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Purification and Characterization of Active Caspase-14 From Human Epidermis and Development of the Cleavage Site-Directed Antibody

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ABSTRACT

Restricted expression of caspase-14 in differentiating keratinocytes suggests the involvement of caspase-14 in terminal differentiation. We purified active caspase-14 from human cornified cells with sequential chromatographic procedures. Specific activity increased 764-fold with a yield of 9.1%. Purified caspase-14 revealed the highest activity on WEHD-methylcoumaryl-amide (MCA), although YVAD-MCA, another caspase-1 substrate, was poorly hydrolyzed. The purified protein was a heterodimer with 17 and 11 kDa subunits. N-terminal and C-terminal analyses demonstrated that the large subunit consisted of Ser^{6}-Asp^{146} and N-terminal of small subunit was identified as Lys^{153}. We successfully developed an antiserum (anti-h14D146) directed against the Asp^{146} cleavage site, which reacted only with active caspase-14 but not with procaspase-14. Furthermore we confirmed that anti-h14D146 did not show any reactivity to the active forms of other caspases. Immunohistochemical analysis demonstrated that anti-h14D146 staining was mostly restricted to the cornified layer and co-localized with some of the TUNEL positive-granular cells in the normal human epidermis. UV radiation study demonstrated that caspase-3 was activated and co-localized with TUNEL-positive cells in the middle layer of human epidermis. In contrast, we could not detect caspase-14 activation in response to UV. Our study revealed tightly regulated action of caspase-14, in which only the terminal differentiation of keratinocytes controls its activation process. J. Cell. Biochem. 109: 487–497, 2010.

KEY WORDS: CASPASE-14; KERATINOCYTE; TERMINAL DIFFERENTIATION

Terminal differentiation of keratinocytes results in formation of the cornified layer that serves as a protective barrier against a hazardous environment [Watt, 1983]. This process is precisely controlled by a differentiation program that begins with proliferating basal cells and leads to the appearance of spinous, granular, and ultimately, cornified cells. During the transition phase from granular to cornified cells, dramatic changes occur both inside and outside of the keratinocytes. Internally, the cells lose their nuclei and cytoplasmic organelles, while they gain a reinforced cytoplasmic membrane called the cornified envelope and a keratin pattern that maintains the flexible, yet sturdy, quality of the interior structure. Externally, the cells are surrounded by lipid layers that contribute to the cell–cell adhesion and permeability barrier. DNA fragmentation and the presence of TUNEL-positive cells, which are usually detected in apoptosis, are frequently observed in differentiating keratinocytes [Lovas, 1986; McCall and Cohen, 1991; Haake and Polakowska, 1993; Maruoka et al., 1997]. However, recent investigations seem to suggest that apoptosis and terminal differentiation of keratinocytes are distinct processes [Gandarillas, 2000; Lippens et al., 2005].

Caspases are well-known executioners of apoptotic cell death [Kumar, 1999]. They are evolutionarily conserved cysteine proteases that cleave their substrates after aspartic acid residues. Proapoptotic

Abbreviations used: inhibitor of caspase-activated DNase, ICAD/DFF45; FMK, fluoromethyl ketone; MCA, 4-methylcoumaryl-7-amide; CHAPS, 3-[(3-cholamidopropyl)-dimethy lammonio]-1-propanesulfonic acid.

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Additional Supporting Information may be found in the online version of this article.

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caspases, such as caspase-3, -6, and -7 are processed and activated by initiator caspases such as caspase-8 and -9. Finally, the processed effector caspases degrade the inhibitor of caspase activated DNase (ICAD/DFF45), resulting in the liberation of CAD as an active nuclease [Enari et al., 1998; Sakahira et al., 1998]. It is suggested that the terminal differentiation of keratinocytes shares some mechanisms with apoptotic cell death such as loss of nucleus. Indeed several caspases are expressed in human keratinocytes and caspase-like activities are observed in cornified cell extracts [Takahashi et al., 1998]. However, typical apoptotic caspases, including caspase-3, are not activated during terminal differentiation of keratinocytes [Lippens et al., 2000].

Caspase-14 is expressed exclusively in differentiating keratinocytes [Eckhart et al., 2000a,b], and its restricted expression suggests possible implication of caspase-14 in keratinocyte terminal differentiation [Pistritto et al., 2002; Rendl et al., 2002]. Recently Mikolajczyk et al. [2004] showed that caspase-14 is active only in high concentrations of kosmotrophic salts. They also showed that recombinant procaspase-14 can be activated with granzyme B, a leukocyte serine proteinase which possesses caspase-like activity. Denecker et al. [2007] suggested that caspase-14 knockout mouse showed some defect in epidermal barrier formation. It was related to incomplete filaggrin degradation, resulting in deterioration of transepidermal water loss. They also showed that caspase-14 deficient epidermis leads to increased levels of UVB-induced apoptosis. However, although the primary structures of mouse and human caspase-14 are highly homologous each other, there are some critical differences between them. For example, mouse capase-14 possesses recognition sequence for caspase-8, whereas human caspase-14 does not [Ahmad et al., 1998]. This may suggest implication of mouse caspase-14 in the apoptotic process. Indeed mouse caspase-14 is activated by caspase-8 in vitro in contrast to humans. However, activation mechanisms of human caspase-14 are totally unknown.

To understand activation mechanisms of caspase-14 in human epidermis, it is important to identify the processing site of caspase-14 obtained from in vivo samples. In this study we purified caspase-14 from human cornified cell extract and determined the cleavage sites. Based on that information, we developed a specific antibody against a cleavage site D146 (anti-h14D146), which recognizes only the active form of caspase-14. Using this antibody we further extended our observation and identified the precise localization of active caspase-14 in human epidermis. In addition, UV radiation study in vivo suggested that human caspase-14 would not be activated under UV-induced apoptosis, which distinguishes caspase-14 from other apoptotic caspases.

MATERIALS AND METHODS

MATERIALS

The caspase substrates Ac-WEHD-methyl-coumarin amide (MCA), Ac-YVAD-MCA, Ac-VDVAD-MCA, Ac-DEVD-MCA, Ac-VEID-MCA, Ac-IETD-MCA, and Ac-LEHD-MCA were purchased from Peptide Institute, Inc. (Osaka, Japan). Benzyloxycarbonyl (Z)-YVAD-fluoromethyl ketone (FMK), ZVDVSD-FMK, Z-DEVD-FMK, Z-VEID-FMK, Z-IETD-FMK, Z-LEHD-FMK, and ZVAD-FMK were purchased from BioVision (Mountain View, CA). The proform and the large subunit of caspase-14 were detected with antibody H99 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against a peptide corresponding to amino acids 24–122 of human caspase-14. Thus, the antibody reacts with the proenzyme and the processed form (large subunit) of caspase-14. The small subunit of caspase-14 was detected with the C-20 antibody (Santa Cruz Biotechnology, Inc.). Anti-caspase-1 antibody was obtained from NeoMarkers (Fremont, CA).

PREPARATION OF CLEAVAGE SITE-DIRECTED ANTIBODY

Cleavage-site directed antibody (ant-h14D146) was prepared with immunizing rabbits using the synthetic hexapeptide, TVGGD, which corresponds to the C-terminal end of the large subunit of the purified caspase-14, according to the method with Kouroku et al. [1998]. Anti-h14D146 antibody was purified with affinity chromatography on TVGGD-coupled Agarose.

MEASUREMENT OF CASPASE-14 ACTIVITY

Caspase-14 activity was measured using Ac-WEHD-MCA as a substrate, according to the method of Mikolajczyk et al. [2004]. Briefly, the assay mixture consisted of 95 μl of 0.1 M HEPES buffer (pH 7.5), 0.06 M NaCl, 0.01% CHAPS, 5 mM DTT, 1.3 M sodium citrate, and 10 μM WEHD-MCA (final concentration). Enzyme samples (5 μl) were added to the mixture and incubated for 10–30 min. Enzyme activity was measured using Fluoroskan Ascent FL (Thermo Electron Co., Wolsam, MA) with 355 nm excitation and 460 nm emission. For inhibitor assays, caspase-14 and peptide inhibitors were incubated in the assay buffer for 15 min at room temperature, and assays were initiated by adding 5 μl of 100 μM Ac-WEHD-MCA.

PURIFICATION OF CASPASE-14

Approximately 14 g of human cornified cells scraped from the heels of healthy individuals were extracted with 0.1 M Tris–HCl (pH 8.0) containing 0.14 M NaCl using a glass homogenizer. Supernatant was obtained after centrifugation at 15,000*g* for 60 min. After concentration with Amicon Ultra (Millipore, Billerica, MA) and desalting with Fast Desalting column HR10/10 (Amersham Biosciences, Piscataway, NJ), the crude extract was applied to a HiPrep 16/10 Q XL column. The column was washed with 20 mM Tris–HCl (pH 8.0) and eluted with a 0–1 M linear NaCl gradient. Fractions were analyzed by Western blot with the anti-caspase-14 antibody (H99). In addition, hydrolytic activity on WEHD-MCA was measured in each fraction. Positive fractions were applied to a Mono Q column equilibrated with the same buffer and eluted with 0–1 M NaCl gradient. Caspase-14-containing fractions were further separated by Mono S cation-exchange chromatography. The column was equilibrated with 20 mM acetate buffer (pH 4.5) and eluted with 0–1 M NaCl gradient. Positive fractions were concentrated and applied to a chromatofocusing Mono P column equilibrated with 25 mM ethanolamine (pH 8.3). Elution was performed using 46 ml of Polybuffer (pH 5.0) forming a pH gradient from 8 to 5 and finally with 2 M NaCl. Caspase-14 was finally purified by Superdex 75 gel chromatography. Protein concentration

was determined with a BioRad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

DETERMINATION OF THE PRIMARY STRUCTURE OF ACTIVE CASPASE-14

Purified active caspase-14 was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and blotted onto a PVDF membrane. After CBB staining, 17 and 11 kDa bands were cut and each amino terminal sequence was determined with a Procise cLC 492 cLC protein sequencer and PTH analyzer (140D) (Applied Biosystems, Foster City, CA). For identification of the large subunit C terminus, the following procedures were carried out. After SDS–PAGE, the caspase-14 protein bands were visualized by staining with CBB. The 17 kDa band was excised from the gel, minced to approximately 1 mm^3 and digested by lysyl-endopeptidase. In-gel digestion was carried out in the presence of 50% (v/v) ^{18}O water to incorporate differentially labeled peptides. Peptides were extracted from the gel, and MALDI-TOF/MS analysis was carried out using BIFLEX III (Bruker Daltonics, Inc., Billerica, MA).

PREPARATION OF RECOMBINANT CASPASE CONSTRUCTS

Caspase-14 cDNA was PCR-cloned into the pQE 30 vector (Qiagen, Inc., Valencia, CA). Recombinant proteins were purified with Ni-NTA Agarose (Qiagen, Inc.) and Mono Q chromatography. cDNA fragments encoding human caspase-14 and human caspase-14D146 were amplified by PCR using the following primers: forward primer for human caspase-14 and human caspase-14D146: 5′-ATGAG-CAATCCGCGGTCTTTGGAA-3′; reverse primer for human caspase-14: 5′-CTAATCTCCACCTACTGTTTCACCGGGGT-3′; and reverse primer for human caspase-14D146: 5′-CTACTGCAGATACAGC-CGTTTCCGGA-3′. PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI) and then subcloned in-frame into the *Eco*RI site of the FLAG tagged (FLAG) CMV expression vector (Kodak, New Haven, CT) and the pEGFP-C1 vector (Clontech, Palo Alto, CA). The FLAG-tagged- or EGFP plasmids (5 μg) were transfected into COS cells using the calcium–phosphate method [Urase et al., 1998]. Cell extracts were used to examine reactivity and specificity of anti-h14D146 by immunoblot analysis. FLAG-tagged large subunits of other caspase members were similarly prepared as described above. They were; rat caspase-2D394; human caspase-3D175; mouse caspase-6D162; mouse caspase-7D198; caspase-8D387 (mcaspase-delta-8D387), lacking the death effector domains (DED), and human caspase-9D330. FLAG-tagged cDNAs were transfected into COS cells and were subjected to immunoblot analysis using anti-FLAG and anti-h14D146 antibodies [Fujita et al., 2001]. The large subunit of human caspase-1 was prepared as a GST fusion protein based on the sequence information (NM_033293).

TISSUE SPECIMENS

Human scalp skin specimens were obtained with informed consent from plastic surgery patients. The study was approved by the Shiseido Committee on Human Ethics. Tissue samples were fixed with 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4) and embedded in paraffin. For the preparation of skin extract, the dermis was excised as much as possible, and the remaining tissue was homogenized with a glass homogenizer, extracted with 50 mM HEPES (pH 7.5) containing 0.5% Triton X-100, 150 mM NaCl, and complete inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were homogenized and extracted as described above. Supernatant was obtained after centrifugation at 15,000*g* for 60 min and analyzed with Western blot.

UV RADIATION STUDY

Healthy male volunteers (age 30–49) received three minimum erythematous doses (MED) of UVB irradiation on the back, using an FL20S-E lamp (290–320 nm; Toshiba, Tokyo, Japan). UV intensity was measured at 305 nm by a UVR-305/365D (II) radiometer (Topcon Corporation, Tokyo, Japan). Irradiation was performed with the lamp 40 cm from the skin. Skin specimens were taken as 3 mm punch biopsy samples 8 h, 1 day and 2 days after UV irradiation. Samples were fixed with 4% PFA in phosphate buffer (pH 7.4) and embedded in paraffin. Non-irradiated skin specimens were obtained from sites away from irradiated skin. Thin sections were prepared and analyzed with hematoxylin and eosin (HE) and immunohistochemical staining.

IMMUNOHISTOCHEMISTRY

Thin sections were incubated with appropriate primary antibody at 4°C overnight. For fluorescence detection of procaspase-14, H99 was used as a primary antibody and Texas Red dye-conjugated donkey anti-rabbit IgG (Nichirei) was used as a secondary antibody. The TUNEL reaction was performed using a fluorescein in situ cell death detection kit (Roche Diagnostics) according to the manufacturer's instructions. For double detection of TUNEL-positive cells and active caspases, Alexa Fluor 555 or 488 (Molecular Probes, Inc., Eugene, OR) was used. To visualize nuclei, samples were immersed in 10 ng/ml DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes) for 5 min and washed three times with PBS. A Leica DMLA microscope was used to observe fluorescence.

WESTERN BLOT ANALYSIS

Proteins were separated with a 5–20% gradient gel by SDS–PAGE. After electrophoresis, proteins were transferred onto a polyvinylidine difluoride membrane (Immobilon-P, Millipore, Bedford, MA) and incubated with anti-caspase-14 antibodies (H99, h14D146, C20). Peroxidase-labeled anti-rabbit IgG (Sigma) or anti-goat IgG was used as a secondary antibody, and immunoreactive proteins were visualized by chemiluminescence using ECL-plus (Amersham Biosciences).

RESULTS

PURIFICATION OF CASPASE-14 FROM CORNIFIED CELL EXTRACT

The majority of the caspase-14 in cornified cells is in the processed form [Eckhart et al., 2000b; Lippens et al., 2000]. We purified the processed fragment of caspase-14 by monitoring its hydrolytic activity on Ac-WEHD-MCA and its immunoreactivity to the H99 antibody (Fig. 1a, see also Supplementary Fig. 1). The final preparation with Superdex 75 chromatography showed a peak with a molecular weight of 30 kDa, which coincided with the hydrolytic activity against Ac-WEHD-MCA. The fractions (No. 25–27) were collected and used for SDS–PAGE, Western blot and sequence

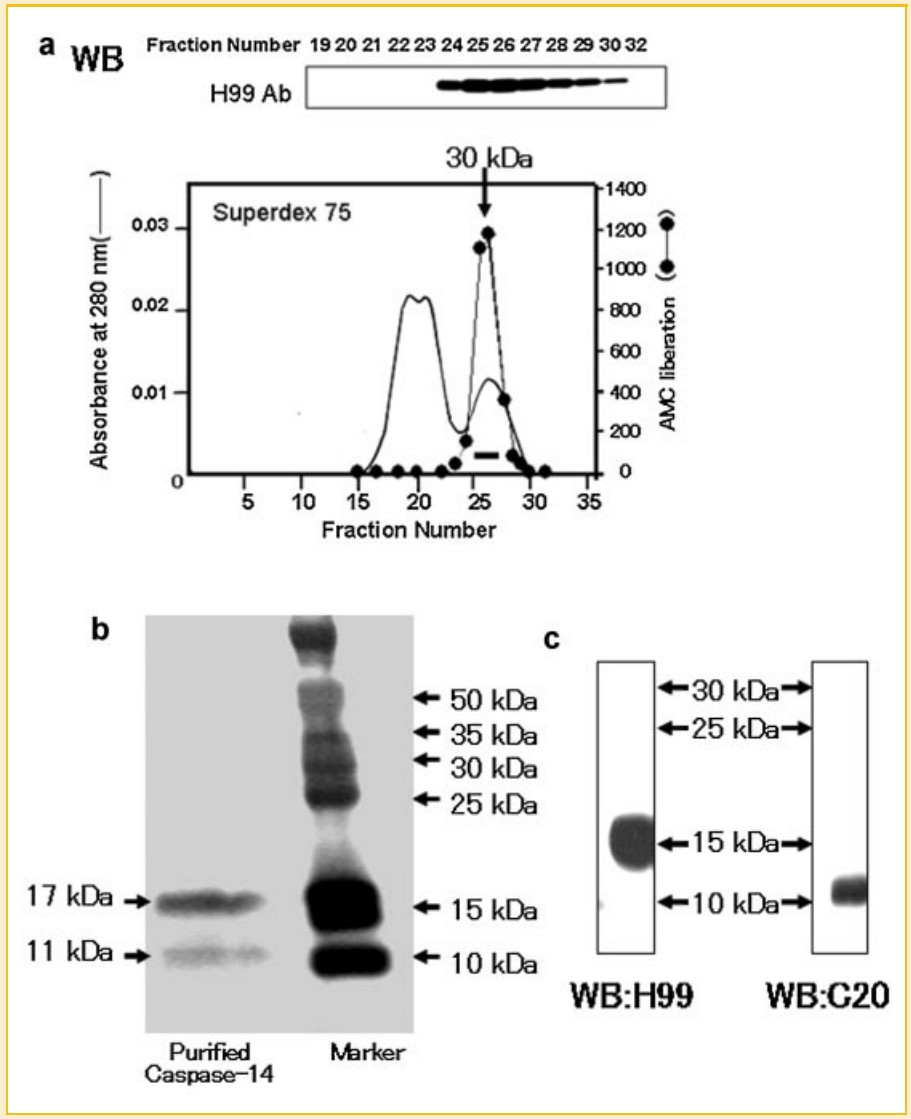

Fig. 1. Purification of caspase-14 from human cornified cell extract. Human caspase-14 was purified from cornified cell extracts with successive chromatographic procedures. a: The final step on Superdex 75 gel chromatography was shown. Hydrolytic activities against Ac-WEHD-MCA were evaluated in each fraction. Western blot analyses with H99 antibody were carried out for the detection of caspase-14. Fractions (25–27) that show both hydrolytic activity against Ac-WEHD-MCA and positive reactivity with H99 antibody are indicated with a bar and used for further analyses. b: SDS–PAGE of the purified caspase-14. Fraction No. 25–27 were collected, electrophoresed and stained with CBB. c: Western blot analysis of the purified fractions. H-99 and C-20 antibodies were used for the large subunit and small subunit detection, respectively.

analyses. The results of sequential chromatographic steps are summarized in Table I. Starting from approximately 100 mg of soluble protein extract, 11.8 μg of purified protein was obtained. The specific activity increased 764-fold and the yield was 9.1%. The preparation included the 17 and 11 kDa fragments, according to SDS–PAGE (Fig. 1b). The 17 kDa band was positive for H99 antibody, which reacted with the 17 kDa fragment at the N-terminal side. The 11 kDa fragment was recognized with the C20 antibody,

TABLE I. Summary of Caspase-14 Purification

	Protein conc. (μg/ml)	Amount (ml)	Total protein (μg)	Activity (AFU (mU))	Specific activity (mU/mg protein)	Total activity (U)	Yield (%)
CC TBS extract	312.0	320.00	99840.0	3.67	11.8	587.34	100.0
Hi Prep Q	1185.0	15.00	17775.0	133.59	112.7	1001.92	170.6
Mono Q	582.0	8.00	4656.0	150.30	258.2	601.19	102.4
Mono S	61.3	8.00	490.4	56.94	928.8	227.75	38.8
Mono P	50.5	1.00	50.5	338.31	6699.2	169.15	28.8
Superdex 75	11.8	1.00	11.8	106.52	9015.1	53.26	9.1

which reacted at the C-terminal region, but not by the H99 antibody (Fig. 1c). Our results indicated that the purified caspase-14 was a heterodimer consisting of large (17 kDa) and small (11 kDa) subunits.

CHARACTERIZATION OF THE PURIFIED HUMAN CASPASE-14

Purified caspase-14 revealed the highest activity on WEHD-MCA (Fig. 2a). This is similar to the profile of granzyme B-activated caspase-14 [Mikolajczyk et al., 2004]. On the other hand, YVAD-MCA, another caspase-1 substrate, was poorly hydrolyzed by epidermal caspase-14, although LEHD- and IETD-MCA were hydrolyzed to some extent. Caspase-14 was strongly inhibited by the caspase-1 inhibitor (YVAD-FMK) and the pan-caspase inhibitor (VAD-FMK) (Fig. 2b). This may reflect structural similarities between caspase-1 and caspase-14. Other caspase inhibitors also showed relatively high level of suppression except VEID-FMK. Class specific inhibitor for cysteine proteinases, iodo acetic acid (IAA) or serine proteinase inhibitor, 4-(2-aminoethyl)benzensulfonyl fluoride (AEBSF), did not show any significant inhibition at the concentrations tested in this study.

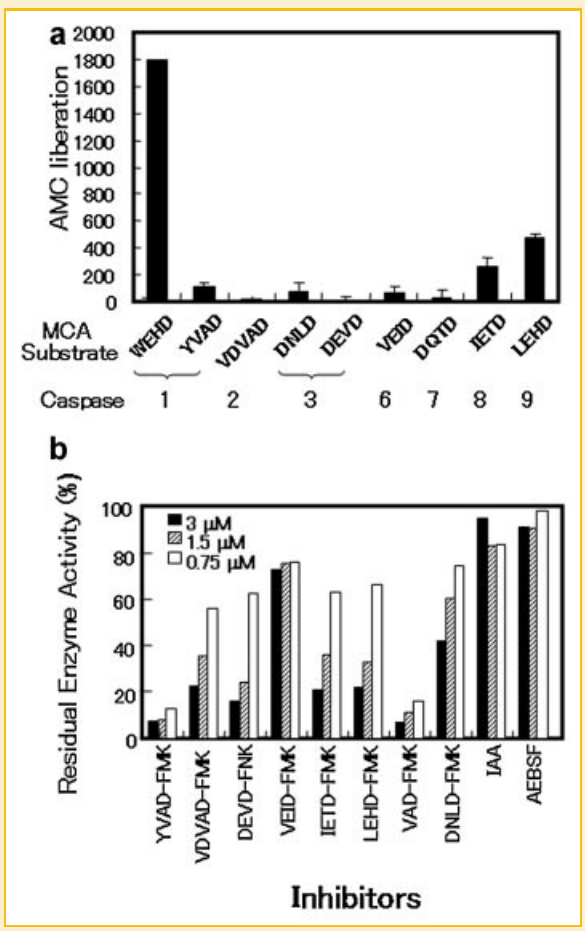

Fig. 2. Enzymatic characterization of the purified caspase-14. a: Substrate specificity of the purified caspase-14 was determined using synthetic caspase substrates Ac-WEHD-MCA, Ac-YVAD-MCA, (casapase-1), Ac-VDVAD-MCA (caspase-2), Ac-DEVD-MCA (caspase-3), Ac-VEID-MCA (csapsae-6), Ac-DQTD-MCA (csapase-7), Ac-IETD-MCA (csapase-8), and Ac-LEHD-MCA (csapase-9). Mean values of triplicate assays with standard deviation were shown. b: Susceptibility to various synthetic inhibitors was tested using the purified caspase-14. Caspase-14 was incubated with peptide inhibitors for caspases or class specific inhibitors for cysteine proteinases (IAA) or serine proteinases (AEBSF). Residual enzyme activity was measured using WEHD-MCA as a substrate in the presence of 1.3 M sodium citrate and 5 mM DTT. Inhibitor concentrations tested in this study was 3, 1.5, and 0.75 μM, respectively. Values represent means of duplicate assays.

CLEAVAGE SITE OF THE ACTIVE FORM OF CASPASE-14 AND PREPARATION OF THE SPECIFIC ANTISERUM AGAINST THE CLEAVAGE SITE

The purified caspase-14 from Superdex 75 chromatography was further separated by SDS–PAGE (Fig. 1b), and the processed fragments were subjected to sequence analyses. When the 17 kDa band was analyzed, the sequence SLEEE, corresponding to amino acids 6–10 of human caspase-14, was identified. The sequence suggests that human caspase-14 is cleaved between R5 and S6 during terminal differentiation. In a separate experiment, the 17 kDa band was excised after SDS–PAGE, and MALDI-TOF/MS analysis was carried out to determine the C-terminal peptide (Supplementary Fig. 2). An isolated peptide with a C-terminal end had a molecular mass of 2368.121, which is almost identical to the theoretical value of 2368.1356 of VYIIQACRGEQRDPGETVGGD. This suggests that human caspase-14 cleaved at D146. On the basis of these results, we concluded that the large subunit of human caspase-14 is a peptide with an amino acid sequence from S6 to D146. N-terminal analysis of the 11 kDa band revealed the sequence KDSPQ, which matches K153 to Q157 of caspase-14 and indicated that cleavage occurred between I152 and K153. Based on the molecular weight we determined with SDS–PAGE, the small 11 kDa subunit starts at K153 and ends with Q242. These lines of evidence suggest that human caspase-14 is activated by three cleavages that remove five amino acids from the N-terminus and six amino acids from the linker region E147-I152 (Fig. 3a).

To confirm cleavage at D146, we prepared a cleavage site-directed antibody (anti-h14D146) according to the procedure described by Kouroku et al. [1998]. Immunoblot analysis showed that anti-h14hD146 reacted with the large p17 (17 kDa) subunit of human caspase-14 at D146 (h14D146) but not the entire caspase-14 proform (30 kDa; Fig. 3b). We also tested reactivity of anti-h14D146 with the large subunit of csapase-1, since caspase-1 is the most closely related enzyme in the family members. This antibody did not show any reactivity with the large subunit of caspase-1 (Fig. 3c). Furthermore, anti-h14D146 did not react with active forms (large subunits) of human caspase-3 (C-terminal; D175), human caspase-9 (D330), mouse caspase-6 (D162), mouse caspase-7 (D198), or mouse caspase-8 (D387) (Fig. 4a). We also evaluated the specificity of anti-h14D146 with immunostaining. Anti-h14D146 showed strong immunostaining in the cytoplasm of h14D146-transfected COS cells but not in the procaspase-14-transfected cells. Thus, anti-h14D146 can be used specifically to detect the active form of human caspase-14 with immunostaining (Fig. 4b).

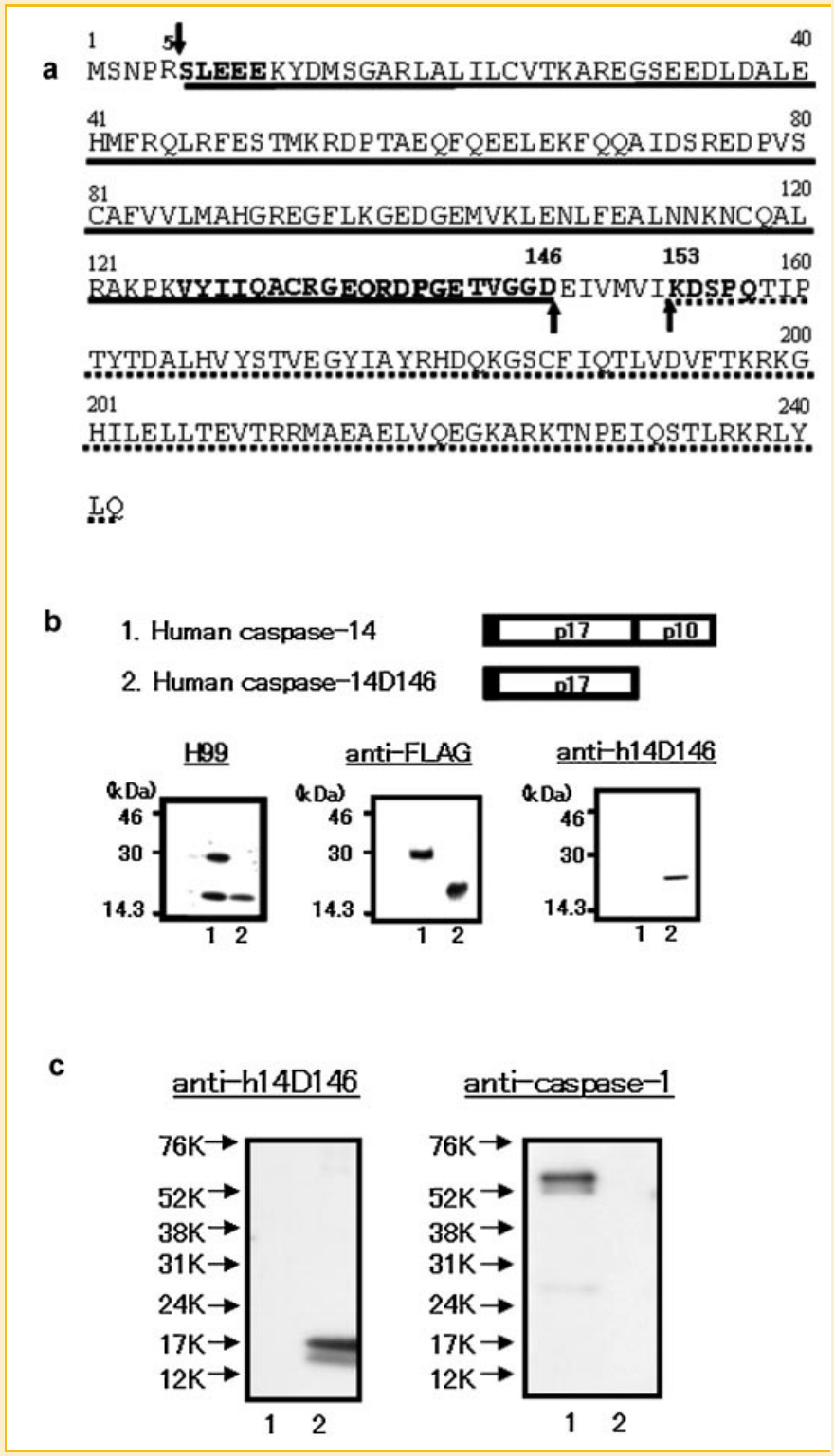

Fig. 3. Primary structure of purified caspase-14 and preparation of the cleavage site-directed antibody. a: Identification of the cleavage sites. N- and C-terminal analyses demonstrated that cleavage occurs at R5 and D146, generating the large subunit (17 kDa) (underlined). The small subunit (11 kDa) (dotted line) begins at K153. Arrows indicate cleavage sites. N- and C-terminal sequences identified in this study were shown in bold letters. b: Specificity of anti-h14D146 against caspase-14. Western blot analysis was performed using the fragment of FLAG-tagged human caspase-14 overexpressed in COS cells. FLAG-human caspase-14 (lanes 1) and FLAG-human caspase-14D146 (lanes 2) were transfected into COS cells, and their expression was examined by immunoblot using H99, anti-FLAG and anti-h14D146 antibodies, 24 h after transfection. Note that anti-h14D146 did not show any reactivity against proform of caspase-14. c: Specificity of anti-h14D146 against caspase-1. GST-fusion caspase-1 large subunit was prepared and reactivity of anti-h14D146 was investigated. The antibody did not recognize caspase-1 activation cleavage site. Lane 1, GST-fusion caspase-1 large subunit; lane 2, purified caspase-14.

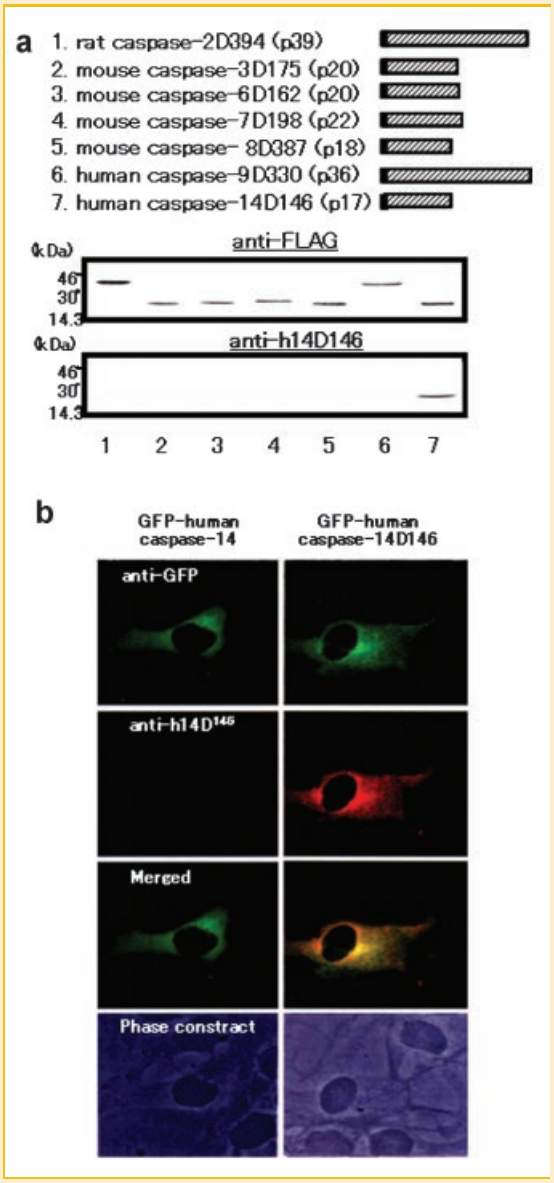

Fig. 4. a: FLAG-tagged human caspase-14D146 and active processing fragments of other caspases were transfected into COS cells, and anti-h14D146 reactivity was examined with immunoblots. Lane 1, rat caspase-2D394; lane 2, human caspase-3D175; lane 3, mouse caspase-6D162; lane 4, mouse caspase-7D198; lane 5, mouse caspase-8D387; lane 6, human caspase-9D330; lane 7, human caspase-14D146. FLAG-tag (shown as black box) was attached to the N-terminal of large subunit of each caspase (shown as shaded box). Note that anti-h14D146 antibody recognized only the large subunit of caspase-14. Immunocytochemical characterization of anti-h14D146. b: COS cells were transfected with GFP-tagged procaspase-14 or the large subunit (D146). Twenty-four hours after transfection, cells were fixed with cold methanol and immunostained with anti-h14D146.

CASPASE-14 PROCESSING AND ITS LOCALIZATION DURING TERMINAL DIFFERENTIATION OF KERATINOCYTES IN HUMAN SKIN

Since granzyme B enzymatically generates active caspase-14 by cleaving procaspase-14 at D146 [Mikolajczyk et al., 2004], we examined immunoreactivity of granzyme B-processed caspase-14 to anti-h14D146. When we incubated recombinant procaspase-14 (30 kDa) with granzyme B, the granzyme B-processed 17 kDa fragment was recognized by anti-h14D146 (Fig. 5a). WEHD hydrolytic activity increased in this preparation, suggesting that cleavage at D146 activates caspase-14, and anti-h14D146 can detect that active form. On the other hand, H99 recognized both the 30 and 17 kDa bands. Next, we tested extracts from human whole skin and cornified cells. While the 30 kDa capsase-14 proform was clearly observed in whole skin extracts (lane 1), the 17 kDa band, which reacted with H99 and anti-h14D146, was detected in the cornified cell extracts (Fig. 5b, lane 2 of right and left panels). We tested the quality of the whole skin extract and cornified cell extracts using antibodies to a basal cell marker, keratin-14, and a granular/cornified cell marker, filaggrin (Fig. 5c). Each extract was found to be rich in its marker protein, respectively. These results suggest that caspase-14 is activated by cleavage at D146 during the final stage of terminal differentiation.

In order to determine the site of caspase-14 activation, we stained human skin with anti-h14D146. Since DNA degradation (loss of nucleus) is an essential step during the terminal differentiation, double staining with TUNEL was also carried out. As shown in Figure 5d, H99-positive caspase-14 mainly localized in a region from the spinous cells to the granular cells in normal human epidermis, as reported previously [Eckhart et al., 2000b]. In contrast, anti-h14D146-reactive caspase-14 was restricted to cornified cells and some granular cells. Cornified layer was heavily stained with anti-h14D146. A small number of TUNEL-positive cells were observed in the granular layer immediately beneath the cornified layer (Fig. 5d, arrows). TUNEL reactivity and anti-h14D146 reactivity was co-localized in granular cells, indicating that DNA fragmentation was occurring in those cells and was associated with caspase-14 activation.

ANALYSIS OF CASPASE ACTIVATION BY UV RADIATION

To determine whether caspase-14 activation in cornified cells and some granular cells is related to cell death, we examined caspase-3 and caspase-14 activation in the skin following UV radiation (Fig. 5). Histological examination showed that sunburn cells with eosin-rich cytoplasm and condensed nuclei appeared 1 day after UV radiation (Fig. 5a). Two days after UV radiation, considerable numbers of sunburn cells were observed in the middle layer of epidermis. Immunohistochemistry demonstrated that anti-h14D146 reactivity was not altered by UV radiation, showing positive staining in the cornified layer (Fig. 5b). On the other hand, immunoreactivity to anti-h3D175 (also called anti-m3D175), an antibody against active human and mouse caspase-3 was observed only in some spinous cells (Fig. 5c). The super-imposed image demonstrated that anti-h3D175-positive cells coincided with sunburn cells, and these cells were TUNEL-positive (Fig. 5c arrows). Caspase-3 activation was not observed during keratinocyte terminal differentiation (Fig. 5d). Thus, in contrast to caspase-3, caspase-14 is not related to UV-induced cell death in vivo in the human epidermis.

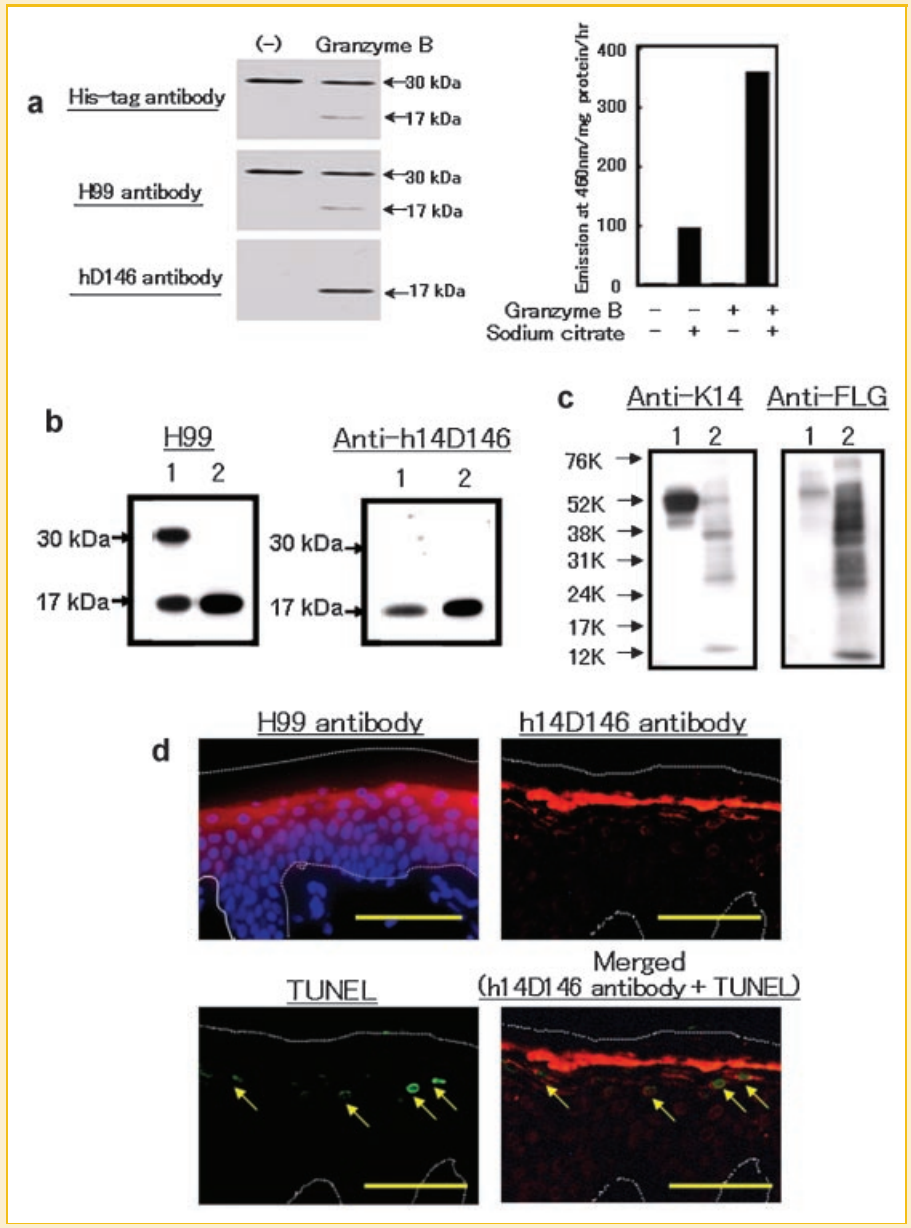

Fig. 5. Caspase-14 processing and localization during keratinocyte terminal differentiation. a: Activation of caspase-14 induced by granzyme B cleavage at D146. Recombinant procaspase-14 was incubated with granzyme B and analyzed with Western blot (left panel). Granzyme B-treated caspase-14 generated a 17 kDa subunit that was recognized by anti-h14D146. Hydrolytic activities of the processed samples were measured using the synthetic substrate, WEHD-MCA (right panel). Granzyme B-treated caspase-14 activity was upregulated in the presence of kosmotropic salt. b: Analysis of the processing fragment of caspase-14 by H99 and h14D146 antibodies. Extracts of whole skin (lane 1), and cornified cells (lane 2) were analyzed for the proform and active form of caspase-14. Ten micrograms of each extract was applied. The H99 antibody recognized both the proenzyme (30 kDa) and the active form (large subunit) of caspase-14. Only the 17 kDa band was detected in the cornified cell extract with the H99 antibody (lane 2), indicating that caspase-14 exists as an active form in the cornified layer. In contrast, anti-h14D146 reacted only with the 17 kDa fragment from each sample. c: Analysis of differentiation marker proteins. Presence of keratin 14 and filaggrin in the samples used in (b) were tested with Western blot. d: Localization of active caspase-14 and TUNEL positive cells. Thin sections of normal human skin were stained with H99 antibody, anti-h14D146, and TUNEL. Immunostaining for H99 was visualized using Texas Red. Dual staining for TUNEL and active caspase-14 were carried out using FITC for TUNEL and Alexa Fluor 555 for anti-h14D146 and is shown as a merged image. Arrows indicate TUNEL-positive cells in the granular layer. All scale bars = 100 μm.

DISCUSSION

Previous reports showed that terminal differentiation of keratinocytes is associated with the processing of caspase-14, which is taken to represent the activation of the caspase family proteinases. In this study we purified active caspase-14 from fully differentiated human keratinocytes and characterized its enzymatic properties. Furthermore we successfully determined the primary structure of the active form using this preparation. Sequence analyses of the purified active form of caspase-14 showed cleavage sites at R5, D146, and I152

(Fig. 3a). Mikolajczyk et al. [2004] demonstrated that caspase-14, which is cleaved at D146 by granzyme B, is enzymatically active in the presence of kosmotropic salt, although granzyme B is not present in epidermis. Thus, it is strongly suggested that cleavage at D146 is involved in the activation of human caspase-14 in vivo and in vitro. In contrast, Chien et al. [2002] reported that caspase-14 immunoprecipitated from foreskin extracts was cleaved at I152. This corresponds to the N-terminal end of the small subunit of the purified caspase-14. Since the linker peptide was very small, consisting of only 6 amino acids, determination of the

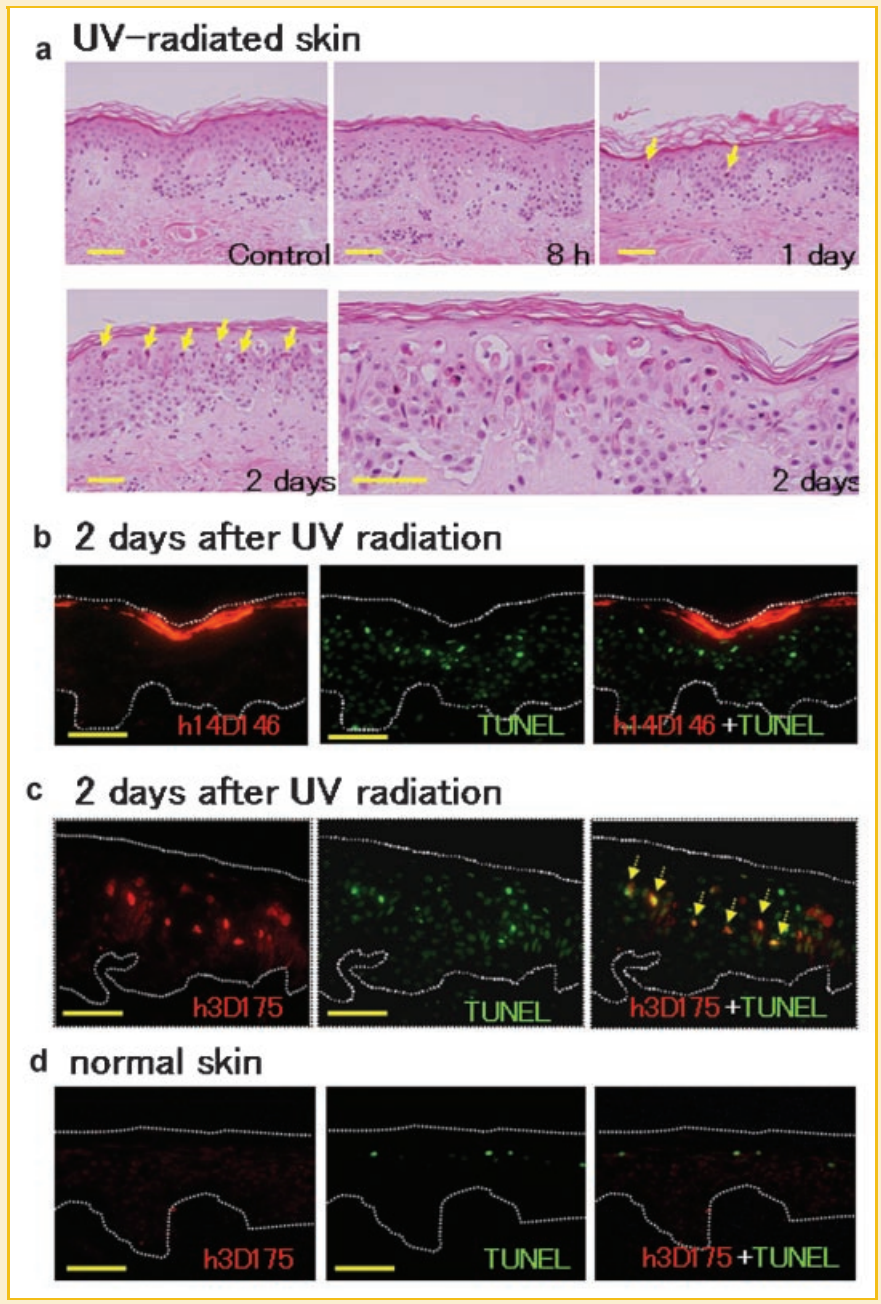

Fig. 6. UV irradiation study. To investigate differences in caspase-14 and caspase-3 activation, we tested the effect of UV irradiation (three MED) on human skin. a: Hematoxylin and eosin (HE) staining of control skin, 8 h, 1 day, and 2 days after UV irradiation. An enlarged image of irradiated skin after 2 days is also shown. Note the appearance of sunburn cells in the middle layer of the epidermis (arrows). b: Localization of active caspase-3, caspase-14, and TUNEL-positive cells in skin with 2 days after UV radiation. Anti-h14D146 showed positive staining mostly in the cornified layer of the 2-day UV-irradiated skin. Significant numbers of TUNEL-positive cells were detected in the middle layer of UV-irradiated skin. A superimposed image demonstrated that the active form of caspase-14 remained unchanged after UV radiation. c: Localization of active caspase-3 and TUNEL-positive cells in skin with 2 days after UV radiation. h3D175 antibody, which recognizes only the active form of caspase-3, showed strong staining in some of the spinous cells of irradiated skin. The superimposed image shows that active caspase-3 co-localized with TUNEL-positive cells (arrows). d: Caspase-3 is not activated in normal epidermis. Although some of the granular cells are TUNEL-positive, activation of caspase-3 is not observed in those cells. All scale bars = 100 μm.

activation-dependent cleavage site would be difficult without obtaining the purified large subunit. To our knowledge, this is the first report to identify the C-terminus of the large subunit of human active caspase-14.

The presence of three distinct cleavage sites (R5, D146, and I152) indicates that activation of caspase-14 requires multiple cleavage steps by multiple enzymes. Many proteinases, including serine, cysteine, and aspartic proteinases are activated during the process of terminal differentiation. Trypsin-like and chymotrypsin-like serine proteinases are suggested to play a role in shedding the outermost cornified cells [Egelrud et al., 1993; Suzuki et al., 1993]. Some of the cysteine proteinases, such as cathepsin B and L are upregulated in differentiated keratinocytes [Tanabe et al., 1991]. Cathespin D, an aspartic proteinase, is suggested to play a role in shedding cornified cells [Horikoshi et al., 1998] and activating transglutaminase [Egberts et al., 2004]. Together, these enzymes may participate in caspase-14 activation.

Based on the sequence of the purified caspase-14, we prepared cleavage site-directed antibody, h14D146. This antibody recognized only the active form of caspase-14, which is confirmed with Western blot, immunocytochemistry and WEHD-MCA hydrolysis. Immunohistochemical analyses using h14D146 antibody clearly revealed that caspase-14 activation occurs at the granular cells during the keratinocyte terminal differentiation. The fact that those cells were also TUNEL-positive may indicate a physiological role of caspase-14 in relation to DNA degradation. Indeed, localization of caspase-14 in the nuclei was demonstrated by other investigators [Eckhart et al., 2000a]. Ultrastructural study also showed that caspase-14 was detected in the nucleus of granular cells and nuclear remnants in the cornified cells [Alibardi et al., 2004].

The requirement of kosmotropic salt may be related to the cellular environment of the granular to cornified layers, causing rapid changes in cell volume and loss of cytoplasmic water during the formation of keratinized cells. Loss of water during terminal differentiation may provide an ideal environment for this enzyme. Furthermore, caspase-3 activation was not observed during keratinocyte terminal differentiation (Fig. 6d). Unlike caspase-3, overexpression of caspase-14 does not induce apoptotic cell death [Pistritto et al., 2002] and it is not activated by various apoptotic stimuli, including UV radiation in the present study (Fig. 6b). Initiator caspases and other caspase family members failed to process procaspase-14, which is in accordance with previous observations [Lippens et al., 2000; Pistritto et al., 2002]. Collectively, our results and those of others indicate that among caspase family members, only caspase-14 is activated according to the differentiation program.

Caspase-14-deficient mice showed impaired filaggrin degradation with the accumulation of smaller degradation products, resulting in reduced skin hydration [Denecker et al., 2007, 2008]. Profilaggrin degradation is complex, multi-step processes. It seemed that filaggrin monomer was produced in the caspase-14-null mice, indicating that mouse caspase-14 plays a role between filaggrin monomer degradation and natural moisturizing factor (NMF) generation [Rawlings and Harding, 2004]. Recent investigation from our laboratory and coworkers identified for the first time that a neutral cysteine protease, bleomycin hydrolase, is responsible for the final stage of filaggrin degradation in order to generate NMF [Kamata et al., 2009]. Caspase-14 was capable to degrade human filaggrin unit, however, bleomycin hydrolase was failed to generate NMF from those degradation products. On the other hand, calpain I-treated degradation products were completely digested to each amino acid by bleomycin hydrolase. It was reported that human filaggrin unit is much longer and shows little homology to the mouse filaggrin [McKinley-Grant et al., 1989]. Further studies are necessary to elucidate the degradation cascade from filaggrin to NMF in the human epidermis.

In conclusion, we purified caspase-14 from human cornified cell extract and determined the primary structure of its active form. Based on this information, we developed an antibody that recognized only the active form of caspase-14. Using this antibody, we clearly showed the site of caspase-14 activation on the human epidermis. The present study reinforces the basis of the caspase-14 research, which will contribute to further understandings for the keratinocyte terminal differentiation.

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