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Stratum corneum-derived caspase-14 is catalytically active

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Abstract Caspase-14, a cysteine protease with restricted tissue distribution, is highly expressed in differentiated epidermal keratinocytes. Here, we extracted soluble proteins from stratum corneum (SC) of human epidermis and demonstrate that the extract cleaves tetrapeptide caspase substrates. The activity decreased to below 10% when caspase-14 was removed by immunodepletion showing that caspase-14 is the predominant caspase in SC. In contrast to normal SC, where caspase-14 was present exclusively in its processed form, incompletely matured SC of parakeratotic skin from psoriasis and seborrheic dermatitis contained both procaspase-14 and caspase-14 subunits. Fractionation of extract from parakeratotic SC revealed that the peak caspase activity coeluted with processed caspase-14 but not with procaspase-14. Our results suggest that during regular terminal keratinocyte differentiation, endogenous procaspase-14 is converted to caspase-14 subunits that are catalytically active in the outermost layers of normal human skin.

Keywords: Caspase-14; Stratum corneum; Enzyme activity; Keratinocyte; Differentiation

1. Introduction

Caspases are cysteine proteases that cleave target proteins at specific aspartate residues. They are expressed as proenzymes that are activated by dimerization and proteolytic processing to a tetrameric complex consisting of two large and two small subunits. 11 human caspases have been described, i.e., caspases-1 to 10 and caspase-14 (for review see [1]). Caspase-11 and caspase-13 represent the murine and the bovine homologs of human caspase-4 [2,3] and caspase-12 is non-functional in humans because of deleterious mutations in its open reading frame [4,5]. Caspases-2, 3, 6, 7, 8, 9, and 10 have been found to act pro-apoptotically by either transducing a cell death signal in the proteolytic caspase cascade or by cleaving various cellular proteins that are required for survival. By contrast, caspases-1, 4, and 5 are implicated in the processing of proforms of interleukin-1β and interleukin-18, two pro-inflammatory cytokines [1].

Caspase-14, the caspase family member identified most recently [6–8], is unique in its restricted tissue expression pattern. It is expressed in differentiated keratinocytes of the epidermis, in hair follicles and in sebaceous glands [9–13], but only at few sites outside the skin, i.e., the Hassall's corpuscules of the thymus, the choroid plexus of the brain and the pigmented layer of the retina. Expression of caspase-14 is suppressed in human epidermal keratinocytes when terminal differentiation is blocked by the pharmaceutical agent all-*trans* retinoic acid [14]. Although it shares structural features with other caspases, caspase-14 is not activated in classical apoptosis after death receptor stimulation or DNA damage [11]. In contrast, caspase-14 is processed into its subunits during the terminal differentiation of keratinocytes in vitro and in vivo [9,15].

A physiological protein substrate of caspase-14 has not been identified so far and controversial data on activity of caspase-14 have been reported. Whereas recombinant caspase-14 produced in bacteria has been found to process the tetrapeptide substrate DEVD [7], other studies could not detect aspartase activity [8]. Recently, Mikolajczyk and colleagues reported that peptidolytic activity of recombinant caspase-14 can be induced by granzyme B cleavage and incubation in the presence of kosmotropic salts such as sodium citrate that had previously been found to dimerize recombinant prodomain-less procaspases-8 and -9 [16,17]. In the present study, we extracted endogenous caspase-14 from stratum corneum (SC) of human epidermis and show for the first time that it is capable of cleaving tetrapeptide caspase substrates.

2. Materials and methods

2.1. Antibodies

A polyclonal rabbit antibody was raised against recombinant His-tagged human procaspase-14 according to standard protocols. The antibody does not cross-react with other caspases as evidenced by the absence of bands: (i) on Western blots of total protein preparations from HeLa cells, which express caspases-1 to 10 and (ii) on protein preparations from non-differentiated keratinocytes, which express all human caspases except caspase-5 and caspase-14 (not shown). Preab-

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Abbreviations: SC, stratum corneum; SCE, stratum corneum extract; p*I*, isoelectric point; mOD_{405}, milli-units of optical density at 405 nm

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sorption of the antibody with purified recombinant caspase-14 prior to Western blot analysis of skin proteins prevents the appearance of bands at the size predicted for caspase-14 [18]. This confirms that the tissue-derived proteins that are recognized by the antibody are caspase-14 protein species. In some experiments, a polyclonal anti-mouse caspase-14 antibody that cross-reacts with human caspase-14 was used for Western blotting [9,11]. A monoclonal mouse anti-human caspase-14 antibody was purchased from Upstate, Lake Placid, NY. An anti-caspase-1 antibody from Biomol (Plymouth, PA) was used for Western blot analysis.

2.2. SC samples and protein extraction

SC was scraped off from the heels of healthy volunteers ($n = 3$) with a scalpel blade. SC scale material was collected from different psoriasis patients ($n = 6$) and from a patient with seborrheic dermatitis ($n = 1$) after obtaining their informed consent. Soluble proteins were extracted with phosphate-buffered saline (PBS). 10 mg SC was incubated with 300 μl buffer on a rotary mixer at 4 °C for 1 h. After vortexing, insoluble components were removed by centrifugation in a table centrifuge at 15 000 rpm for 10 min. The final concentration of total proteins was approximately 0.8 mg/ml. Alternatively, proteins were extracted by incubation in a buffer containing 1% SDS and 50 mM Tris, pH 7.5, at 95 °C for 10 min.

2.3. Anion exchange chromatography

Parakeratotic SC from a patient with seborrheic dermatitis was extracted with a buffer containing 20 mM Tris–HCl, pH 8.0, and 1 mM EDTA. After filtration through a 0.45 μm filter, the extract was loaded on a HiTrap QFF anion exchange column with a bed volume of 1 ml that had been equilibrated with the extraction buffer. Protein elution was achieved with a 0–1 M NaCl gradient. Fractions of 1 ml were collected and analyzed further.

2.4. Immunochemical analyses

For immunohistochemical analysis, SC squames were scraped off from the skin with a scalpel and embedded in cryomatrix (Thermo-Shandon, Pittsburgh, PA). Immunohistochemistry was performed on cryosections that were fixed with acetone:ethanol (1:1) using the indirect immunoperoxidase staining technique as described [19]. Endogenous peroxidase was blocked with methanol/H_2O_2. The sections were stained with a mouse monoclonal anti-human caspase-14 antibody (Upstate) diluted 1:2000 in Tris-buffered saline, pH 7.5, plus 2% BSA overnight at 4 °C. After washing, slides were incubated with biotinylated sheep anti-mouse IgG (1:10 000) and exposed to streptavidin biotin complex obtained from DAKO (Glostrup, Denmark). 3-Amino-9-ethylcarbazole was used as chromogen. The slides were counterstained with hematoxylin. The specificity of the staining was confirmed by preabsorption of the first step antibody with recombinant caspase-14, which blocked the staining. Western blot analysis was performed according to a previously described protocol [9].

2.5. Immunodepletion

10 μl of the polyclonal antibody against human procaspase-14, 30 μl Ultralink Immobilized Protein A Plus beads (Pierce, Rockford, IL) and 300 μl PBS were incubated on a rotary mixer at 4 °C for 2 h. Subsequently, the beads were washed with PBS and incubated with 300 μl stratum corneum extract (SCE) (1 mg SC/100 μl) at 4 °C for 2 h. After centrifugation, both the supernatant and the material bound to the beads were analyzed further. For all experiments, control reactions were performed with pre-immune rabbit serum instead of the anti-caspase-14 antiserum.

2.6. Caspase activity assay

The catalytic activity of SCEs was determined in an assay system similar to the one described by Boatright and colleagues [17] for recombinant caspases-8 and -9 with modifications. In brief, 30 μl PBS-extract from SC was added to microtiter plate wells containing 70 μl reaction mixture so that the final concentrations were 1.3 M sodium citrate pH 7.0, 5 mM dithiothreitol, and 100 or 200 μM tetrapeptide-*p*-nitroaniline (pNA). Then, the plate was incubated at 37 °C and the absorbance at 405 nm was measured. DEVD-pNA, IETD-pNA, WEHD-pNA, and YVAD-pNA were purchased from Apotech, Vienna, Austria. zVAD-fmk was obtained from Alexis Corp., Vienna, Austria.

3. Results

3.1. Processing of caspase-14 is complete in normal SC but not in parakeratotic SC

Since SC is the most superficial layer of the epidermis and contains the subunits of caspase-14 [9], it is an ideal source for endogenous caspase-14. SC was collected from the heels of healthy volunteers, from psoriatic lesions and from affected skin of a patient with seborrheic dermatitis. Immunohistochemical analysis of SC squames revealed the presence of caspase-14 in all samples (Fig. 1A–C). The staining was inhomogeneous, which may reflect either uneven distribution of caspase-14 throughout SC or a partial loss of the antigen during the immunohistochemical procedure. Hematoxylin staining revealed that in contrast to normal orthokeratotic SC (Fig. 1A), SC from psoriatic skin and from seborrheic dermatitis skin was parakeratotic, i.e., it contained nuclei (Fig. 1B, C). Protein extraction with PBS from SC of normal skin yielded the caspase-14 subunits p17 and p11 but not procaspase-14 as demonstrated by Western blotting (Fig. 1D, lane 1). In contrast, significant amounts of procaspase-14 together with caspase-14 subunits were present in parakeratotic SC (Fig. 1D, lanes 2 and 3). Essentially, the same results were obtained when instead of PBS an extraction buffer containing SDS was used (Fig. 1D, lanes 4–6). These results indicate that during normal terminal keratinocyte differentiation in healthy skin, the entire pool of procaspase-14 of keratinocytes is converted to caspase-14 subunits, whereas incomplete differentiation manifesting as parakeratosis leads only to partial processing of procaspase-14.

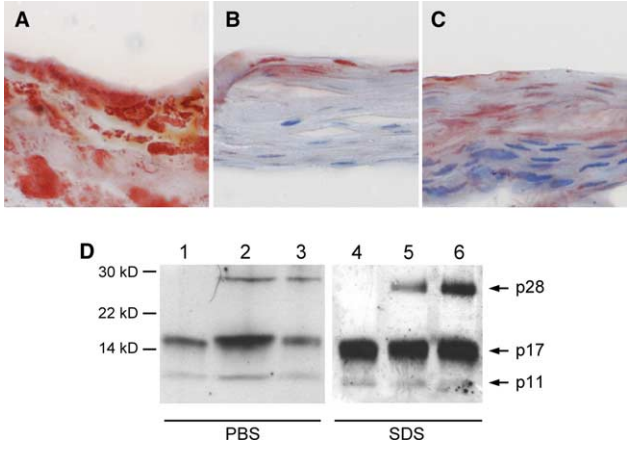

Fig. 1. SC of normal skin contains only the subunits of caspase-14, whereas parakeratotic SC also contains unprocessed procaspase-14. Stratum corneum (SC) squames from the heels of a healthy individual (A), from psoriatic plaques (B), and from a patient with seborrheic dermatitis (C) were immunostained for caspase-14 (red). Nuclear counterstaining was done with hematoxylin (blue). Absence of nuclei established normal differentiation (orthokeratosis) in plantar SC (A), whereas retention of nuclei indicated incomplete cell differentiation (parakeratosis) (B,C). Original magnifications, 1000×. Caspase-14 protein species were determined by Western blot analysis of SC squames extracted with PBS (D, left panel) or SDS-buffer (D, right panel). Bands corresponding to procaspase-14 as well as caspase-14 subunits p17 and p11 are indicated by arrows on the right. Lanes 1 and 4, normal SC; lanes 2 and 5, psoriatic SC; lanes 3 and 6, seborrheic dermatitis SC.

3.2. SC-derived caspase-14 is an active caspase

To test for catalytic activity of epidermal caspase-14, we extracted SC of normal human skin with PBS and incubated the extract with the synthetic tetrapeptide substrate WEHD-pNA under various assay conditions. In standard caspase activity buffers, minimal to undetectable catalytic activity was observed, indicating that conventional caspases with high activity towards WEHD were not present in significant amounts in the extract (not shown). Western blot analysis for caspase-1, i.e., the caspase with the highest affinity for WEHD [20], confirmed that it was absent from the extract (not shown). However, when a high concentration of sodium citrate was used, strong caspase activity was detected (Fig. 2A). The reaction was completely blocked by addition of the pan-caspase inhibitor zVAD-fmk (10 μM), confirming the specificity of a caspase-mediated cleavage (Fig. 2A). To test whether this activity was attributable to caspase-14, we removed caspase-14 from the extract by immunoprecipitation and analyzed the residual WEHDase activity. Immunodepletion of caspase-14 virtually abolished WEHDase activity (Fig. 2A), whereas a control reaction performed with immunobeads coated with pre-immune serum instead of anti-caspase-14 serum did not decrease the activity (Fig. 2A). Western blot analysis showed that the untreated SCE contained the same amount of the caspase-14 p17 subunit as the supernatant of the control depletion, whereas the supernatant of the caspase-14 immunodepletion contained only minimal amounts thereof (Fig. 2B). Accordingly, caspase-14 protein was found on beads coated with anti-caspase-14 serum but not on control beads (Fig. 2B). The proteins that bound to beads coated with anti-caspase-14 antibody displayed significant WEHDase activity, whereas proteins adhering to the control beads were catalytically inactive (not shown). In addition to WEHD-pNA, the extract was also active against IETD-pNA and YVAD-pNA, whereas only minimal activity against DEVD-pNA was observed (Table 1). IETDase and YVADase could be removed by caspase-14 depletion, whereas the weak DEVDase activity remained unchanged. These results show for the first time that endogenous caspase-14 present in SC is catalytically active. Furthermore, the fact that most of the caspase activity could be removed by immunodepletion of caspase-14 suggests that caspase-14 is the predominant active caspase in normal SC.

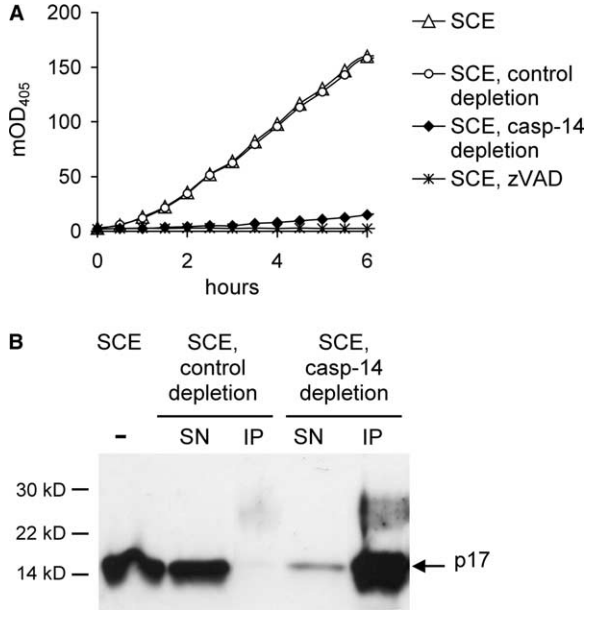

Fig. 2. Caspase-14 extracted from SC is catalytically active. SCE was prepared from plantar skin by incubation of SC squames with PBS. Part of SCE was subjected to immunoprecipitation with anti-caspase-14 serum (casp-14 depletion) or with preimmune control serum (control depletion). Untreated SCE, immunodepleted SCE and SCE preincubated with the pan-caspase inhibitor zVAD-fmk (10 μM) were analyzed for caspase activity using the colorimetric tetrapeptide substrate WEHD-pNA under kosmotropic conditions (A). Cleavage of the substrate was determined by milli-units of optical density at 405 nm (mOD_{405}). The efficiency of immunodepletion of caspase-14 was evaluated by Western blot analysis with a monoclonal antibody that reacts with procaspase-14 and with the large subunit of caspase-14 (p17) (B). Untreated SCE as well as the supernatants (SN) of the immunobeads and the proteins bound to the immunobeads (IP) were analyzed. The position of the p17 subunit of caspase-14 is indicated by an arrow. Procaspase-14 was not present.

Table 1
Substrate specificity of endogenous caspase-14

Substrate	SCE (aU)	SCE–C14 (aU)	Activity depleted (aU)	Depletion (%)
WEHD	137 ± 7	13 ± 1	124	91
IETD	22 ± 1	2 ± 1	20	91
YVAD	17 ± 0	4 ± 0	13	76
DEVD	5 ± 4	6 ± 4	0	0

SCE was analyzed for cleavage activity against the tetrapeptide substrates, WEHD-pNA, IETD-pNA, YVAD-pNA, and DEVD-pNA, as described in Section 2. For the determination of the contribution of caspase-14 to the observed activities, SCE was immunodepleted of caspase-14 prior to the activity assay (SCE-C14). The catalytic activity was determined by measuring mOD_{405} after 6 h incubation with each substrate (aU, arbitrary units). The activity depleted by removal of caspase-14 was calculated as absolute value and as percentage of the activity of untreated SCE.

3.3. In parakeratotic SC, caspase-14 activity is associated with processed caspase-14 subunits but not with procaspase-14

Next, we investigated the composition and activity of caspase-14 complexes in tissue samples that contain both processed and unprocessed caspase-14. An extract from parakeratotic SC was separated by anion exchange chromatography and the fractions were analyzed for the presence of caspase-14 protein species and WEHDase activity. As shown in Fig. 3, procaspase-14 was eluted at lower salt concentrations than the subunits of caspase-14. Significant signals of the p28 band (procaspase-14) were detected in fractions 29–35, whereas the p17 band (large subunit) was present in fractions 31 through 37. Despite the big difference in isoelectric points (p*I*) of the large (p*I* 4.78) and the small subunit (p*I* 9.13) of caspase-14, they were eluted in the same fractions of anion exchange chromatography. This indicated that the subunits are present as a complex in SCEs. WEHDase activity was analyzed under kosmotropic conditions and correlated strictly with the intensity of the caspase-14 p17 band (Fig. 3), whereas it did not correlate with the amount of procaspase-14. This activity profile was apparently not influenced by other caspases, since there was no WEHDase activity in the fractions containing caspase-14 when standard caspase buffers were used (not shown). Furthermore, caspase-1 was not detected by Western blotting (not shown). Our data therefore suggest that the catalytic activity of processed caspase-14 is much higher than that of procaspase-14.

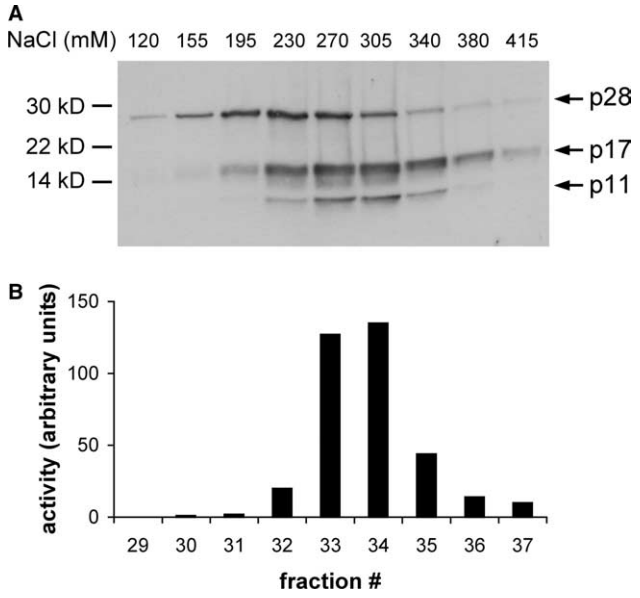

Fig. 3. Caspase-14 activity is associated with processed caspase-14 subunits but not with procaspase-14. Parakeratotic SC was extracted with Tris-EDTA buffer and fractionated by anion exchange chromatography as described in "Materials and Methods". The concentration of sodium chloride corresponding to the eluted fractions is shown at the top. Aliquots were subjected to SDS-PAGE and immunoblotted with a polyclonal anti-caspase-14 antibody that reacts with procaspase-14 and with the subunits, p17 and p11 (A). WEHDase activity of the fractions was measured as described in Fig. 2A (B). Arbitrary activity units shown here are defined as mOD_{405} after 1 hour incubation with WEHD-pNA under kosmotropic conditions.

4. Discussion

The present investigations were based on our observation that endogenous caspase-14 can be extracted from SC by incubation with detergent-free buffer. This suggests that caspase-14 subunits either are released from the cornified keratinocyte remnants (corneocytes) during the extraction process or are present in the intercorneocyte space. Previously, it was shown that procaspase-14 remains uncleaved as long as SC formation is not induced [9,11]. However, it is presently unclear whether procaspase-14 is processed in differentiating keratinocytes prior to the transition into the SC or only thereafter, i.e., within SC. Our finding that all caspase-14 is processed in orthokeratotic SC whereas a fraction of procaspase-14 remains unprocessed in SC containing parakeratotic areas suggests that incomplete caspase-14 activation is associated with diseased skin phenotypes. Intriguingly, a recent study shows that a Vitamin D_3 analog that is used for treatment of psoriasis is able to enhance caspase-14 processing in an in vitro skin model [21]. Whether the potential effect of psoriasis therapies on caspase-14 processing correlates with treatment efficiency remains to be determined.

The observation of catalytic activity in SCEs containing only cleaved caspase-14 and the correlation of caspase activity with the amount of caspase-14 subunits but not with the amount of procaspase-14 in chromatography fractions indicate that the subunits of caspase-14 are catalytically active, whereas the proenzyme has weak or no activity. In addition, our data strongly argue against the hypothesis that the physiological form of activated caspase-14 is a complex of procaspase-14 and the subunits, as proposed by Chien and colleagues [15]. Whereas the proenzyme and the large subunit of caspase-14 could be separated partially by anion exchange chromatography, the large and the small subunit coeluted under the same conditions. This indicates that the subunits are present in the form of a complex in SCEs.

Enzymatic activity of SC-derived caspase-14 was only observed at a high concentration of citrate. Since equal concentrations of NaCl did not induce activity (not shown), this effect does not seem to be mediated by high salt concentrations in general but rather by the kosmotropic properties of citrate. Kosmotropes, a term coined by Washabaugh and Collins for "water structure making" ions [22], bind water molecules in aqueous solutions more effectively than proteins, thereby establishing conditions that favor interactions between protein domains over interactions between protein and water [22]. Conditions with comparable impact on enzyme activity may be present in SC. The water content of SC is much lower than in other skin compartments and decreases strongly from the inner to the outer layers where it reaches 15 to 25% [23,24]. Furthermore, various ions including citrate are present at elevated concentrations in SC as compared to other tissues [25]. The conditions in aqueous microdomains of SC [26], where hydrolytic enzyme reactions are likely to take place, are largely unknown at present. Further studies will be necessary to determine which particular parameters or cofactors present in skin facilitate caspase-14 activity in vivo.

Our data regarding requirement for kosmotropic reaction conditions and substrate preference for WEHD are in accordance with results recently reported for recombinant caspase-14 [16]. Therefore, recombinant caspase-14 appears to be a valid model for the investigation of caspase-14 activity, even when it is not processed by its endogenous activator, which is currently unknown, but by granzyme B at an unphysiological site between the subunits [16].

In conclusion, the present study demonstrates that caspase-14 is present in its active form in SC and that it is the major, if not only, caspase active at this site. As to the biological function of this enzyme, our data indicate that the search for potential substrates of caspase-14 should be focused on proteins of the outermost skin layers. Our demonstration that large quantities of active caspase-14 can be obtained easily by non-invasive procedures from SC provides a good starting point for further studies.

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