Changing Pattern of Deiminated Proteins in Developing Human Epidermis

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Peptidylarginine deiminases are widely distributed, calcium-ion-dependent enzymes that convert arginine residues of proteins into citrulline residues. This reaction, deimination, is thought to be an important event during the final stage of epidermal differentiation, possibly associated with integration and disintegration of keratin filaments. To elucidate the possible roles of protein deimination during human epidermal development we investigated localization of deiminated proteins using anti-citrulline peptide antibody, which preferentially recognizes citrulline residues in the V subdomains of keratin 1, and anti-chemically modified citrulline antibody, which enables detection of citrulline residues independent of amino acid sequences. Anti-chemically modified citrulline antibody, but not anti-citrulline peptide antibody stained the periderm in two-layered epidermis of 49 d and 57 d estimated gestational age. In the stratified epidermis of 88 d, 96 d, and 108 d estimated gestational age fetal skin, anti-citrulline peptide antibody and anti-chemically modified citrulline antibody staining was seen in the periderm and intermediate cell layers. After periderm cells regressed and keratinization began in the interfollicular epidermis, anti-citrulline peptide antibody and anti-chemically modified citrulline antibody were restricted to the cornified cell layers of the interfollicular epidermis, similar to the distribution patterns of that in adult epidermis. Postembedding immunoelectron microscopy showed anti-citrulline peptide antibody immunogold labeling over the cytoplasmic intermediate filament network in the periderm and the intermediate cell layers. These results demonstrate an orderly formation of deiminated proteins in different layers of embryonic epidermis and suggest important roles for peptidylarginine deiminases in human epidermal morphogenesis. ***Key words: anti-citrulline peptide antibody/epidermal development/fetal skin/keratinization/peptidylarginine deiminase. J Invest Dermatol 120:817–822, 2003***

Peptidylarginine deiminases (PAD) are a group of enzymes that convert protein arginine residues to citrulline residues. This reaction is called "deimination". There are five known PAD subtypes so far reported: type I in the epidermis (Takahara *et al*, 1989; Ishigami *et al*, 1998; Rus'd *et al*, 1999); (ii) type II in the brain and muscle (Takahara *et al*, 1989; Watanabe and Senshu, 1989); (iii) type III in the hair follicles (Nishijyo *et al*, 1997; Rogers *et al*, 1997; Rus'd *et al*, 1999; Kanno *et al*, 2000); (iv) type IV originated from rat epidermis (Ishigami *et al*, 1998; Yamakoshi *et al*, 1998); and (v) type V induced in differentiated human promyelocytic leukemia HL-60 cells (Nakashima *et al*, 1999). All these PAD show a definite requirement for calcium ions. Deimination is one of the most important post-translational modifications during epidermal terminal differentiation. As the positive charge of arginine residues is irreversibly lost, deimination of even a single arginine residue can cause significant changes in the structure and function of target proteins. For this reason, PAD activities are thought to be under strict regulation *in vivo*.

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Abbreviations: PAD, peptidylarginine deiminase; ACP, anti-citrulline peptide antibody; AMC, anti-chemically modified citrulline antibody.

In the human epidermis, deiminated proteins are localized in the cornified cell layers (Senshu *et al*, 1996). The major protein substrate of PAD is keratin (K) 1 and minor substrates include filaggrin and K10 (Senshu *et al*, 1995, 1996), suggesting deimination of these proteins might be important during the final stages of epidermal differentiation.

Virtually, no data are available regarding the possible involvement of protein deimination in the developing human fetal epidermis. The purpose of this study was to immunocytochemically examine when, where, and what kinds of epidermal proteins are deiminated during human epidermal development. We have used (i) anti-citrulline peptide antibody (ACP), which preferentially reacts with citrulline residues in the V subdomains of keratin 1 in human epidermis (Ishida-Yamamoto *et al*, 2000, 2002), and (ii) anti-chemically modified citrulline antibody (AMC), which recognizes chemically modified citrulline residues in proteins regardless of the amino acid sequence.

Here we show a highly regulated orderly formation of deiminated proteins in different layers of embryonic epidermis suggesting an important role for deiminated proteins in epidermal morphogenesis. In addition, we discuss similarity of the regression of the embryonic periderm with the keratinization of adult epidermis in terms of protein deimination.

MATERIALS AND METHODS

Samples Human embryonic and fetal skin specimens were obtained from abortuses of 49 d, 57 d, 88 d, 96 d, 108 d, and 163 d estimated

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gestational age (EGA) through the Central Laboratory of Human Embryology at the University of Washington, Seattle with the approval of the Human Subjects Review Board and in accordance with the United States Department of Health, Education, and Welfare policies. For immunoelectron microscopic observation, skin specimens from a fetus of 105 d EGA was used. EGA was determined from the maternal history, fetal measurements, and comparative histologic appearance of epidermis (Shepard, 1975; Holbrook, 1979; Mercer *et al*, 1987). Normal adult human skin was obtained at the time of skin surgery.

Cell culture Normal human epidermal keratinocytes (NHEK) and an immortalized keratinocyte cell line (HaCaT cells) were cultured on eight-chamber glass slides each in low concentration calcium medium ($Ca^{2+} = 0.15$ mM in keratinocyte growth medium). In order to induce keratinization, both cells were cultured with high concentration calcium medium ($Ca^{2+} = 1.2$ mM) for 48 h. Cultured epidermal sheets over a fibroblast feeder layer (Japan Tissue Engineering Co. J-TEC, Aichi, Japan) were also used for the study. Immediately before the immunolabeling, all specimens were fixed with precooled 1:1 acetone/methanol for 10 min, and treated with 0.05% Triton X at 37°C for 10 min.

Antibodies Preparation and affinity purification of the rabbit polyclonal antibody (ACP) against a deiminated undecapeptide corresponding to the identified deimination site in the V2 subdomain of mouse K1 (amino acid residues 545GSSGGGRGGSS555) have been described previously (Senshu *et al*, 1999). ACP was shown to react not only with deiminated mouse K1, but also with deiminated human K1 (Ishida-Yamamoto *et al*, 2000, 2002). A monospecific antibody to chemically modified citrulline residues (AMC) was prepared as described previously (Senshu *et al*, 1992). Other primary antibodies were mouse monoclonal antibody, 34βB4, which recognizes human suprabasal keratins (Novocastra, Newcastle upon Tyne, U.K.), and an anti-human filaggrin antibody (Biomedical Technologies Inc., Stoughten, MA). The secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H + L) antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and tetrarhodamine isothiocyanate-conjugated anti-mouse IgG1 antibody.

Immunolabeling Cryostat sections of skin specimens and cultured keratinocytes on glass slides were incubated with ACP (3.8 μg per ml) alone or with a mixture of ACP and 34βB4 (1 : 10 dilution in phosphate-buffered saline), or a mixture of ACP and anti-human filaggrin (1 : 500 dilution in phosphate-buffered saline). After phosphate-buffered saline washing, the bound antibodies were detected using FITC-conjugated goat anti-rabbit IgG (H + L) antibody and tetrarhodamine isothiocyanate-conjugated goat anti-mouse IgG1 antibody. For all labeling, specimens were incubated with the secondary antibody alone, and incubation with irrelevant primary antibodies or nonimmunized sera served as negative controls. In some cases, nuclei were counterstained with propidium iodide (Dojindo Laboratories, Kumamoto, Japan). Image collection of the immunohistochemical staining was performed by a confocal laser scanning microscope with software Fluoview version 2.0 (Olympus America Inc., Melville, NY). AMC staining was performed as previously described (Senshu *et al*, 1995). Briefly, skin cryostat sections were fixed with 5% glutaraldehyde in phosphate-buffered saline for 15 min. Sections were then 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 chemically the citrulline residues. Control sections were incubated in the medium depleted of diacetyl monoxime and anti-pyrine. Immunoperoxidase staining of the modified proteins was performed with AMC and a Histfine SAB-PO kit (Nitirei, Tokyo, Japan) using 3,3′-diaminobenzidine as a chromogenic substrate.

Immunoelectron microscopy Fetal skin samples were cryofixed, cryosubstituted, and embedded in Lowicryl K11M resin (Chemische Werke Lowi, Waldkraiburg, Germany) as previously described (Shimizu *et al*, 1989). Ultrathin sections were cut, collected on formvar-coated nickel grids, and immunostained as described previously (Ishida-Yamamoto *et al*, 1996). ACP was used as the primary antibody and 5 nm gold-conjugated goat anti-rabbit IgG was used as the secondary antibody.

RESULTS

Deiminated proteins were detected in developing epidermis Although no ACP antibody staining was observed in the early two-layered epidermis (49 d and 57 d EGA) (**Fig 1**), significant AMC staining was detectable in the periderm cytoplasm at 49 d EGA (**Fig 2**). In the three-layered or more stratified epidermis (88 d, 96 d, and 108 d EGA), both the periderm and intermediate cells became ACP and AMC positive for both in the cytoplasm, but the basal cells remained negative for both techniques. After the regression of periderm and the commencement of keratinization in the interfollicular epidermis (163 d EGA), staining of ACP and AMC became restricted to the cornified cells, and were absent from the spinous and basal cells. In adult epidermis, both ACP and AMC staining was restricted to the cornified cell layers as described previously (Ishida-Yamamoto *et al*, 2000).

Colocalization of ACP-positive proteins with suprabasal keratins in the intermediate cells In the ACP-negative two-layered epidermis, neither 34βB4 nor anti-filaggrin staining was observed (data not shown). In the stratified epidermis, ACP-positive intermediate layer cells were also positive for 34βB4 demonstrating the colocalization of ACP-positive proteins with the suprabasal keratins (**Fig 3**, 108 d EGA). During this period, filaggrin was not yet expressed.

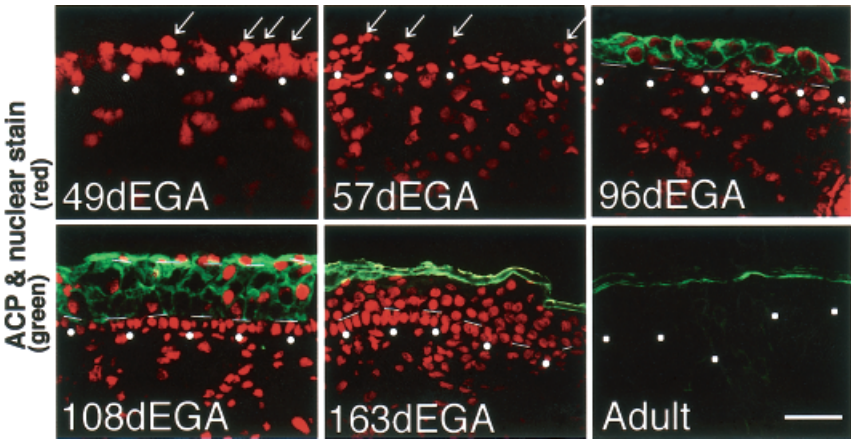

Figure 1. ACP staining became detectable from the three-layered epidermis during human epidermal development. In the early two-layered epidermis of 49 d and 57 d EGA, no staining for ACP was observed. In the stratified epidermis of 96 d and 108 d EGA, staining for ACP was observed (green, FITC) in the cytoplasm of the periderm and the intermediate cells, but not in the basal cells. After the regression of periderm cells of 163 d EGA, staining for ACP was restricted to the stratum corneum. In normal adult skin, ACP staining was restricted to the lowermost part of the stratum corneum. Nuclei were counterstained with propidium iodide in fetal skin. *Arrows*: periderm cells; *white dots*: basement membrane zone; *white thin bars*: junction of basal cell layer and intermediate cell layer. *Scale bars*: 50 μm.

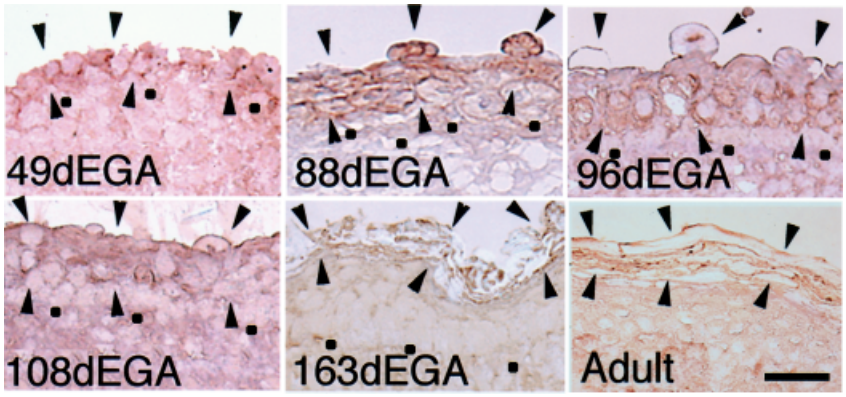

Figure 2. AMC staining was detected in the periderm of two-layered epidermis. In the early two-layered epidermis at 49 d EGA, staining for AMC was observed in the cytoplasm of the periderm (*arrowheads*; brown, immunoperoxidase). In the stratified epidermis of 88 d, 96 d, and 108 d EGA, staining for AMC was observed both in the cytoplasm of the periderm and intermediate cells. After the regression of periderm cells on 163 d EGA, staining for AMC was restricted to the stratum corneum. In normal adult skin, AMC staining is limited in the stratum corneum. Black dots: basement membrane zone. *Scale bars*: 50 μm.

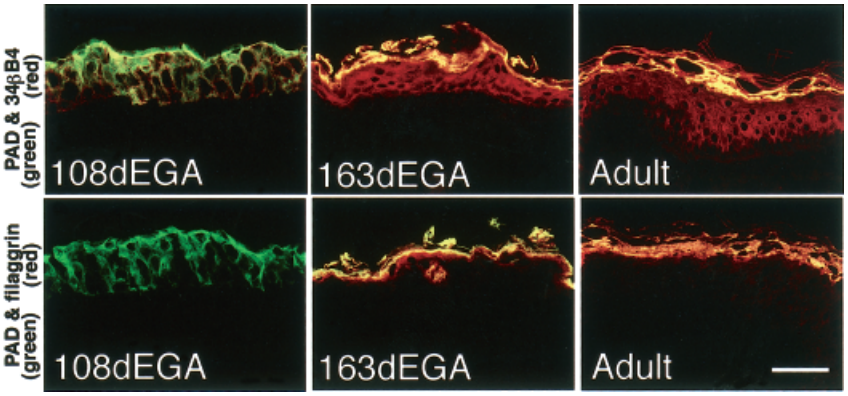

Figure 3. Both deiminated proteins and suprabasal keratins were seen in the intermediate cells and cornified cells, whereas filaggrin was expressed in the granular and cornified cells late in the development of human fetal epidermis. In the stratified epidermis of 108 d EGA, ACP-positive (green, FITC) intermediate cells were also positive for suprabasal keratin antibodies, 34βB4 (red, rhodamine) (upper, left). After the periderm cells have regressed by 163 d EGA, ACP immunoreactivity became restricted to the cornified cells along with suprabasal keratins (top, middle center). At this period, the staining patterns of 34βB4 and ACP were similar to those of adult epidermis (upper, right). ACP staining (green, FITC) was seen in the stratified epidermis of 108 d EGA but filaggrin (red, rhodamine) was not yet expressed (bottom, left). After the periderm cells had regressed by 163 d EGA, anti-filaggrin antibody stained the upper part of granular cell layers and the cornified cell layers along with ACP staining (bottom, middle center). These distribution patterns of filaggrin and ACP immunoreactivity on 163 EGA were similar to those of adult epidermis (bottom, right). Green (FITC), ACP; red (rhodamine), suprabasal keratins or filaggrin. Overlapping of green and red fluorescence resulted in yellow fluorescence. *Scale bars*: 50 μm.

Deiminated proteins, suprabasal keratins, and filaggrin were all seen in the cornified cells after the regression of periderm After the periderm cells regressed, ACP staining was restricted to cornified cells and partly overlapped with 34βB4 keratin staining (**Fig 3**, 163 d EGA). At this point, the anti-filaggrin antibody began to stain the upper part of granular cell layers and the cornified cell layers. This staining profile of the keratinized fetal epidermis resembled that of the adult epidermis (**Fig 3**, adult).

ACP immunogold labeling was observed over the cytoplasmic filament network in the periderm and the intermediate cells In immunoelectron microscopy, ACP immunogold labeling was observed over the cytoplasmic filament network in the periderm (**Fig 4*a***) and the intermediate cells (**Fig 4*b***) at 105 d EGA. No ACP labeling was observed in the basal cells (data not shown). In the negative control specimens, almost no immunogold labeling was seen.

ACP-positive proteins were induced in the upper layer of the epidermal sheet grown on a fibroblast feeder layer Neither ACP nor 34βB4 staining was observed in NHEK or HaCaT cells cultured in low calcium medium. When they were induced to differentiate by culturing in high calcium medium, weak 34βB4 staining only became detectable in large flattened cells, which remained negative for ACP (data not shown). ACP-positive staining, however, was detectable in cells covering the upper surface of human epidermal sheets cultured on a fibroblast feeder layer (**Fig 5**). These cells were also stained with 34βB4 and anti-filaggrin (data not shown).

DISCUSSION

In this study, we investigated the formation of deiminated proteins in developing human epidermis using two antibodies against deiminated proteins. One is ACP, which preferentially recognizes citrulline residues in the V subdomains of keratin and the other is AMC, which enables the detection of citrulline residues independent of amino acid sequences. The V subdomains of K1 contain only a few arginine residues that are the target sites for PAD in highly glycine-rich sequences containing scattered phenylalanine and tyrosine residues. An important finding in this study was the cytoplasmic staining of the periderm in the two-layered epidermis with AMC, both layers of which were not stained with ACP at all (**Fig 6**). This suggests some deimination

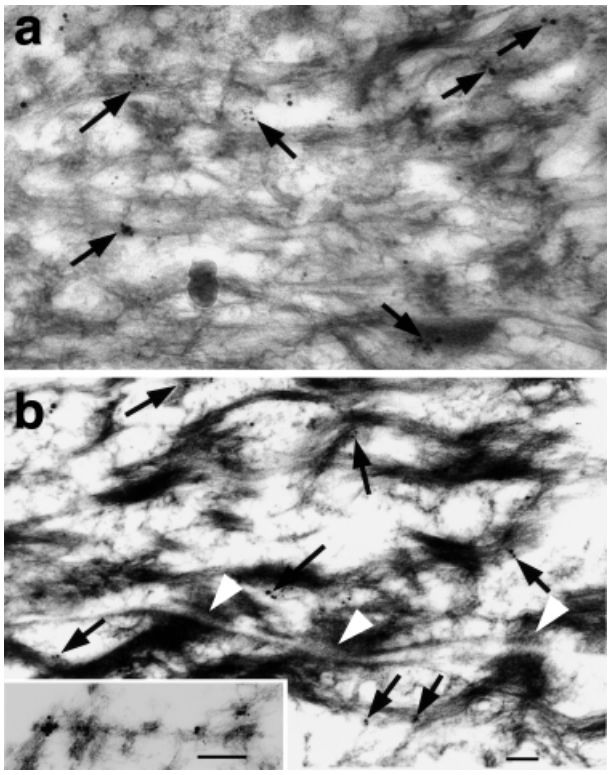

Figure 4. ACP immunogold labeling was observed over the cytoplasmic intermediate filament network in the periderm and the intermediate cells. At 105 d EGA, immunogold labeling for ACP is seen on the cytoskeletal filament bundles in the periderm (*a*) and intermediate cells (*b*), but not over the cell membrane, including desmosomes. *Inset*: high-power view of the gold labeling on the cytoskeletal filament bundles. *Arrows*: ACP immunogold labeling; *arrowheads*: desmosome. *Scale bars*: 100 nm.

of periderm proteins at this developmental stage. Judging from the specificity of ACP, AMC-positive proteins are probably deiminated at arginine residues with specific amino acid sequences unrelated to the V subdomains of K1. In adult human epidermis, AMC-positive deiminated proteins include K1/K10 and filaggrin (Senshu *et al*, 1996). Actually, neither layer stained with the monoclonal antibody 34βB4 against the suprabasal keratins (K1 and K10). In rat newborn epidermis, AMC staining revealed that K6 (56 kDa)/K16 (48 kDa) (the hyperproliferative keratins), trichohyalin, and filaggrin were deiminated (Senshu *et al*, 1995). In the periderm of human fetal epidermis, neither K6/K16 nor filaggrin are expressed, although 40 kDa and 52 kDa simple epithelial keratins are expressed (Moll *et al*, 1982; Dale *et al*, 1985). Thus, AMC-positive, ACP-negative deiminated protein(s) in early periderm are unlikely to be deiminated K6/K16 or filaggrin, however, this remains to be clarified. The staining pattern with AMC (**Fig 2**) suggested that there was some cornified cell envelope reactivity in addition to cytoplasmic staining. As the AMC antibody is not specific for the V domain of K1, we cannot exclude the possibility that AMC-positive deiminated proteins in developing human epidermis may include certain cornified cell envelope proteins; however, there is no known evidence that cornified cell envelope proteins, including involucrin, are deiminated by PAD.

Interestingly, in the three or more layered stratified epidermis, both the cytoplasm of periderm and intermediate cells became AMC and ACP positive. This was confirmed by immunoelectron microscopy showing ACP immunogold particles over the cytoplasmic filament networks of both periderm and intermediate cells. The expression of suprabasal keratins was observed in the intermediate cells as previously reported (Moll *et al*, 1983; Dale *et al*, 1985). Thus, the major substrate of PAD in the intermediate cell layer could be K1 as in adult epidermis. Alternatively, the

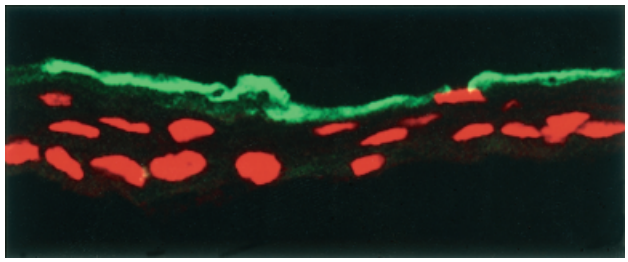

Figure 5. ACP staining was observed in the cultured epidermal sheet. Uppermost cells of epidermal sheet cultured on the fibroblast feeder layer were positive for ACP (green, FITC). Nuclei were counterstained with propidium iodide (red).

ACP staining may reflect deimination of other cytoskeletal proteins bearing regional sequence similarities to the V subdomains of K1. The ACP-positive staining of the periderm cells at the three or more layered epidermis may also be accounted for, by such deiminated proteins. Similar, though as yet unidentified ACP-positive proteins were reported in fetal and early neonatal mouse epidermis (Senshu *et al*, 1999). Using a database search, the only known cytoskeletal or envelope proteins that have any sequence similarity to the K1 V subdomain were K10, K2e, and loricrin, but loricrin has no arginine residues in its amino acid sequence. Thus, as the ACP-positive deiminated protein(s) in the intermediate cells, K10 and K2e could be the candidate molecules. In periderm cells, neither K10 nor K2e (65.5 kDa) are expressed (Dale *et al*, 1985; Collin *et al*, 1992) and there are likely to be other candidate substrates yet to be identified in the periderm. After the regression of periderm and the commencement of keratinization in the interfollicular epidermis (163 d EGA), deiminated proteins were localized in the cornified layers as observed in adult human epidermis.

The regulated expression of deiminated proteins in different layers of embryonic epidermis reported here represents an interesting feature of developing human epidermis that distinguishes from normal adult epidermis. In the latter, deiminated proteins are only observed in cornified cell layers, highlighting the fact that deimination is under strict regulation during the keratinization of normal adult epidermis. Formation of deiminated proteins in the periderm and intermediate cells of the fetal skin suggests not only gene expression but also activation of the as yet unidentified PAD. Interestingly, this appears to occur in cells partially lacking morphologic features of keratinization. It has recently been shown that several proteins essential for the formation of the cornified cell envelope appear in the periderm and the intermediate layer before the onset of keratinization (Lee *et al*, 1999; Akiyama *et al*, 1999). Cornified cell envelope precursor proteins, involucrin and loricrin are restricted to periderm cells. Periderm cells are destined to regress during the epidermal development. Periderm regression has been postulated to be similar in terms of cell loss and cornified cell envelope formation to epidermal keratinization (Akiyama *et al*, 1999). Protein deimination in the periderm may be an important process in the regression of the periderm cell as it is in the epidermal keratinization. In addition, protein deimination in intermediate cells may be a preparatory process for keratinization as the expression of proteins involved in the formation of cornified cell envelope. Types of PAD expressed and their target proteins remain to be identified and may provide fruitful areas for future research.

The present keratinocyte culture system cannot be considered as a model of fetal epidermal morphogenesis and the culture system simply corresponds to near-normal epidermal differentiation. Keratinocytes cultured in low Ca^{2+} conditions showed neither suprabasal keratin expression nor deiminated protein formation. Cells cultured in the high Ca^{2+} condition expressed suprabasal keratins, without the formation of deiminated proteins. It has been shown by immunoelectron microscopy that K1 is not deiminated in the first few cornified cell layers of adult human

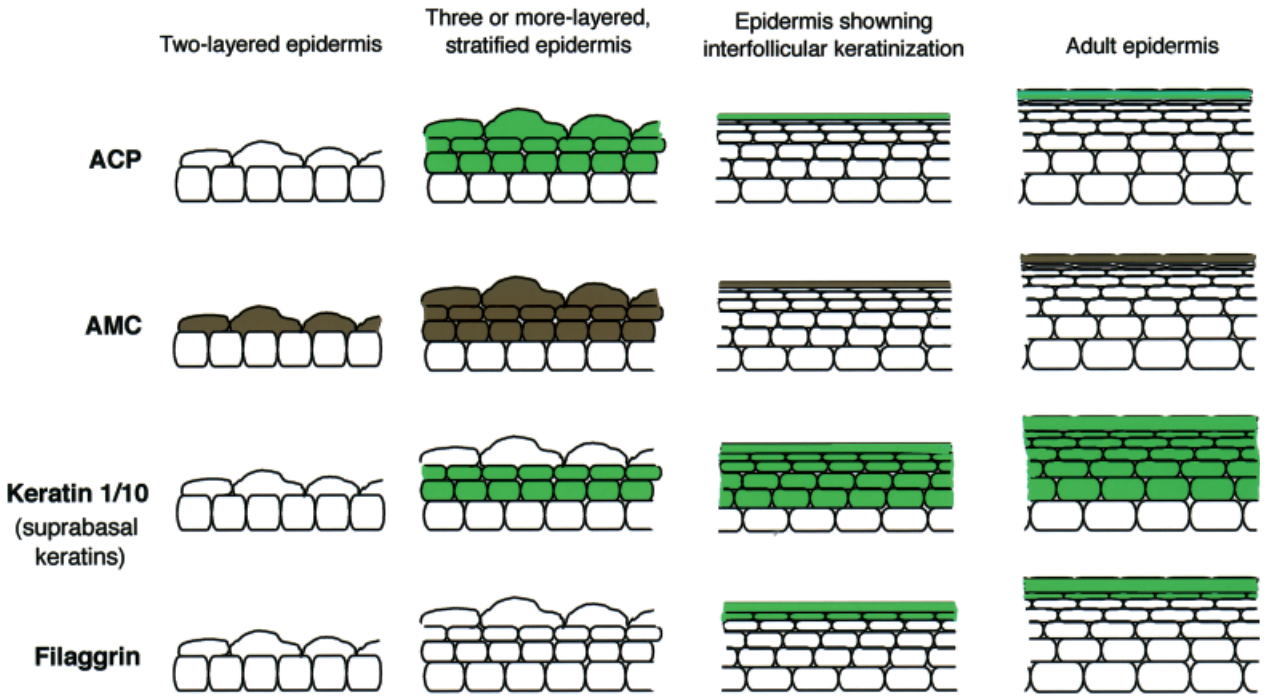

Figure 6. Scheme of the expression patterns of deiminated proteins, suprabasal keratins (keratin 1/10) and filaggrin. AMC staining represents distribution of chemically-modified citrulline residue of deiminated proteins and ACP staining is for the deiminated keratin 1. AMC was positive in the two-layered epidermis while ACP, suprabasal keratins (keratin 1/10) and filaggrin were not observed. In the three or more-layered stratified epidermis, ACP and AMC were positive in the periderm and the intermediate cell layers and were colocalized with keratin 1/10 in the intermediate cell layers. After the periderm regressed and interfollicular keratinization started, expression of ACP and AMC were restricted to the cornified cell layers where keratin 1/10 and filaggrin were also expressed. This expression pattern was similar to that of adult epidermis.

epidermis (Ishida-Yamamoto *et al*, 2002). In this context, keratinized cultured cells under high Ca^{2+} conditions might be at a similar stage of differentiation to the first few cornified cell layers. Whereas, deiminated K1 was detected in keratinocytes covering the uppermost surface of the epidermal sheet cultured on a fibroblast feeder layer. Thus, the superficial cells in the epidermal sheet are thought to be more differentiated and closer to the *in vivo* keratinizing epidermal cells, compared with cultured cells without a feeder layer.

In conclusion, the results of this study reveal a highly ordered sequence of deiminated proteins in different layers of developing human epidermis. These findings indicate that the keratinization corresponding to that of the adult epidermis occurs in the periderm during the periderm regression phase and suggests that deimination by PAD may be a key event in the development of epidermal differentiation.

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