence Analyzer (Bio-Rad). In order to make all citrulline residues in the separated proteins detectable, the blot was next incubated in 0.0125% $FeCl_3$, 2.3 M H_2SO_4, 1.5 M H_3PO_4, 0.25% diacetyl monoxime, and 0.125% anti-pyrine (modification medium) at 37°C for 3 h to modify citrulline residues chemically. This treatment also abolished the reactivities of the antibodies bound in the preceding step. The blot was then incubated with a monospecific antibody to chemically modified citrulline (AMC) (Senshu *et al*, 1992) and the labeled second antibody for chemi-luminescence detection in a similar manner. The blot was finally stained with Amido Black 10B to visualize proteins. The data were presented together with superimposed color images, that were prepared using an Atto Spot Screener (Atto Corporation, Tokyo, Japan).

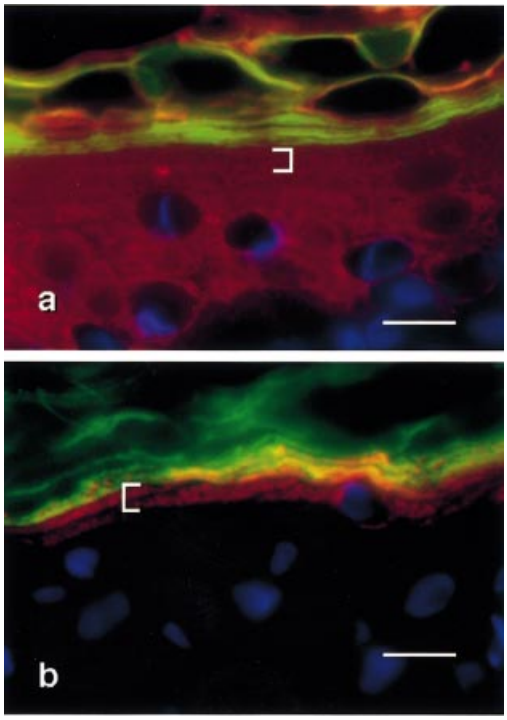

Figure 1. The superficial epidermal layers of the normal human skin are ACP positive. Cryostat sections are labeled for ACP (fluorescein isothiocyanate) and 34βB4 (Texas red) (*a*) or ACP (fluorescein isothiocyanate) and filaggrin (Texas red) (*b*). Enucleated flat cells (bracket) are possible lower cornified cells that are ACP negative, 34βB4 positive (*a*), and filaggrin positive (*b*). Nuclei are stained with 4′,6-diamidino-2-phenylindole dihydrochloride. *Scale bar*: 10 μm.

RESULTS

The first few cornified cell layers were ACP negative The ACP antibody raised against the deiminated mouse K1 peptide strongly stained epidermal cornified cells of the normal human skin as reported previously (Ishida-Yamamoto *et al*, 2000). Some ACP-negative, 34βB4-positive, and filaggrin-positive cells were detected at the lower cornified layers (**Fig 1**). As detailed nature of these

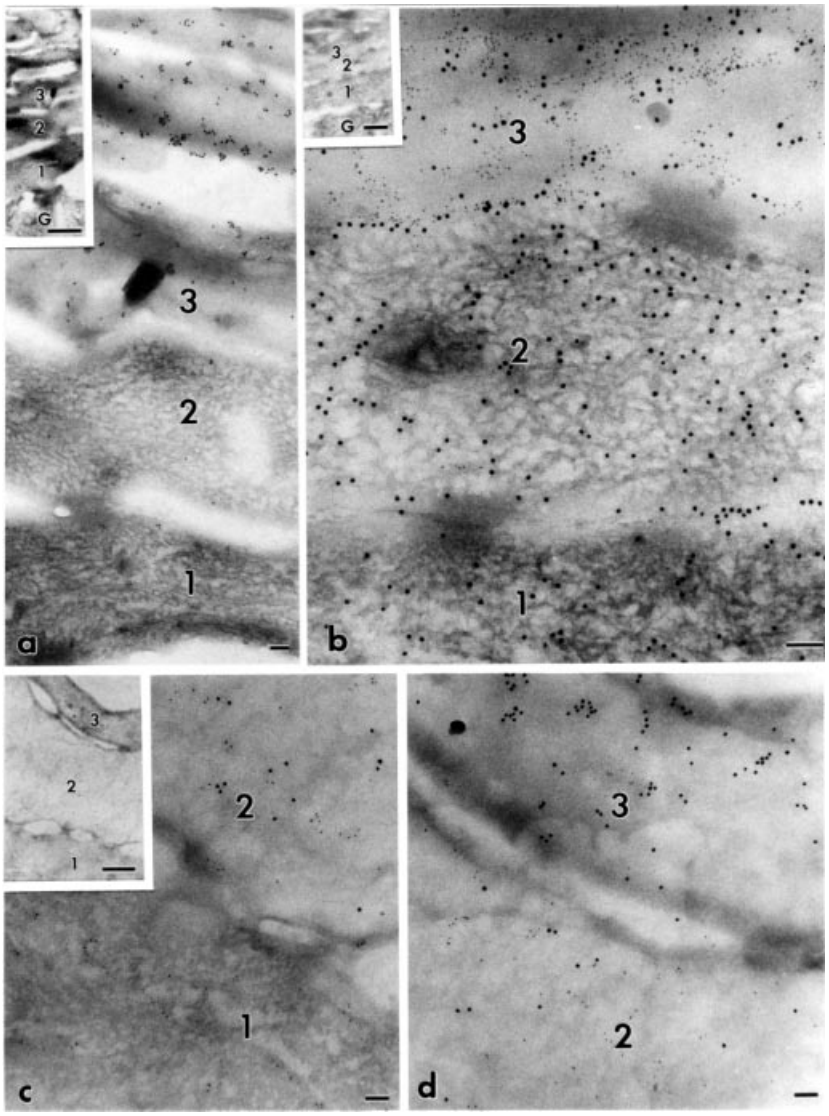

Figure 2. Immunoelectron microscopy shows sequential reorganization of cornified cell keratin filaments. (*a*) The first (1) and second (2) cornified cell layers are ACP negative, whereas the third layer (3) is positive. Note that the cytoplasm of negative cells shows a reticular pattern, whereas that of positive cells is amorphous in texture. Five nanometer gold labels have been enhanced using a silver staining method. The inset is to show these cells at lower magnification. G, granular cell. (*b*) Double staining with ACP (5 nm gold) and 34βB4 (10 nm gold). All the cornified cells are similarly positive to 34βB4. ACP staining is not detected in the first layer (1), only a few labels are seen in the second layer (2) and many labels in the third layer (3). These are shown in a lower magnification in the inset. G, granular cell. (*c*, *d*) Double staining with anti-filaggrin (5 nm gold) and ACP (10 nm gold). The first cornified cell (1) is filaggrin positive/ACP negative. The second cell (2) is double positive, and the third cell (3) is filaggrin negative/ACP positive. The inset in *c* is to show cells 1–3 in *c* and *d* at lower magnification. *Scale bars*: 0.1 μm; 1 μm in the inset.

thin layers of epidermal cells was difficult to assess by immunofluorescence microscopy, we performed immunoelectron microscopy. We confirmed that the first few cell layers of stratum corneum were ACP-negative (**Fig 2*a***). A difference in the texture of cytoplasm between the ACP-positive cells and ACP-negative cells was also appreciated. Namely, positive cells had homogeneous cytoplasm, whereas negative cells showed some fine reticular patterns. In double staining with 34βB4, all cornified cells were 34βB4-positive, including ACP-negative lower cornified cells (**Fig 2*b***). In double staining with filaggrin, the first few layers were filaggrin positive, but ACP negative (**Fig 2*c***). The next few layers were double positive, and the more superficial cell layers were filaggrin negative/ACP positive (**Fig 2*d***).

Heterogeneous and poor ACP-staining in BCIE We next examined skin from patients with BCIE. This is a genetic skin disease caused by K1/K10 mutations. In BCIE skin, we have found that keratin filaments were abnormally clumped throughout the differentiated cell layers (Ishida-Yamamoto *et al*, 1992) and some clumps in the stratum corneum were poorly associated with filaggrin (Ishida-Yamamoto *et al*, 1994). This led us to speculate that K1 deimination might be altered in BCIE. Immunofluorescence showed that 34βB4 was positive in all the differentiated epidermal cells, whereas ACP labeling was noted only in a portion of stratum corneum (**Fig 3**). Immunoelectron microscopy revealed that amorphous keratin clumps were less labeled with ACP (**Fig 4*a***) and filaggrin antibodies than the filamentous keratin (**Fig 4*b***; Ishida-Yamamoto *et al*, 1994). To clarify the difference between clumped and filamentous keratins, we compared the labeling over the keratin clumps and keratin filaments. The numbers of ACP-positive gold particles per μm^2 were 17.5 ± 2.0 (SEM) and 79.2 ± 5.9, respectively ($p < 0.0001$). Those of filaggrin labels were 94.3 ± 5.6 and 174.1 ± 13.4, respectively ($p < 0.0001$).

Decreased ACP-positive K1 derivatives in BCIE The BCIE scale extracts were subjected to western blotting analyses in parallel with that of stripped normal cornified cells (**Fig 5**). Keratins derived from viable epidermal cells were used as references. Amido Black staining visualized four major bands, that were estimated to be K1, K5, K10, and K14 from their mobilities (**Fig 5*a***, *lane 1*). The cornified cells extracts from all normal subjects examined showed broad bands migrating slightly ahead of K1 and K10 (a representative example is shown in **Fig 5*a***, *lane 2*), corresponding to partially degraded K1 and K10, respectively, as described (Senshu *et al*, 1996; Ishida-Yamamoto *et al*, 2000). Those from three BCIE patients displayed more heterogeneous profiles containing a band comigrating with undegraded K1 and those migrating ahead of K10, suggesting aberrant expression and/or processing of keratins (**Fig 5*a***, *lanes 3–5*). In the normal control ACP visualized a strong major signal comigrating with partially degraded K1 and a few more minor bands (45–51 kDa) (**Fig 5*b***, *lane 2*), that were not

detectable in the stained total profile. Similar bands were detected in the keratome biopsy samples (Ishida-Yamamoto *et al*, 2000). The intensity of these ACP-positive bands was decreased greatly in all three BCIE scales examined, suggesting decreased deimination of specific arginine residues in K1 as well as in 45–51 kDa proteins (**Fig 5*b***, *lanes 3–5*). The same blot was incubated in the modification medium followed by probing with AMC to detect all citrulline residues in the separated proteins. This treatments visualized diffuse bands comigrating with partially degraded K1 and K10 in the normal control (**Fig 5*d***, *lane 2*) as described (Senshu *et al*, 1996; Ishida-Yamamoto *et al*, 2000). An AMC-positive band comigrating with partially degraded K1 was also detected in the BCIE scales at intensities no less than that of the normal control (**Fig 5*d***, *lanes 3–5*). It should be noted that all three BCIE scales showed heterogeneous profiles, and that the overall intensity of AMC-positive signals in these samples was comparable or even greater than that of the normal control.

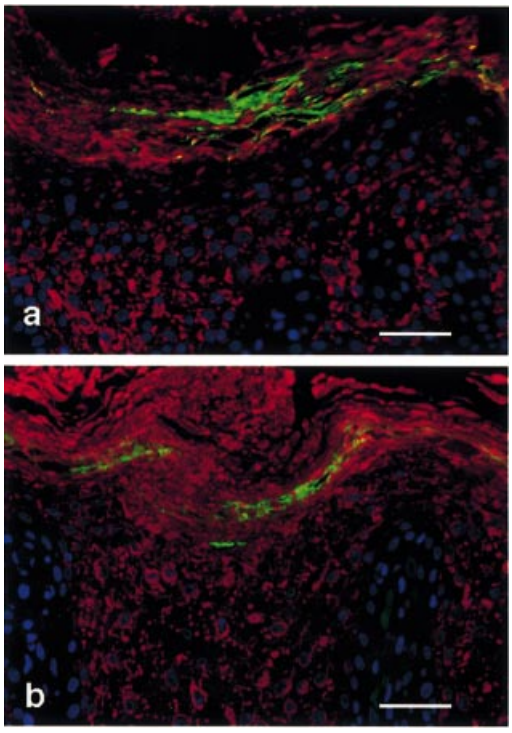

Figure 3. BCIE cornified cells are only partially positive to ACP. Case 1 (*a*) and 2 (*b*). Semithin sections of Lowicryl K11M blocks are labeled for ACP (fluorescein isothiocyanate) and 34βB4 (R-phycoerythrin). Nuclei are stained with 4′,6-diamidino-2-phenylindole dihydrochloride. *Scale bar*: 50 μm.

DISCUSSION

We have studied the possible role of V subdomain specific deimination of K1. To address these questions, we analyzed immunohistochemical and ultrastructural localization as well as immunoblotting profiles of the deiminated keratins in normal human skin and in BCIE using ACP and AMC. ACP reacts preferentially with deiminated peptide sequences in V subdomains of K1, whereas AMC detects all citrulline residues present in resolved proteins on the blot. The present immunohistochemical study showed that deimination of specific arginine residue(s) in the V subdomains of K1 remained below the limit of detection in the first few layers of the stratum corneum. This indicates that K1 deimination is not completed at the initial stage of cornification. We observed a marked ultrastructural change in the texture of corneocyte cytoplasm, namely from a reticular form in the ACP-negative lower cells to the homogeneous one in the ACP-positive upper layers. This raises an interesting possibility that deimination of the V subdomains of K1 accounts for the ultrastructural change, at least in part. Other types of keratin modifications such as cross-linking by transglutaminases or sulfhydryl oxidases and proteolysis might also be involved in the conformational alterations of K1. As the V1 and V2 subdomains of K1 contain a few arginine residues as the only positively charged amino acids (Johnson *et al*, 1985; Steinert *et al*, 1985), deimination of even a single arginine residue would markedly decrease the net positive charge of K1 as well as the hydrophilicity of these subdomains.

Although very little is known about substrate configuration required for the susceptibility to PAD, Tarcsa *et al* (1996) found that the degree and rate of modification of arginine residues to citrulline residues directly correlated with the structural order of the substrate. Proteins such as filaggrin having only simple β-turn secondary structures were rapidly modified by PAD III *in vitro*, whereas those with high α-helical contents such as trichohyalin were modified less readily (Tarcsa *et al*, 1996). Arginine residues in the V subdomains of keratins are probably more susceptible to PAD type I, which is presumed to be a major epidermal PAD, than those in the rod domain. Filaggrin bundles keratin filaments into

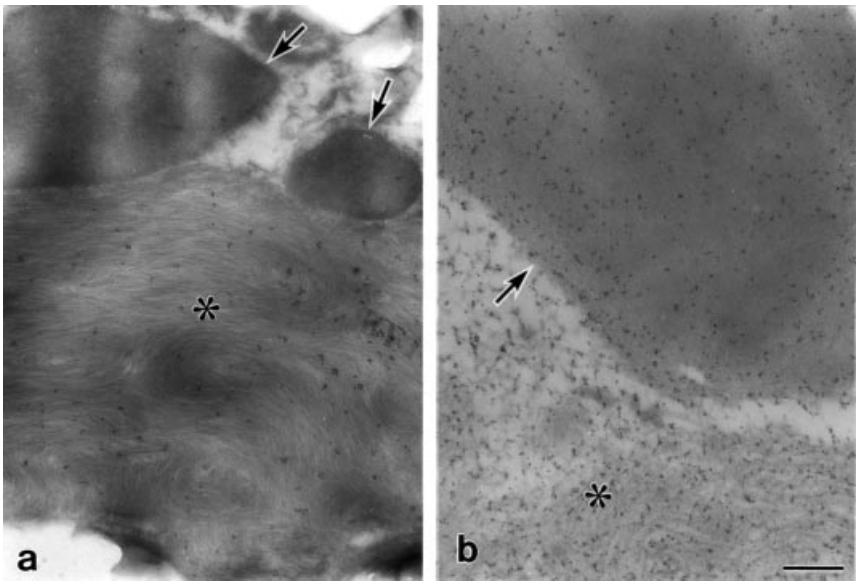

Figure 4. Immunoelectron microscopy shows that deimination and association with filaggrin are heterogeneous in BCIE keratins. More labels for ACP (*a*) and for filaggrin (*b*) (silver enhanced 5 nm gold) are seen over filamentous keratin (*) than over round amorphous keratin clumps (*arrows*). *Scale bar*: 0.5 μm.

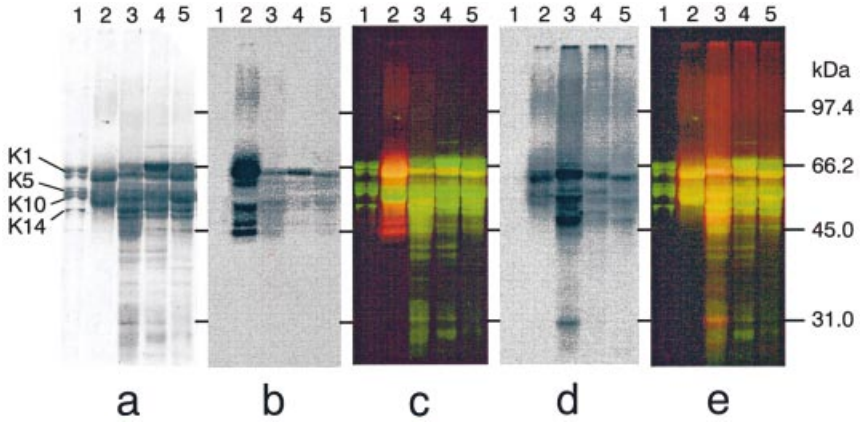

Figure 5. Decreased V subdomain deimination of K1 in BCIE. Proteins extracted from tape-stripped outermost cornified cells of normal skin (*lane 2*) and from scraped scales of three patients with BCIE (cases 3, 4, and 5) (*lanes 3*, *4*, and *5*, respectively) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotted for sequential probing with ACP (*b*) and AMC (*d*) followed by staining total proteins (*a*) as described in the text. Keratins in the viable layers of epidermis were loaded to *lane 1* as references. Keratin types were estimated by their mobilities. The amounts of proteins loaded were about 10 μg. Superimposed color images of ACP (red) and protein (green) (*c*) and AMC (red) and protein (green) (*e*) were also included for comparison.

macrofibrils (Dale *et al*, 1990). In the normal human skin, aggregation of keratins with filaggrin was detected earlier than K1 deimination in the V subdomains (**Fig 2**). In BCIE, deimination in the V subdomain occurred poorly (**Fig 5**), particularly in clumped keratins (**Fig 4*a***) that had failed to interact properly with filaggrin (**Fig 4*b***) (Ishida-Yamamoto *et al*, 1994). Interestingly, although the intensity of ACP-positive signals was markedly decreased in BCIE scales, the overall intensity of AMC-positive signals in these samples was comparable or even greater than that of the normal control. These data suggest that PAD are not deficient in BCIE epidermis, but cause aberrant deimination of K1 and possibly other epidermal proteins. Therefore, it might be that the deimination of specific arginine residues in the V subdomains occurs preferentially on keratin filaments compacted with filaggrin, and that the decreased ACP signals in BCIE may be due to decreased susceptibility of certain arginine residues to the action of PAD in abnormally clumped keratins. In addition, the decreased ACP staining in BCIE could be due to its hyperproliferative condition, as a similar decrease has been observed in psoriasis vulgaris epidermis (Ishida-Yamamoto *et al*, 2000). The minor ACP-positive bands (45–51 kDa) found in normal cornified cells, which were thought to have a similar deiminated peptide sequence, remain to be identified.

In summary we have presented evidence suggesting that cornification, the final step of keratinocyte differentiation, is not completed instantaneously. It proceeds as sequential steps of intermolecular interactions and modifications involving keratin, filaggrin, and probably many other proteins. Alteration of these events would hamper the normal keratinization process and might be responsible for some keratinization disorders.

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