Caspase-14 Expression by Epidermal Keratinocytes is Regulated by Retinoids in a Differentiation-associated Manner

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Caspase-14 is the only member of the caspase family that shows a restricted tissue expression. It is mainly confined to epidermal keratinocytes and in contrast to other caspases, is not activated during apoptosis induced by ultraviolet irradiation or cytotoxic substances. As it is cleaved under conditions leading to terminal differentiation of keratinocytes we suggested that caspase-14 plays a part in the physiologic cell death of keratinocytes leading to skin barrier formation. Here we show that retinoic acid, at concentrations inhibiting terminal differentiation of keratinocytes, strongly suppressed caspase-14 mRNA and protein expression by keratinocytes in monolayer culture and in a three-dimensional in vitro model of differentiating human epidermis (skin equivalent). By contrast, the expression of the caspases 3 and 8, which are both activated during conventional apoptosis, was increased and unchanged, respectively, after retinoic acid treatment. In addition to inhibition of differentiation in skin equivalents, retinoic acid treatment led to keratinocyte apoptosis and activation of caspase-3, both of which were undetectable in differentiated control skin equivalents. As this occurred in the absence of detectable caspase-14, our data demonstrate that caspase-14 is dispensable for keratinocyte apoptosis. The fact that in contrast to caspase-3 and caspase-8, caspase-14, similarly to other keratinocyte differentiation-associated proteins, is downregulated by retinoids, strongly suggests that this caspase, but not caspase-3 and -8, plays a part in terminal keratinocyte differentiation and skin barrier formation. Key words: apoptosis/differentiation/epidermis/skin. J Invest Dermatol 119:1150–1155, 2002

During their turnover from the basal to the uppermost epidermal layers primary keratinocytes (KC) differentiate into corneocytes, thereby forming the tight barrier that protects the organism against transepidermal water loss and from external damaging influences (Haake and Holbrook, 1999; Latkowski and Freedberg, 1999). During the transition to corneocytes, commonly referred to as terminal differentiation, KC die in a tightly controlled fashion (for review Gandarillas, 2000; Melino et al, 1998). This type of cell death is in some aspects, such as the loss of the nuclei, reminiscent of programmed cell death in other cells; however, the fact that the end-point of this process is not complete disintegration of cells, but formation of cellular ghosts, that are resistant to biochemical and biophysical degradation, i.e., corneocytes, sets it apart from conventional apoptosis, which aims at the complete removal of cells without leaving a trace.

Caspases are the key enzymes of apoptosis. They cleave their substrates specifically at 4 amino-acid motifs leading to the inactivation and degradation of a large number of regulatory and structural proteins (Chang and Yang, 2000). In addition, caspases can cleave and activate each other thereby regulating and amplifying apoptotic signals. Caspase activity was detected in epidermal preparations (Takahashi et al, 1998) and activation of caspase-3 was localized to cells at the transition zone between the granular and cornified layers of the epidermis (Weil et al, 1999) and related to the formation of the skin barrier. Recently, we and others have reported that caspase-14, the latest member of the caspase family (Ahmad et al, 1998; Hu et al, 1998; Van de Craen et al, 1998) is almost exclusively expressed in the epidermis (Eckhart et al, 2000a; Lippens et al, 2000). We could show that the activation of human caspase-14 occurs during the terminal differentiation of KC in vitro (Eckhart et al, 2000) and we have suggested that it plays a part in the terminal differentiation of KC but not in conventional apoptosis.

To expand our knowledge of the role of caspases in the terminal differentiation of KC we have analyzed here the regulation of caspase-14, -3, and -8 expression by retinoids, which are potent modulators of KC differentiation (Fisher and Voorhees, 1996). Our finding that caspase-14, but not caspase-3 and -8, is regulated by retinoids in a differentiation-dependent fashion, supports the role of caspase-14 in the terminal differentiation of KC and sets it further apart from the classical pathways of apoptosis.

MATERIALS AND METHODS

Cell culture Human neonatal foreskin-derived KC were obtained from Clonetics (San Diego, CA) and cultured as described by the manufacturer.
under low Ca\(^{2+}\) conditions (0.15 mM) in serum-free keratinocyte growth medium (KGM), i.e., modified MCDB 153 medium containing 0.1 ng human recombinant epidermal growth factor per ml, 5 µg insulin per ml, 0.5 µg hydrocortisone per ml, 0.4% bovine pituitary extract, 50 µg gentamicin per ml, and 30 ng amphotericin B per ml. Second to fourth passage KC were used for all experiments. For differentiation-promoting confluence assays KC were seeded at low density in six-well plates, grown to confluence (designated as "day 0") and cultured for up to 5 d postconfluence (designated as "day 5"). Retinoids or dimethyl sulfoxide were added to fresh KGM at indicated days and medium changes were performed daily.

Human dermal fibroblasts were obtained by explant cultures of de-epidermized skin specimen derived from routine breast reduction surgery. Fibroblasts were subcultured in Dulbecco's Modified Eagle medium ( Gibco, BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and were used between passages 3 and 10.

Skin equivalents In vitro reconstituted skin equivalents were generated essentially as described by Stark et al. (1999) with modifications. Briefly, 2.5 ml of a collagen solution containing 2.4 mg/ml bovine collagen type I (Vitrogen 100, Collagen Corporation, CA), 1 x Hank's buffered saline solution (Gibco), 10% fetal bovine serum and 1 x 10^5 fibroblasts per ml, adjusted to pH 7.4 with 2 M NaOH, were poured into 24 mm cell culture inserts. After gelation at 37°C in a humidified atmosphere for 2 h in the absence of CO\(_2\), gels were equilibrated in 16 ml prewarmed KGM (2 ml inside, 14 ml outside the insert) for 4 h at 37°C in a 5% CO\(_2\)/95% air environment for a humidified incubator. The medium inside the insert was then removed and replaced by 2 ml KGM containing 3 x 10^5 KC per cm\(^2\) (1 x 3 x 10^6 cells). After submerged culture overnight, the medium was changed to 10 ml serum-free keratinocyte defined medium (SKDM) outside the insert, and the cultures were kept at the air–liquid interface from that time onward. SKDM (1 ml) containing 0.1% bovine serum albumin (Sigma, St Louis, MO) and 20 µg L-ascorbic acid per ml. Culture medium for skin equivalents was replaced by fresh prewarmed SKDM every 2–3 d and culture was continued for up to 6 d. At indicated days samples were taken by punch biopsy, frozen and processed for either hematoxylin and eosin (H&E) staining or immuno-fluorescence. Simultaneously, the detection of apoptosis was confirmed by terminal deoxy nucleotidy l transferase-mediated deoxyuridine triphosphate nick end-labeling with the "in situ cell death detection kit" (Boehringer Mannheim, Vienna, Austria) according to the manufacturer's instructions.

**Western blot analysis** For Western blot analysis all samples were lysed in sodium dodecyl sulfate lysis buffer (62.5 mM Tris–HCl, pH 6.8, 6 M urea, 2% sodium dodecyl sulfate, 0.01% (w/v) bromophenol blue, 5% (v/v) mercaptoethanol). After ultrasonication and removal of insoluble cell debris, 40 µg of protein were electrophoresed through an 8–18% gradient polyacrylamide gel and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were blocked for 2 h in blocking buffer (phosphate-buffered saline with 7.5% nonfat dry milk, 2% bovine serum albumin, 0.1% Tween) and incubated with caspase-14 anti-serum (1: 1000) (Lippens et al., 2000), caspase-8 anti-serum (1: 1000, kind gift from Peter Krammer (Scaffidi et al., 1997), caspase-3 (1: 100) (Pharmingen, San Diego, CA), and filaggrin (1: 200, Biomedical Technologies) antibodies in blocking buffer overnight at 4°C. Subsequently, membranes were washed in phosphate-buffered saline with 5% nonfat dry milk, 0.1% Tween, incubated with peroxidase-conjugated goat anti-rabbit IgG Fc antibody (Pierce, Rockford, IL 1: 10000 in blocking buffer) for 1 h at room temperature, washed in phosphate-buffered saline and developed using the ECL chemiluminescence detection system (Amersham Buckinghamshire, UK).

**Northern blot analysis** Northern blot analysis was performed as previously described (Eckhart et al., 2000b). The probe for caspase-14 was generated by 32P-deoxycytidine triphosphate random labeling of the polymerase chain reaction product amplified from human KC DNA with the primers 5'-ATAGGTCAGGTCCGCGTCTGGTGGTGGGGCGCCCCAGGCACCAGGGC-3' (sense) and 5'-CCTTTGTTGACACACAGCTTAGTACCAGCT3' (anti-sense)

- RESULTS

When primary KC were allowed to differentiate by culturing them at confluence (Lee et al., 1998), expression of the differentiation-associated marker profilaggrin was induced and increased substantially over the following days (Fig 1A). The addition of 10^-6 M RA, a potent inhibitor of KC differentiation (Fisher and Voorhees, 1996), nearly completely inhibited this induction (Fig 1A) (Asselineau and Darmon, 1995). Similarly, the induction of pro-caspase-14 (p28), which as we have recently shown is induced in differentiating conditions (Eckhart et al., 2000b), was inhibited by RA treatment (Fig 1A). In contrast to caspase-14 protein levels, which after RA treatment stayed steady throughout the culture period, caspase-14 mRNA was reduced by RA treatment to nearly undetectable levels within the first 24 h, indicating its downregulation at the transcriptional level (Fig 1A). Treatment with different concentrations of RA demonstrated the concentration dependence of the effect on pro-caspase-14 protein expression (Fig 1B). Similar inhibition of pro-caspase-14 expression was observed with retinol (not shown), the physiologic precursor that is converted to active RA after uptake into cells (Fisher and Voorhees, 1996), as well as with the all-trans RA isomers 13-cis RA and 9-cis RA (not shown).

In analogy to the absence of profilaggrin processing (Fig 1A), no cleavage of pro-caspase-14 was detected in confluent KC cultures (Fig 1A) (Eckhart et al., 2000b). This is most likely due to the fact that in submersed cultures KC differentiate only incompletely (Asselineau et al., 1989). In contrast to KC in monolayer cultures, KC in skin equivalents (skin equivalents) form a stratified, regularly differentiated epidermis under the stimulus of air exposure (Stark et al., 1999). Under these conditions profilaggrin is processed (Asselineau and Darmon, 1995) and also pro-caspase-14 must be activated, as cleavage products become detectable (Fig 2A). In skin equivalents retinoids prevent regular epidermal differentiation (Asselineau and Darmon, 1995). We could confirm that RA treatment of skin equivalents differentiated in a manner similar to that of profilaggrin induction and processing (Fig 2A), and we found that the expression of pro-caspase-14 (p28) was strongly reduced by RA treatment (Fig 2A) without the appearance of cleavage products, which would be indicative of its activation. Analogous to KC in monolayer culture,
Northern blot analysis revealed a strong inhibition of caspase-14 mRNA expression (Fig 2).

To understand better the regulation of caspase-14 expression and its temporal and spatial association with the inhibition of KC differentiation by RA, we studied skin equivalents by H&E staining and immunofluorescence analysis of the in situ expression of caspase-14 and KC differentiation markers. At 4 and 6 d of culture, control skin equivalents showed all features of regular epidermal differentiation (Fig 3a,c), including strong expression of filaggrin (Fig 3k) and caspase-14 (Fig 3g). Treatment with $10^{-6}$ M RA for 4 d prevented the formation of both granular and cornified KC layers (Fig 3b). After 6 d, epidermal thickness had strongly increased, stratification was disturbed, KC appeared to lose their orientation, and no cornified layer was formed (Fig 3d). Both caspase-14 and filaggrin expression were strongly reduced after 4 d (Fig 3j) and only found in scattered cells after 6 d (Fig 3l). The possibility that filaggrin and caspase-14 downregulation was due to an overall reduction of protein synthesis was excluded by the demonstration that de novo expression of the "wet epithelial" keratin 19 was strongly induced by RA treatment (Asselineau and Darmon, 1995).

As activation of caspase-3 has been suggested to be involved in the transition of viable KC into corneocytes (Weil et al., 1999) and caspase-8 has been shown to associate with caspase-14 (Hu et al., 1998) we also analyzed the expression, regulation, and activation of these caspases during differentiation and its inhibition by RA. In KC monolayer culture we found that, in contrast to caspase-14, both caspase-3 and -8 were strongly expressed at the time of culture initiation and that the expression levels during confluence slightly decreased and did not change, respectively (Fig 4a). As opposed to the potent downregulation of caspase-14 by RA treatment, no regulation of caspase-8 was observed and caspase-3 levels slightly increased after addition of RA (Fig 4a). The results on caspase-3 and -8 expression obtained in monolayer culture
were comparable with those found in skin equivalents: both caspases were strongly expressed and caspase-3 pro-enzyme levels were strongly increased by RA treatment, whereas those of caspase-8 remained unchanged (Fig 4b). In contrast to the activation of caspase-14 in regularly differentiated skin equivalents, no cleavage of caspase-3 or -8 could be detected (Fig 4b) under control conditions; however, RA treatment of skin equivalents for 6 d, led to caspase-3 activation as determined by the occurrence of the 17 kDa large subunit in western blot analysis, indicative of ongoing apoptosis (d, arrowheads). Simultaneously, as compared with untreated control samples (e,g), caspase-14 expression was nearly completely inhibited after 4 d (f, arrows), and after 6 d only scattered KC stained weakly positive (h, arrows). Downregulation of the differentiation marker filaggrin served as a control for the inhibitory effects of RA treatment on epidermal differentiation (i-l). By contrast, keratin 19, characteristic for “wet epithelia”, was expressed after 4 d (n) and 6 d (p) of RA treatment, and was absent in control samples (m,o). Bars = 50 μm.

**DISCUSSION**

The late stages of epidermal differentiation comprise several well defined changes such as the reorganization of the cytoskeleton, the appearance of granules containing cornified envelope precursor proteins, the assembly of the cornified envelope, and the degradation of the nucleus (Haake and Holbrook, 1999; Latkowski and Friedberg, 1999). In recent years many investigators have sought to clarify the relationship between KC death occurring during these final steps of epidermal differentiation and conventional apoptosis (Gandarillas, 2000). Depending on the methods
Figure 4. Caspase-3 and -8 are not regulated in a differentiation dependent manner in primary KC and differentiating skin equivalents. Primary KC were differentiated by culturing at confluence (a) or in stratified skin equivalents (b) and differentiation was inhibited by addition of RA. Expression of caspase-3 and -8 was analyzed by Western blot. (a) Unlike caspase-14, both caspase-3 and -8 expression were neither up-regulated during differentiation nor inhibited by RA treatment. Rather, caspase-3 pro-enzyme levels slightly decreased after extended culture at confluence and slightly increased by additional RA treatment. Expression levels of pro-caspase-8 were unchanged under any conditions tested. (b) No differentiation-associated regulation of caspase-3 and -8 by RA treatment was detectable. Moreover, no cleavage cloud be detected in regularly differentiated SE, indicating its dispensability for regular epidermal differentiation. However, caspase-3 activation, as demonstrated by the appearance of the cleaved 17 kDa large subunit, was readily detectable after RA treatment.

Figure 5. Apoptosis of KC in skin equivalents after RA-mediated abrogation of differentiation involves activation of caspase-3. Skin equivalents were cultured at the air–liquid interface for 6 d and their differentiation was inhibited by treatment with RA. In situ analysis of caspase-3 activation by immunofluorescence with an anti-serum specific for activated caspase-3 was performed. (a) In control samples, caspase-3 activation was detected only in few scattered apoptotic cells (arrow) but not in the terminal differentiating layers. (b) In addition to inhibition of differentiation, RA treatment strongly increased the number of active caspase-3-positive cells indicative of apoptosis. Bar = 50 µm.

used and the cell culture conditions, some common and some distinguishing features of KC death in the course of their transition to corneocytes and apoptosis were identified (Melino et al, 1998; Gandarillas, 2000). In the chain of events leading to apoptosis, caspases are key enzymes that are able to amplify both apoptotic signals and execute late steps of the cell death program by cleaving crucial structural and regulatory cellular target proteins (Chang and Yang, 2000). The involvement of caspases in the formation of the cornified layer was recently suggested by Takahashi et al (1998) and Weil et al (1999), who detected caspase activity in preparations of the stratum corneum and blocked epidermal differentiation in vitro using caspase inhibitors, respectively. We have recently demonstrated that a new caspase, i.e., caspase-14, which belongs to the group of executioner caspsases (Chang and Yang, 2000), is almost exclusively expressed in KC of the epidermis, the hair follicles, and the sebaceous glands (Eckhart et al, 2000a, b; Lippens et al, 2000). In contrast to other executioner caspsases it is dispensable for and not activated during conventional KC apoptosis; however, cultivation of KC under conditions leading to the formation of a cornified layer leads to activation of caspase-14 (Eckhart et al, 2000b), suggesting its participation in this process. Here we show that in contrast to caspase-3 and -8, which are involved in classical apoptotic pathways, caspase-14 is downregulated in KC by retinoids under conditions that prevented formation of a cornified layer. The finding that caspase-14 is regulated analogously to proteins involved in the formation of the skin barrier such as filaggrin and involucrin (Fisher and Voorhees, 1996) is an additional piece of evidence that caspase-14 also plays a part in this process. The most likely explanation regarding the mechanisms of RA-mediated downregulation of caspase-14 expression is transrepression of AP-1-mediated gene activation, as has been shown for several differentiation associated genes (Fisher and Voorhees, 1996). Preliminary data on the caspase-14 promoter show that it contains at least two potential AP-1 binding sites that could participate in transrepression (unpublished observations).

In contrast to caspase-14, caspase-3 was not downregulated in skin equivalents by RA treatment. In addition, whereas activation of caspase-3 was not detectable in untreated controls by western blot analysis, it became readily detectable after RA treatment. In situ staining with anti-sera specific for activated caspase-3 corroborated these data. Whereas only few scattered cells expressing activated caspase-3 were found in untreated skin equivalents, up to 20% of cells were positive after treatment of skin equivalents for 6 d. By light microscopy, a similar percentage of cells showed the typical “sunburn cell” morphology (Bayerl et al, 1995) of apoptotic KC. Our data on the RA-mediated KC apoptosis in skin equivalents are in accordance with what has been reported previously (Haake and Cooklis, 1997). As this KC apoptosis occurred in the virtual absence of caspase-14 our data provide further evidence that this caspase is dispensable for conventional KC apoptosis. On the other hand, our results on the presence of activated caspase-3 are in contrast to the observation of the activation of caspase-3 in terminal differentiating KC at the transition zone to the cornified layer (Weil et al, 1999). In agreement with these authors we detected a faint staining of single transitional cells in untreated skin equivalents (not shown); however, we found that this staining was nonspecific as it could not be blocked by preincubation of the serum with active recombinant human caspase-3
Hodam JR, Creek KE: Uptake and metabolism of \( ^{3}H \) retinoic acid delivered to human foreskin keratinocytes either bound to serum albumin or added directly to the culture medium. Biochem Biophys Acta 1311:102–110, 1996
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