Decreased Deiminated Keratin K1 in Psoriatic Hyperproliferative Epidermis

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Citrulline-containing proteins, mainly originating from keratin K1 and formed by enzymatic deimination of arginine residues, have been identified in the cornified layers of human epidermis. We analyzed the localization and nature of the deiminated proteins in psoriatic epidermis. Immunostaining based on chemical modification of citrulline residues showed that the normal and psoriatic uninvolved epidermis contained deiminated proteins diffusely in the cornified cell layer, whereas the involved epidermis had no detectable or markedly reduced levels of deiminated proteins. Immunolabeling with polyclonal antibodies against a synthetic citrulline-containing peptide corresponding to a deiminated sequence of mouse K1 also suggested markedly decreased deiminated K1 in psoriatic involved lesions. Keratin analyses indicated that deiminated K1 present in normal and psoriatic uninvolved epidermis was not detected in the psoriatic involved epidermis. Double staining with a monoclonal antibody, 34βB4, and the polyclonal antibodies demonstrated that epidermis with low suprabasal keratin expression was negative for deiminated K1. In contrast, intralesional acrosyringia showing decreased suprabasal keratin immunoreactivity like that of the surrounding psoriatic epidermis showed strong deiminated K1 staining. This suggests that abnormal keratin deimination is restricted to the psoriatic hyperproliferative epidermis, without affecting sweat ductal epithelia. ***Key words: glycine loop/loricrin/peptidylarginine deiminase/protein deimination. J Invest Dermatol 114:701–705, 2000***

During the normal keratinization process a series of biochemical changes is observed, among which are the post-translational modifications of various proteins, such as disulfide bonding, isopeptide cross-linking (Ishida-Yamamoto and Iizuka, 1998), and a relatively unexplored modification "protein deimination." The deimination is catalyzed by peptidylarginine deiminases (EC 3.5.3.15) that deiminate arginine residues in calcium-ion-dependent ways, generating citrulline residues (Rogers *et al*, 1977; Kubilus *et al*, 1980; Fujisaki and Sugawara, 1981). The deiminated proteins show lowered isoelectric points, affecting protein interactions dependent on ionic charge. There may also be interference with the stability of H bonds (Tarcsa *et al*, 1996).

There are several substrate proteins of peptidylarginine deiminase in the epidermis, including filaggrin and trichohyalin (Tarcsa *et al*, 1996). Recent evidence indicates that major deiminated proteins in mammalian epidermis are partially degraded/disulfide-cross-linked keratin K1, based on detection of chemically modified citrulline residues with a monospecific antibody (Senshu *et al*, 1995; 1996). Interestingly, two arginine residues identified as preferred target sites of peptidylarginine deiminase are located in its V subdomains (Senshu *et al*, 1999a), which are characterized by marked enrichment with glycine residues (Steinert *et al*, 1991). We also developed a new immunochemical probe elicited with one of the deiminated peptide sequences identified in mouse keratin K1 (Senshu *et al*, 1999b). Previous immunohistochemical analysis of deiminated protein(s) based on the conventional detection method indicated that they are located in normal human epidermis mainly in the lowermost cornified layer (Senshu *et al*, 1996). No information is available, however, regarding the distribution of the deiminated proteins in diseased skin. Here we report the altered distribution of the deiminated protein(s) in psoriatic hyperproliferative epidermis. Both the conventional and newly developed detection methods indicated markedly diminished deiminated K1 in psoriatic involved epidermis.

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Abbreviations: ACP, polyclonal antibodies to a citrulline-containing undecapeptide corresponding to the identified deimination site in the V2 subdomain of mouse keratin K1; AMC, monospecific antibody to detect chemically modified citrulline residues.

MATERIALS AND METHODS

Antibodies A monospecific antibody to chemically modified citrulline residues (AMC) has been described previously (Senshu *et al*, 1992). Preparation and affinity purification of polyclonal antibodies to a citrulline-containing undecapeptide (ACP) corresponding to the identified deimination site in the V2 subdomain of mouse keratin K1 (amino acid residues 545 GSSGGGRGGSS 555) have also been described (Senshu *et al*, 1999a). A monoclonal antibody, 34βB4, that detects suprabasal keratins (Gown and Vogel, 1984; Shah *et al*, 1987) was the product of Enzo Diagnostics (New York, NY). Monoclonal antibodies AE1 and AE3 are generous gifts from Dr. Tung-Tien Sun (New York University School of Medicine).

Immunohistochemical analyses of deiminated proteins of normal and psoriatic epidermis Biopsy samples for immunohistochemical staining were obtained from 10 psoriatic plaques including perilesional uninvolved skin. All specimens were cut in halves and one half of each was fixed with 10% formalin, dehydrated with ethanol, embedded in paraffin, and stained with hematoxylin and eosin to confirm the diagnosis. The other halves from five plaques were processed for staining deiminated proteins on the basis of the conventional detection method using AMC (Senshu *et al*, 1995). Briefly, the tissues were soaked overnight at 4°C in a Bouin Hollande Sublimate solution (10% saturated $HgCl_2$ in 2.5% cupric acetate, 4% picric acid, 4% paraformaldehyde, and 1% acetic acid). They were then soaked in a graded series of sucrose solutions before being embedded in O.C.T. compound 4583 (Sakura Finetechnical, Tokyo, Japan) for cryosectioning. The sections were mounted on gelatin-coated slides and postfixed with 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate-buffered saline. Endogenous peroxidase was blocked with 1% H_2O_2. Sections were briefly digested with trypsin and 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% antipyrine (modification medium) at 37°C for 3 h to chemically modify citrulline residues. Immunoperoxidase staining of the modified proteins was performed with AMC and a Histofine SAB-PO kit (Nitirei, Tokyo, Japan) using 3,3′-diaminobenzidine as a chromogenic substrate (Senshu *et al*, 1995). In addition, the paraffin-embedded samples were cut, de-paraffinized, and stained with a monoclonal antibody, 34βB4.

The remaining halves of the other five plaques were directly embedded in O.C.T. compound. Frozen sections were cut from them and incubated with ACP. The sections were further incubated with fluorescein isothiocyanate conjugated swine antirabbit IgG antibodies (Dako, Glostrup, Denmark). For simultaneous detection of ACP-positive proteins and suprabasal keratins, the sections were first incubated with a mixture of ACP and 34βB4 and were then incubated with a mixture of fluorescein isothiocyanate conjugated swine antirabbit IgG antibodies and Texas Red conjugated sheep antimouse IgG antibodies (Amersham Life Science, Buckinghamshire, U.K.).

Biochemical analyses of deiminated keratins Normal and psoriatic epidermis was obtained by keratome biopsy (set at 0.3 mm). The epidermal samples were crushed to frozen powder using a Cryopress (Microtech Nichion, Chiba, Japan) under cooling with liquid nitrogen. Differential extraction of urea-soluble and urea/2-mercaptoethanol (2-ME)-soluble fractions was performed as described previously (Senshu *et al*, 1996). These fractions were enriched with undegraded keratins derived from the viable epidermal cells and partially degraded keratins in the cornified cells, respectively. They were resolved by nonequilibrium pH gradient gel electrophoresis (pH 3.5–10) (O'Farrell *et al*, 1977) and/or sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) for western blotting. The blot was incubated with ACP and horseradish peroxidase-labeled goat antirabbit IgG (Bio-Rad, Hercules, CA) for detection by the enhanced luminol reaction using Renaissance (Dupont NEN). The chemiluminescence image was recorded using a chilled charge-coupled device camera as described previously (Senshu *et al*, 1996). The blot was incubated in the modification medium and then with AMC and the labeled second antibody for the detection of deiminated proteins by the conventional method. The incubation in the strongly acidic modification medium was sufficient to eliminate the antigenic properties of bound antibodies in the preceding step. The blot was finally stained with Amido Black 10B to visualize proteins. The resulting image was recorded using a high performance charge-coupled device. The data were presented as superimposed color images using an Atto Spot Screener (Atto, Tokyo, Japan).

RESULTS

Decreased deiminated proteins characterize psoriatic involved cornified cell layer We first conducted immunocytochemical staining of deiminated proteins by the conventional method. AMC-positive staining was noted in the cornified cell layer of perilesional uninvolved psoriatic epidermis (**Fig 1*C***). Essentially the same staining pattern was observed in normal epidermis (data not shown). On the other hand, psoriatic lesional epidermis showed very faint staining of deiminated proteins (**Fig 1*A***). When present, the deiminated proteins were only focally detected (**Fig 1*A***). Thus the psoriatic hyperproliferative epidermis was characterized by decreased deiminated proteins in the cornified cell layer. The psoriatic lesional epidermis showed decreased staining of suprabasal keratins with the monoclonal antibody 34βB4 (**Fig 1*B***) compared with the perilesional epidermis (**Fig 1*D***).

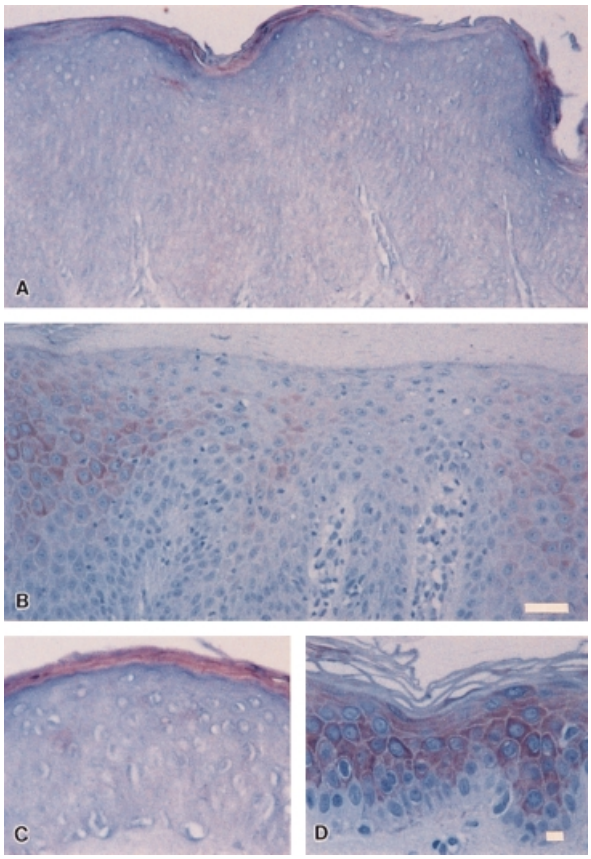

Figure 1. Decreased expression of deiminated proteins in psoriatic involved epidermis. Both deiminated proteins detected by the conventional method using AMC (*A*, *C*) and suprabasal keratin(s) stained with 34βB4 (*B*, *D*) are decreased in psoriatic lesional skin (*A*, *B*) compared with perilesional uninvolved skin (*C*, *D*). *Scale bars*: (*A*, *B*) 50 μm, (*C*, *D*) 10 μm.

Decreased staining of the psoriatic involved cornified cell layer was more evident when equivalent sections were stained with ACP. Double staining with 34βB4 and ACP demonstrated that the psoriatic involved epidermis with low suprabasal keratin expression showed negative or only poor immunoreactivity to ACP (**Fig 2*A*, *B*** compared with ***C***, ***D***). Intralesional acrosyringia showing decreased suprabasal keratin immunoreactivity like that of the surrounding psoriatic epidermis, however, showed strong ACP staining (**Fig 2*E-G***).

Decreased deiminated K1 in the psoriatic involved epidermis Four major keratin bands (about 67, 59, 57, and 51 kDa) were detected in the stained protein profiles (**Fig 3*A*, *B***; green), and were identified as K1, K5, K10, and K14, respectively, using the monoclonal antibodies AE1 and AE3 (data not shown). The urea-soluble fractions of normal and psoriatic uninvolved and involved epidermis consistently showed no positive signals upon immunoblotting with either AMC or ACP. The urea/2-ME-soluble fractions of normal epidermis (**Fig 3*A***, *lane b*) and psoriatic uninvolved epidermis (*lanes d*, *h*) showed a major AMC-positive signal (orange to yellow) co-migrating with K1. The normal sample showed a minor signal co-migrating with K10. This signal was less obvious in the psoriatic samples. Immunoblotting with ACP visualized a major signal (orange to yellow) in all these urea/2-ME-soluble fractions also co-migrating with K1 together with several minor signals (**Fig 3*B***, *lanes b*, *d*, *h*). Interestingly, the normal sample gave a distinct signal (about 47 kDa; red), which was hardly visible in the stained protein profile. The virtual absence of ACP-positive proteins in the urea-soluble fraction and the fact that the major ACP-positive band in the urea/2-ME-soluble fraction co-migrated with AMC-positive keratin K1 suggest that a major part

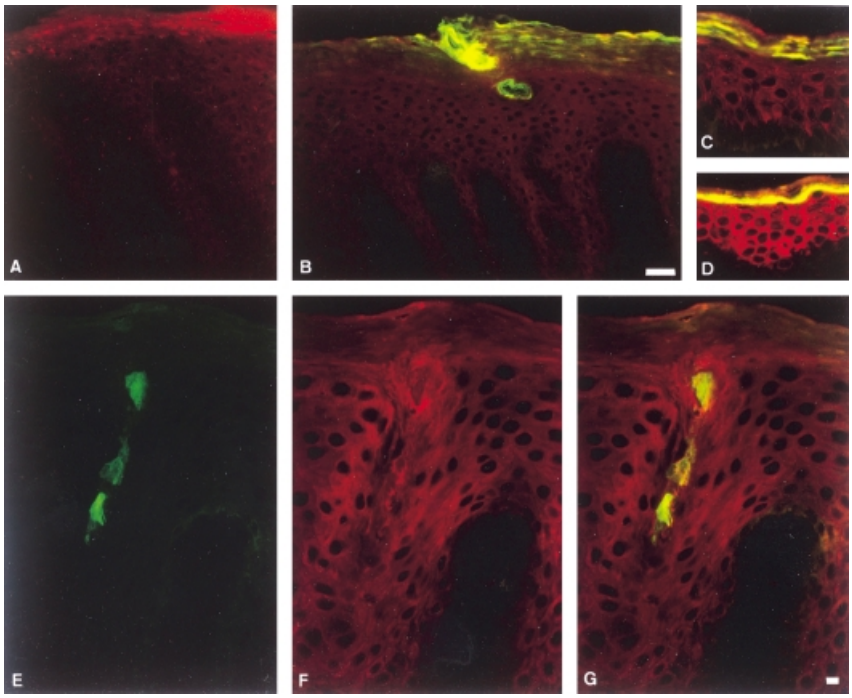

Figure 2. Decreased expression of ACP-positive proteins in psoriatic involved epidermis. Lesional psoriatic skin shows absence (*A*) or marked reduction (*B*) of ACP staining compared with intralesional acrosyringium (*B*, in the middle), perilesional (*C*) and normal skin (*D*). (*A*)-(*D*) are double staining for ACP-positive proteins (green) and suprabasal keratins (red). (*E*)-(*G*) are the same section showing staining for ACP-positive proteins, suprabasal keratins, and both, respectively. Note that although acrosyringium and surrounding epidermis show similar 34βB4 staining intensity (*F*), the former is ACP-positive but the latter is negative (*F*). *Scale bars*: (*A*, *B*) 50 μm, (*C*-*G*) 10 μm.

of ACP-positive immunoreactivity in the histologic sections may be accounted for by deiminated K1.

We also analyzed the urea/2-ME-soluble fraction from normal epidermis by two-dimensional western blot analyses. The major ACP-positive signal found on the one-dimensional blot was resolved into multiple charged isomers derived from K1 (**Fig 4*A***; orange-yellow), among which relatively acidic components coincided with the spots detected with AMC (**Fig 4*B***; orange to yellowish green). Similar results were obtained with the psoriatic uninvolved extracts (data not shown). The 47 kDa ACP-positive component was detected close to the anodic edge (**Fig 4*B***).

DISCUSSION

Recent evidence indicates that not only filaggrin and trichohyalin but also keratins are the substrates of peptidylarginine deiminase (Senshu *et al*, 1996, 1999a, b). Actually Senshu *et al* have shown that the major citrulline-containing proteins in human epidermis are partially degraded/disulfide-cross-linked keratin K1 by the conventional detection method using AMC (Senshu *et al*, 1996). The results obtained using AMC in this study indicate that psoriatic involved epidermis is characterized by decreased deiminated K1. Immunohistochemical analysis showed decreased deiminated proteins in the psoriatic involved cornified cell layer (**Figs 1, 2**). Keratin analysis demonstrated that the deiminated protein of normal (**Fig 3*A*, *B***, *lane b*) and psoriatic uninvolved epidermis (**Fig 3*A*, *B***, *lanes d*, *h*) is mostly derived from K1, which is markedly diminished in the psoriatic involved epidermis (**Fig 3*A*, *B***, *lanes f*, *j*). This is consistent with the biochemical data indicating that the major deiminated epidermal protein is K1 (Senshu *et al*, 1996).

ACP was elicited with the deiminated undecapetide corresponding to the identified deimination site in the V2 subdomain of mouse K1 (545 GSSGGGRGGSS 555). It decorated the cornified layers specifically in normal and psoriatic uninvolved epidermis. Moreover, it visualized multiple charged isomers derived from keratin K1 localized in the cornified layer (**Fig 4**). Relatively basic charged isomers were not detected with the conventional method using AMC. This was reasonable as ACP detected the immunizing peptide antigen at much higher sensitivity than AMC (Senshu *et al*, 1999b). We presume that ACP is useful for detecting citrulline residues in glycine- and/or serine-rich sequences. The ACP-positive 47 kDa protein remains to be identified.

As intralesional acrosyringia showed strong deiminated K1 staining (**Fig 2*E*-*G***) despite the decreased suprabasal keratin immunoreactivity like that of the surrounding psoriatic epidermis, the activity of peptidylarginine deiminase itself seems to be retained in acrosyringia. This and the decreased deimination of K1 in psoriatic epidermis might indicate that abnormal keratin deimination in psoriasis is restricted to the epidermis, without affecting sweat ductal epithelia.

Peptidylarginine deiminase affects ionic interactions of proteins by lowering isoelectric points of substrate proteins. The preferential deimination sites of K1 identified in the V1 and V2 subdomains (Senshu *et al*, 1999a) have been implicated in the association with desmosomal proteins such as desmoplakin (Kouklis *et al*, 1994; Meng *et al*, 1997). Interestingly, these are also the sites of interaction with loricrin by a putative Velcro mechanism (Steinert *et al*, 1991), where keratin and loricrin associate together by hydrophobic glycine loop interaction. Deimination of arginine residues by decreasing the isoelectric point of K1 especially at the site of glycine loop configuration would diminish the electrostatic repulsion of keratins with their basic partner, loricrin. Note that loricrin does not have any arginine residues and is not modified by peptidylarginine deiminase.

On the other hand, filaggrin, another substrate of peptidylarginine deiminase, is supposed to be dissociated from keratin bundles and to undergo proteolytic degradation eventually to free amino acids in the cornified cell layer.

The deimination of K1 by lowering the isoelectric point would hamper the dissociation of filaggrin, which is a basic protein. In this circumstance the deimination of filaggrin, which has long been documented (Tarcsa *et al*, 1996), might be critical to the dissociation of filaggrin from the keratin bundles (Harding and Scott, 1983). Thus the post-translational deimination of arginine residue(s) facilitates interaction between keratin and loricrin, and dissociation of keratin and filaggrin. In this context it is interesting to note that K1, filaggrin, and loricrin are all reportedly decreased in the psoriatic hyperproliferative epidermis (Baden *et al*, 1978; Bernard *et al*, 1988; Stoler *et al*, 1988; Thewes *et al*, 1991; Ishida-Yamamoto *et al*, 1996).

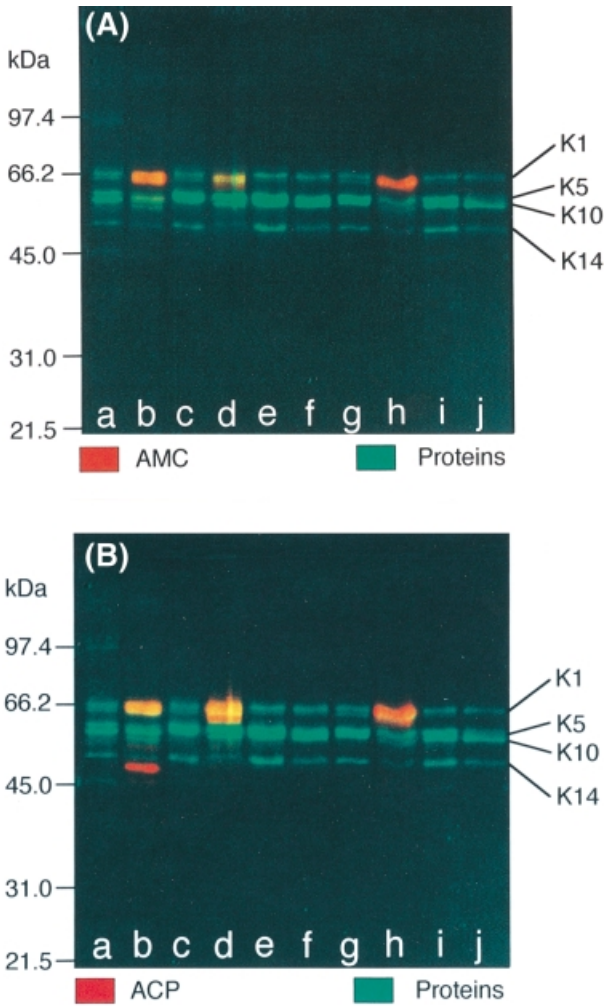

Figure 3. Deficiency of deiminated K1 in psoriatic involved epidermis. The urea-soluble (*lanes a, c, e, g, i*) and urea/2-ME-soluble (*lanes b, d, f, h, j*) fractions obtained from normal and psoriatic epidermis were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotted to polyscreen PVDF (Dupont NEM) for sequential detection using AMC (*A*) and ACP (*B*) followed by poststaining with Amido Black 10B. The data are displayed as superimposed color images. Orange to yellow colored bands indicate positive double staining of protein (green) and AMC or ACP (red). *a, b*, normal epidermis; *c, d*, psoriatic uninvolved epidermis (case 1); *e, f*, psoriatic involved epidermis (case 1); *g, h*, psoriatic uninvolved epidermis (case 2); *i, j*, psoriatic involved epidermis (case 2). Keratin bands of K1, K5, K10, and K14 (in order of increasing mobility) were identified by their molecular weights and confirmed by using monoclonal antibodies. Note the major immunopositive signals co-migrating with K1 in the urea/2-ME-soluble fractions of normal and psoriatic uninvolved epidermis (*lanes b, d, h*). Such bands were hardly detected in the urea/2-ME-soluble fraction of psoriatic involved epidermis (*lanes f, j*).

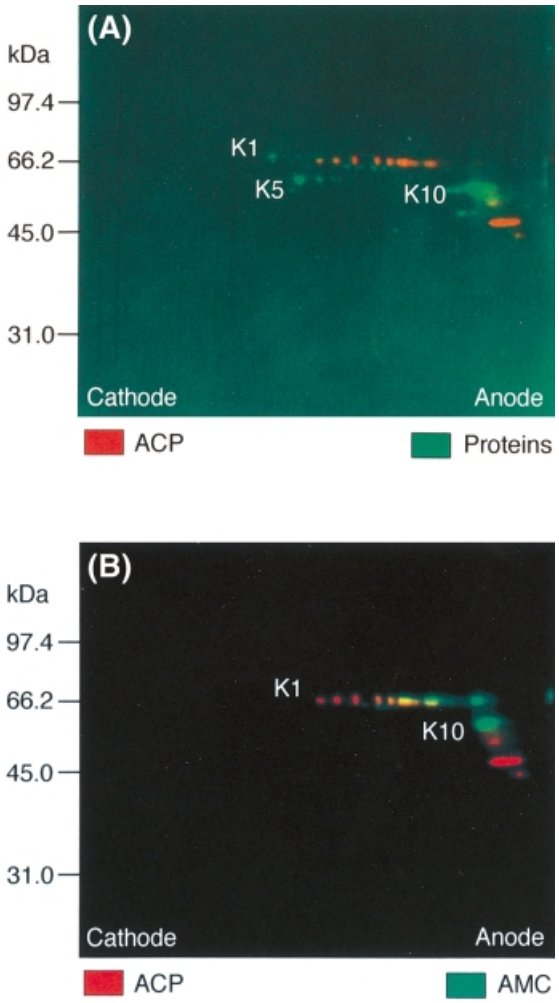

Figure 4. ACP visualizes multiple charged isomers of K1 in normal epidermis. The urea/2-ME-soluble extract of normal epidermis was resolved by two-dimensional gel electrophoresis and western blotted for sequential detection using ACP and AMC followed by poststaining with Amido Black 10B. The data are displayed as superimposed color images. (*A*) ACP-positive spots (red) and stained proteins (green); (*B*) ACP- and AMC-positive spots (red and green, respectively). Orange to yellowish green colored spots indicate positive double staining by two different detection methods. Note the multiple ACP-positive charged isomers migrating as K1.

In conclusion our results have demonstrated for the first time that psoriatic hyperproliferative epidermis is characterized by decreased deiminated proteins, especially K1. The finding adds further evidence for the deranged keratinization process in psoriatic lesional epidermis. As K1 is the major citrulline-containing protein in the normal human cornified cell layer, with possible interactions with other structural proteins, the alteration would have a significant effect on terminal keratinization processes. Whether the finding is associated with a defect of peptidylarginine deiminase(s) is currently under investigation in our laboratories.

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