Terminal Differentiation of Human Keratinocytes and Stratum Corneum Formation is Associated with Caspase-14 Activation

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Programmed cell death of epidermal keratinocytes (KC) results in the formation of cornified cells, which constitute the outermost skin layer, the stratum corneum. Here we show by reverse transcription-polymerase chain reaction, western blot, and immunohistochemistry that epidermal KC express caspase-14, a member of the caspase family of pro-apoptotic proteases, in a tissue-specific manner. Caspase-14 protein abundance strongly increases during terminal differentiation of KC *in vivo* and *in vitro*. Under conditions that lead to stratum corneum formation caspase-14 cleavage products, which indicate proenzyme activation, appeared in the KC lysates. Cleavage of the enzyme was also detected in lysates from normal human epidermis and in extracts of stratum corneum. Our findings demonstrate that caspase-14 is activated during KC differentiation and strongly suggest that it is involved in the formation of the human skin barrier. ***Key words: apoptosis/epidermal equivalent/epidermis/skin barrier. J Invest Dermatol 115:1148–1151, 2000***

The principal function of the epidermis is to form a tight barrier, i.e., the stratum corneum, which protects against environmental insults from the outside and loss of water from the inside (Haake and Holbrook, 1999). This barrier consists of tightly interconnected flat polyhedral-shaped cell ghosts, the corneocytes, which are enclosed by a specialized membrane, the cornified envelope, and, under normal conditions, lack nuclei and other cell organelles (Haake and Holbrook, 1999). The formation of corneocytes from epidermal keratinocytes (KC) represents a tissue-specific form of programmed cell death that differs from classical apoptosis because it does not result in fragmentation of the cell into apoptotic bodies and subsequent phagocytosis (Kerr *et al*, 1994); however, the recent finding that a pan-caspase inhibitor prevents cornification indicated that caspases and possibly other elements of the molecular machinery of apoptosis are involved in that process (Weil *et al*, 1999).

Caspases are conserved cysteine-proteases that function as central mediators of apoptosis (Thornberry and Lazebnik, 1998). They are synthesized as catalytically inactive proenzymes composed of an N-terminal prodomain followed by two domains, which form the active subunits after cleavage of the proenzyme. The active enzymes cleave functionally diverse protein substrates containing caspase-specific 4 amino acids motifs that end with an aspartate residue (Wolf and Green, 1999). The list of substrates includes other regulators of apoptosis, structural proteins, and various factors necessary for cell survival (Wolf and Green, 1999).

The latest member of the caspase family, i.e., caspase-14, was recently cloned in the mouse (Ahmad *et al*, 1998; Hu *et al*, 1998; Van de Craen *et al*, 1998). It shares little sequence homology with other caspases and its role in apoptosis has not been clarified yet (Ahmad *et al*, 1998; Hu *et al*, 1998; Van de Craen *et al*, 1998). Caspase-14 expression in several adult organs was reported by Ahmad *et al* but was not confirmed by others (Ahmad *et al*, 1998; Hu *et al*, 1998; Van de Craen *et al*, 1998). The presence of caspase-14 in mouse skin (Van de Craen *et al*, 1998) suggested a role for this enzyme in skin physiology.

Here we investigate caspase-14 expression in humans and provide evidence that caspase-14 is associated with the terminal differentiation of epidermal keratinocytes.

MATERIALS AND METHODS

Cell culture Normal human epidermal KC (NHEK; Clonetics, San Diego, CA) were cultured in KC growth medium (KGM Bullet Kit) provided by the supplier. For analysis of caspase-14 expression, confluent NHEK were cultured in basal medium for 4 d because in preliminary experiments we had observed induction of caspase-14 mRNA and protein under these conditions (data not shown). *In vitro* reconstituted epidermis, i.e., epidermal equivalents (EE) were purchased from MatTek (Ashland, MA). EE consist of several layers of differentiating KC and form a stratum corneum comparable with that of normal human epidermis *in vivo* and can therefore be considered an *in vitro* model for terminal differentiation of KC (Kubilus *et al*, 1996). For the analysis of the time course of caspase-14 processing, EE were either left submerged in culture medium or lifted to the air–liquid interface after onset of stratification.

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Abbreviations: EE, epidermal equivalents; KC, keratinocytes; KGM keratinocyte growth medium; NHEK, normal human epidermal keratinocytes; PBL, peripheral blood leukocytes.

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Samples obtained at different time points were stored frozen on dry ice until further analysis.

Human tissue Epidermis was separated from underlying dermis by dispase II (Boehringer Mannheim, Germany) incubation of split-skin samples derived from mammary reduction plastic surgery. Stratum corneum preparations were obtained from the plantar skin of healthy volunteers by scraping it off the skin surface with a scalpel blade. Care was taken not to wound the underlying epidermis.

Reverse transcription-polymerase chain reaction (RT-PCR) Analysis of caspase-14 expression was performed on cDNA from several human tissues purchased from Origene (Rockville, MD) and Clontech (Palo Alto, CA). RNA from human epidermis and from KC was prepared as described previously (Tschachler *et al*, 1989). RT-PCR were performed according to a published protocol (Ballaun *et al*, 1995). For the amplification of human caspase-14, the primers c14-s1, 5′-ATATGATATGTCAGGTGCCCG-3′ and c14-a1, 5′-CTTTGGTGACACACAGTATTAG-3′ were designed on the basis of stretches of nucleotide sequence homology of cosmid R31973 (Ac004699) with murine caspase-14 cDNA (Van de Craen *et al*, 1998). The caspase-14 specific PCR involved 35 cycles of DNA denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min 15 s. For PCR-amplification of caspase-3, the primers c3-s1, 5′-ATTTGG-AACCAAAGATCATACAT-3′, and c3-a1, 5′-GTTTCTTTCATG-AAAAGTAGC-3′ were used with a thermocycling program identical to that for caspase-14 except for 55°C annealing temperature and 1 min 30 s extension time. Transcripts of glyceraldehyde-3-phosphate dehydrogenase were detected with specific sense (5′-ACCACGTCCATGCCATCAC-3′) and antisense (5′-TCCACCACCCTGTTGCTGTA-3′) primers in 28 cycles. PCR products were electrophoresed through a 1.5% agarose gel containing ethidiumbromide.

Immunostaining Paraffin sections of human epidermis and of EE were immunostained according to a previously published protocol (Weninger *et al*, 1999). In brief, dewaxed sections were boiled in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 500 W for 10 min, cooled to room temperature, and incubated with 10% goat serum (DAKO, Glostrup, Denmark). A rabbit antiserum raised against the entire recombinant murine caspase-14 (Lippens *et al*, 2000) was applied at a dilution of 1:1000 at room temperature for 1 h followed by 30 min incubation with 4 ng per ml Alexa 546-goat anti-rabbit antibody (Molecular Probes, Leiden, Netherlands). Sections were evaluated with a confocal laser microscope (LSM 410, Zeiss, Oberkochen, Germany). For control purposes the anti-caspase-14 antiserum was preincubated with 10 μg per ml recombinant mouse caspase-14 for 1 h at room temperature.

Western blot analysis For western blot analysis all samples were lyzed in SDS-lysis buffer (62.5 mM Tris/HCl, pH 6.8, 6 M urea, 2% SDS, 0.00125% bromophenolblue, 5% β-mercaptoethanol). After ultrasonication and removal of insoluble cell debris, 40 μg of protein were electrophoresed through a 8% to 18% gradient polyacrylamide gel and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were blocked for 3 h in blocking buffer (PBS with 7.5% nonfat dry milk, 2% BSA, 0.1% Tween) and incubated with caspase-14 specific antiserum diluted 1:1000 in blocking buffer overnight at 4°C. Subsequently, membranes were washed in PBS with 5% nonfat dry milk, 0.1% Tween, incubated with peroxidase-conjugated goat antirabbit IgG Fc antibody (Pierce, 1:10000 in blocking buffer) for 1 h at room temperature, washed in PBS, and developed using the ECL chemiluminescent detection system (Amersham). As control the anticaspase-14 antiserum was preincubated with 10 μg per ml recombinant mouse caspase-14 for 1 h at room temperature.

RESULTS

Human caspase-14 is expressed in epidermal keratinocytes PCR screening of cDNA derived from several human tissues revealed that caspase-14 mRNA is expressed at significant levels only in the epidermis and in epidermal KC (**Fig 1**). Weak signals were detected in brain and thymus. By contrast, caspase-3 was detected in all tissues examined (**Fig 1**).

Immunohistochemical analysis of human epidermis using a polyclonal antibody raised against the recombinant murine caspase-14 showed that caspase-14 protein is confined to the differentiated layers of the epidermis (**Fig 2**) and to terminally differentiating

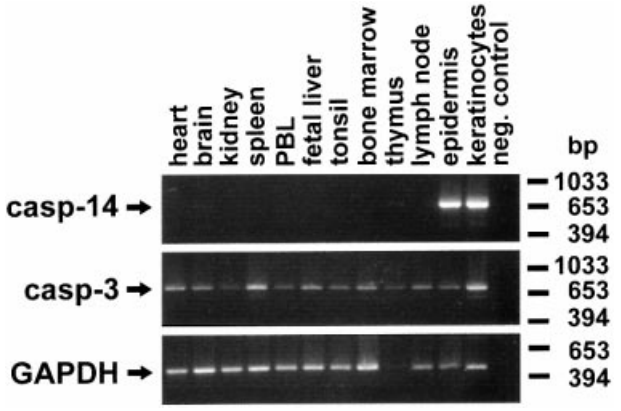

Figure 1. Human epidermis is the major site of caspase-14 mRNA expression. cDNA from human tissues were subjected to PCR with primers specific for caspase-14 (*upper panel*), caspase-3 (*middle panel*), and GAPDH (*lower panel*). *Arrows* indicate the expected size of the respective PCR products. Sizes of a DNA length marker are indicated at the right side. PBL, peripheral blood leukocytes.

epidermal appendages, i.e., hair follicles and sebaceous glands (not shown). In normal epidermis, caspase-14 abundance increased from the first suprabasal layer to the granular layer where it was most strongly expressed (**Fig 2*a***). Positive staining was also observed in the stratum corneum (**Fig 2*a***). A similar expression pattern was detected when we analyzed *in vitro* reconstituted human EE (**Fig 2*b***). The less intensive staining of the stratum corneum of EE probably reflects a lower abundance of caspase-14 in the corneocytes as compared with normal epidermis. Pre-incubation of the antiserum with recombinant caspase-14 to confirm specificity of the immunoreaction resulted in complete abrogation of the staining (**Fig 2*c***, ***d***).

When we analyzed protein lysates from NHEK cultured as a confluent monolayer, caspase-14 proenzyme at 28 kDa (**Fig 3*a***, *third lane*) corresponding to the size predicted from the amino acid sequence (GenBank accession number AF097874) but no cleavage products indicative of enzyme activation were detectable. By contrast, both proenzyme and cleavage products were readily detectable in lysates from human total epidermis (**Fig 3*a***, *first lane*). The size of these caspase-14 fragments matched with the predicted size of the catalytic subunits of about 11 and 17 kDa (**Fig 3*a***, *arrows* at the right margin), respectively, indicating the presence of active caspase-14 in the epidermis. Identical bands were detected in lysates from stratum corneum (**Fig 3*a***, *lane 2*) as well as in EE (**Fig 3*a***, *fourth lane*). The specificity of the reaction was confirmed by preincubating the antiserum with recombinant caspase-14, which leads to abrogation of the immunostaining of both proenzyme and cleavage products (**Fig 3*a***, *last lane*).

To determine the kinetics of caspase-14 activation during epidermal differentiation we monitored the appearance of its cleavage products during the formation of EE. In this system, the exposure of the culture to air is a strong stimulus for the formation of a regular epidermis (Asselineau *et al*, 1985). Therefore we compared EE cultured at the air–liquid interface with EE left submerged in the medium. Under submerged conditions, EE failed to differentiate and did not form a regular stratified epidermis. After 7 d the uppermost KC layers still contained nuclei (**Fig 3*b***, *arrows*). Although high amounts of the caspase-14 proenzyme (28 kDa) were detectable at all time points, only minute amounts of caspase-14 cleavage products could be detected after 7 d (**Fig 3*b***). By contrast, in EE, which were lifted to the air–liquid interface, KC differentiation proceeded to the formation of a well structured stratum corneum devoid of nuclei (**Fig 3*b***, *arrowheads*). In lysates of these EE, subunit bands of caspase-14 demonstrating its activation

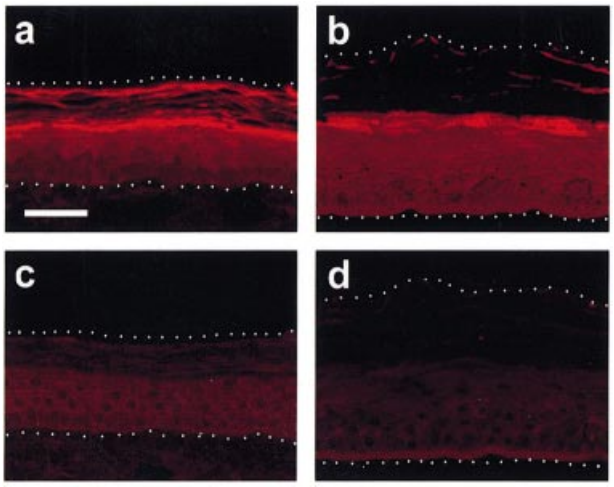

Figure 2. Caspase-14 accumulates in the granular layer and in the stratum corneum of normal and *in vitro* differentiated epidermis. Sections of normal human skin (*a*, *c*) and EE (*b*, *d*) were immunostained with anticaspase-14 antiserum (*a*, *b*). For control purposes the antiserum was preincubated with recombinant caspase-14 (*c*, *d*). The white dotted lines indicate the borders of the epidermis. *Scale bars*: 50 μm.

were present already 2 d after lifting EE to the air–liquid interface (**Fig 3*b***) and strong processing was observed on day 7. The slight decrease of caspase-14 expression on day 4 as compared with day 2 is most likely due to variability between individual samples.

DISCUSSION

The epidermis is a permanently renewing tissue governed by the balance of cell proliferation in the basal layer and cell differentiation resulting in the transformation of KC to corneocytes that form the stratum corneum. This last differentiation step leads to the death of KC and involves the coordinated removal of cell organelles and the cross-linking of cellular proteins (Haake and Holbrook, 1999). Recently it was found that DNA degradation during the transition of KC to corneocytes has features of apoptosis-associated DNA breakdown (McCall and Cohen, 1991; Ishida-Yamamoto *et al*, 1998) and that a caspase inhibitor is able to block the loss of the nuclei during stratum corneum formation *in vitro* (Weil *et al*, 1999). Two protein moieties with caspase-like activity were partially purified from human cornified cell extracts (Takahashi *et al*, 1998), but their molecular identity remained unclear. Together, these observations indicated that components of the apoptosis machinery might participate in the differentiation-associated death of KC. Here we demonstrate that the formation of a regular stratum corneum is associated with expression and activation of caspase-14, an epidermis-specific member of the caspase family.

As we demonstrate by RT-PCR, human caspase-14 is highly abundant in the epidermis but is not expressed in most other organs. Low caspase-14 expression was detectable also in thymus where its presence was confined to Hassall's corpuscles (data not shown) and in the brain where its source remains to be determined. Within human skin, caspase-14 expression was restricted to epidermal KC, hair follicle cells and sebocytes (our unpublished data, not shown; and Lippens *et al*, 2000). As detected by immunohistochemistry, caspase-14 accumulates strongest in the granular layer where the terminal steps of the transformation of KC to corneocytes occur. Confluent culture of primary KC contained large amounts of caspase-14; however, we did not observe activation of the proenzyme. Similarly no activation was observed in EE under conditions that did not result in the development of a regular stratum corneum. These data suggest that accumulation of high proenzyme concentrations is not sufficient to induce autocatalytic processing of caspase-14 but rather that another factor associated with late KC differentiation triggers activation of

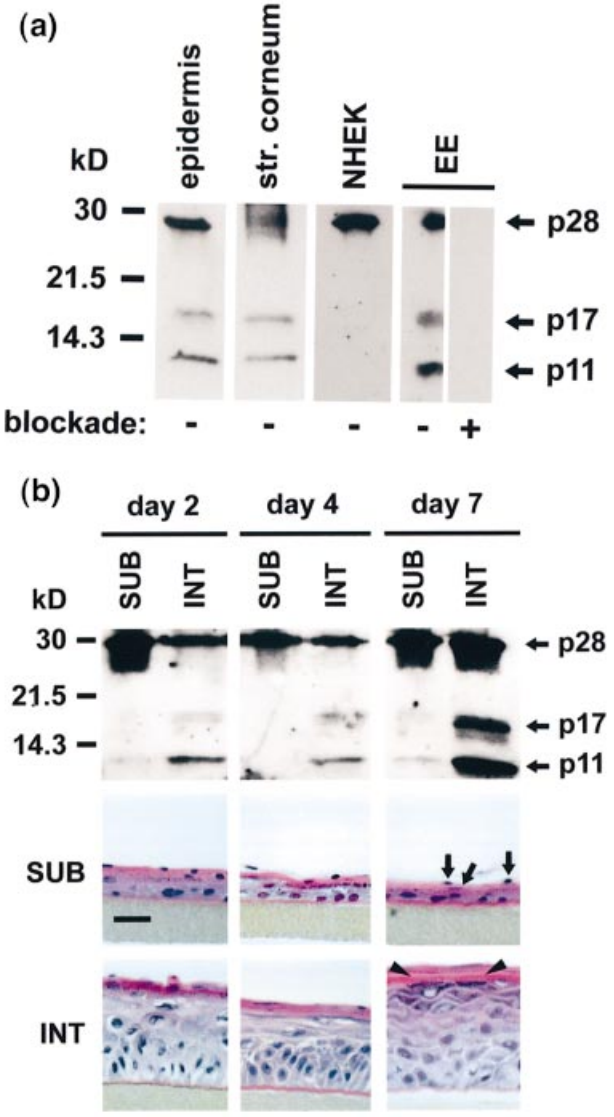

Figure 3. Caspase-14 is cleaved in normal epidermis and in *in vitro* differentiated epidermal equivalents. (*a*) Western blot analysis for caspase-14 was performed with lysates of total human epidermis, isolated stratum corneum, cultured NHEK, and EE. Specificity of the antibody was controlled by preincubation with recombinant caspase-14 (EE, *right lane*). *Arrows* on the right-hand side indicate the size of the proenzyme (28 kDa) and the respective cleavage products at 11 and 17 kDa. (*b*) Kinetics of caspase-14 cleavage were studied by analyzing EE at 2, 4, and 7 d after lifting at the air–liquid interface (INT). In parallel EE maintained submerged in culture medium (SUB) were tested. Western blot analysis for caspase-14 is depicted in the uppermost three panels. *Arrows* on the right-hand side indicate the size of the proenzyme (28 kDa) and the respective cleavage products at 11 kDa and 17 kDa. Morphology of submerged EE (SUB) and EE lifted to the interface (INT) was analyzed at the indicated time points by hematoxylin and eosin staining of paraffin sections. *Arrows* indicate the presence of nuclei in the uppermost epidermal layer of the submerged EE after 7 d. *Arrowheads* indicate the zone of regular stratum corneum in the EE cultured at the air–liquid interface for 7 d. *Scale bar*: 25 μm.

procaspase-14. The fact that caspase-14 contains only a short prodomain suggests that it is a caspase-3-like effector caspase that depends on external activation (Van de Craen *et al*, 1998). *In vitro*, caspase-8 and caspase-10 were the only members of the caspase family capable of caspase-14 processing (Ahmad *et al*, 1998; Van de Craen *et al*, 1998); however, data by Lippens and coworkers (2000) on the lack of caspase-14 cleavage after caspase-8 activation in KC

argue against the relevance of this activation pathway in the epidermis.

The substrates of caspase-14 are currently unknown. Caspase-14 might participate directly or indirectly in the maturation of proforms of KC proteins such as profilaggrin and transglutaminase 1, which are involved in KC cornification (Haake and Holbrook, 1999). Alternatively, the role of caspase-14 might be to induce the degradation of the nuclei during the transition of KC into the stratum corneum. Strikingly, processing of caspase-14 coincided with the removal of nuclei from the corneocytes in the *in vitro* differentiation model. Currently we are screening expression libraries from differentiated KC for interaction partners of caspase-14 in order to identify substrates and/or regulators of enzyme activity.

In conclusion we have demonstrated in this study that human caspase-14 has a restricted tissue distribution and that it is predominantly expressed in the epidermis. Our observation that caspase-14 accumulates in the granular layer together with the findings that caspase-14 is activated during the formation of stratum corneum *in vitro* and that caspase-14 cleavage products are present in normal epidermis and in stratum corneum *in situ* strongly suggest that this enzyme is involved in the terminal differentiation of KC.

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