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Expression and transcriptional regulation of caspase-14 in simple and complex epithelia

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Abstract

Caspase-14 is a recent addition to the caspase family of aspartate proteases involved in apoptotic processes. Human caspase-14 appears to be only weakly processed during apoptosis, and it does not cleave classical caspase substrates. Post partum, caspase-14 is prominently expressed by human keratinocytes and reportedly participates in terminal differentiation of complex epithelia. Here we provide evidence challenging the view that caspase-14 expression or processing is linked exclusively to terminal keratinocyte differentiation. We demonstrate that caspase-14 expression extended to multiple cell lines derived from simple epithelia of the breast, prostate, and stomach. In keratinocytes and breast epithelial cells, caspase-14 expression was upregulated in high-density cultures and during forced suspension culture. These effects were primarily due to transcriptional activation as indicated by reporter gene assays using a 2 kb caspase-14 promoter fragment. Importantly, caspase-14 was not cleaved during forced suspension culture of either cell type although this treatment induced caspase-dependent apoptosis (anoikis). Forced expression of caspase-14 in immortalized human keratinocytes had no effect on cell death in forced suspension nor was the transfected caspase-14 processed in this setting. In contrast to postconfluent and forced suspension culture, terminal differentiation of keratinocytes induced in vitro by Ca^{2+} treatment was not associated with increased caspase-14 expression or promoter activity. Our results indicate that (1) caspase-14 is expressed not only in complex but also simple epithelia; (2) cells derived from complex and simple epithelia upregulate caspase-14 expression in conditions of high cell density or lack of matrix interaction and; (3) in both cell types this phenomenon is due to transcriptional regulation.

Introduction

Caspases (cysteine-dependent aspartate-specific proteases) are proteolytic enzymes with prominent roles in programed cell death in organisms ranging from nematodes to man.[1–3] Membership in the caspase protein family is defined by sequence homology with Ced-3, a key effector protein in programed cell death in *Caenorrhabditis elegans*.[4] In non-apopotic cells caspases are present as inactive proenzymes. Proteolytic cleavage of these zymogens during apoptosis generates catalytically active p20/p10 heterodimers.[5–8] An important feature of caspases is the presence of either long or short N-terminal prodomains. Based on their interaction with the apoptosome, long prodomain caspases appear to initiate caspase activation by cleaving short prodomain caspases, which are widely considered as downstream 'executioner' caspases. Caspases-1, -2, -4, -5, -8, -9, -10, -11, -12 and -13 contain long prodomains, whereas caspases-3, -6, -7, and -14 have short prodomains.[9,10] It is believed that caspase-dependent cleavage of key intracellular proteins contributes to the sequence of events necessary to complete the apoptotic process.[11,12] However, some enzymes with features typical of caspases serve roles unrelated to cell death. For example, the prototypical caspase interleukin-1β-converting enzyme (ICE, caspase-1) derives its name from the capacity to cleave and thereby activate IL-1β during inflammatory or regenerative processes.[13,14]

Recently, our laboratory and others identified and characterized caspase-14, a new member of caspase family with a short prodomain.[15–17] Mouse procaspase-14 is only weakly processed *in vitro* by caspase-8, caspase-10 and *in vivo* by granzyme B.[16] Human caspase-14 is not processed by any known caspase nor does it cleave other caspases or classical caspase substrates (our unpublished data). Thus, the role of caspase-14 in apoptotic processes is presently

unclear. A recent report implicated caspase-14 expression and processing in the terminal differentiation program in stratifying epidermis.[18] Terminal differentiation of keratinocytes shares some features with classical apoptosis including DNA fragmentation, nuclear condensation and induction of cross-linking transglutaminases in the outer layers of the differentiating epidermis.[19,20] Several lines of evidence point to caspase activation during epidermal differentiation.[21] Specifically, caspase-3 activation has recently been observed during terminal differentiation of *in vitro* reconstituted epidermis.[22] Furthermore, Eckhart *et al.*[18] described upregulation of caspase-14 expression an *in vitro* model for keratinocyte differentiation, i.e., growth to postconfluency. These investigators also described caspase-14 cleavage in intact epidermis and epidermal reconstructs.

Here we re-examine caspase-14 expression and function in epithelial cells. We report expression of caspase-14 in cells derived from simple (breast, prostate) and complex (epidermis) epithelia. We confirm induction of caspase-14 expression in human epidermal keratinocytes grown to high density and extend this observation to keratinocytes grown in forced suspension culture. We provide evidence that upregulation of caspase-14 expression in these experimental settings was not unique to keratinocytes but was shared by breast epithelial cells representative of simple epithelia. In both cell types, regulation of caspase-14 expression was due, at least in part, to transcriptional activation of the caspase-14 gene. Furthermore, induction of terminal keratinocyte differentiation by Ca^{2+} did not affect caspase-14 expression or cleavage. Finally, forced expression of caspase-14 in immortalized human keratinocytes did not affect either differentiation or survival of these cells in forced suspension culture. Taken together, our results suggest that expression and regulation of caspase-14 in epithelial cells is not exclusively linked to the terminal differentiation program as observed in complex epithelia.

Results

Characterization of caspase-14 mAb

Unlike MCF-7, 293 cells do not express any detectable caspase-14 mRNA as determined by RT–PCR (see Figure 2B, and data not shown). Thus, to determine the specificity of the anti-caspase-14 mAb (clone # 1–71), which was raised against a recombinant human caspase-14 protein, we used total extracts from 293 and MCF-7 for Western blot analysis. Caspase-14 proenzyme was detected as a 29 kDa band in the MCF-7, but not in the 293 extracts (Figure 1A, left panel). A similar size band was detected in the 293 extracts only after transient transfection of these cells with a construct encoding full-length human procaspase-14 (Figure 1A, third lane). Preincubation of the caspase-14 mAb with purified recombinant human caspase-14 protein blocked its ability to immunostain the caspase-14 bands in the MCF-7 and caspase-14-transfected 293 extracts (Figure 1A, middle panel). This indicates that the 29 kDa band in these extracts is procaspase-14. To further verify that the 29 kDa band in MCF-7 extracts is indeed procaspases-14 we used a second caspase-14 mAb (clone #1–52) which can only immunopre-

cipitate caspase-14 but not immunostain caspase-14 bands on Western blots. This antibody was able to precipitate the same 29 kDa procaspases-14 band from the MCF-7 and caspase-14-transfected 293 extracts but not from the empty vector-transfected 293 extract (Figure 1B). The caspase-14 mAb can also detect the large subunit of caspase-14 (p17, residues 1–146) (Figure 1A, right panel), indicating that its epitope is located within the large subunit.

Caspase-14 expression in cells derived from simple and complex epithelia

Caspase-14 is expressed in tissue-specific fashion in adult mice as previously determined by Northern blot analysis.[16] Whereas some investigators found caspase-14 expression exclusively in epidermal keratinocytes,[17] we observed caspase-14 mRNA expression also in the liver and, to a lesser extent, in the brain and kidneys of mice.[16] Here we describe that caspase-14 expression extends to multiple carcinoma cell lines derived from simple, i.e. breast, prostate, bladder and stomach epithelia (Figure 1C–G). As determined by Western blot analyses using the caspase-14 mAb, caspase-14 was expressed in the prostate carcinoma cell line PC3, in immortalized benign and malignant prostate cell lines[23] but not in LNCAP and DU145 cells (Figure 1D). Furthermore, the breast carcinoma cells MCF-7, HBL100, T47-D, BT-20, MDA-MB-175, MDA-MB-436 and MDA-MB-453, SKBR-3, SKBR-5, SUM-102, SUM-149, SUM-229 were caspase-14-positive whereas, MDA-MB-231, MDA-MB-415, and ZR-75 were not (Figure 1E). Stomach and bladder carcinoma cell lines were also positive for caspase-14 expression (Figure 1F,G). Similarly, a panel of keratinocyte-derived squamous carcinoma cells revealed heterogeneous expression of caspase-14 with three cell lines (SCC9, 12, and 13) positive and two cell lines negative (HaCaT and A431) (Figure 1C). As caspase-14 expression was described earlier in HaCaT keratinocytes[18] we assessed caspase-14 mRNA expression in multiple HaCaT RNA isolates by RT–PCR, which revealed no caspase-14-specific PCR products (Figure 2B). By contrast and in agreement with the results of the Western blot analyses caspase-14 PCR products were obtained using RNA preparations derived from MCF-7 cells and normal keratinocytes (Figure 2A). Immunohistochemical analysis of caspase-14 expression in normal epidermis showed weak staining in the suprabasal, differentiating layers (Figure 3A). By comparison, psoriatic skin (Figure 3B) demonstrated stronger immunoreactivity with the caspase-14 antibody predominantly in the outer subcorneal layer of the stratifying epidermis. Various squamous carcinomas revealed heterogeneous reactivity with the caspase-14 antibody similar to the heterogeneous pattern observed for caspase-14 mRNA expression in SCC lines (not shown).

Lack of procaspase-14 processing during keratinocyte apoptosis

To determine whether caspase-14 was involved in apoptotic death of normal keratinocytes, apoptosis was induced by UVB exposure of normal human keratinocytes as described previously.[24] Within 48 h after UVB radiation subconfluent
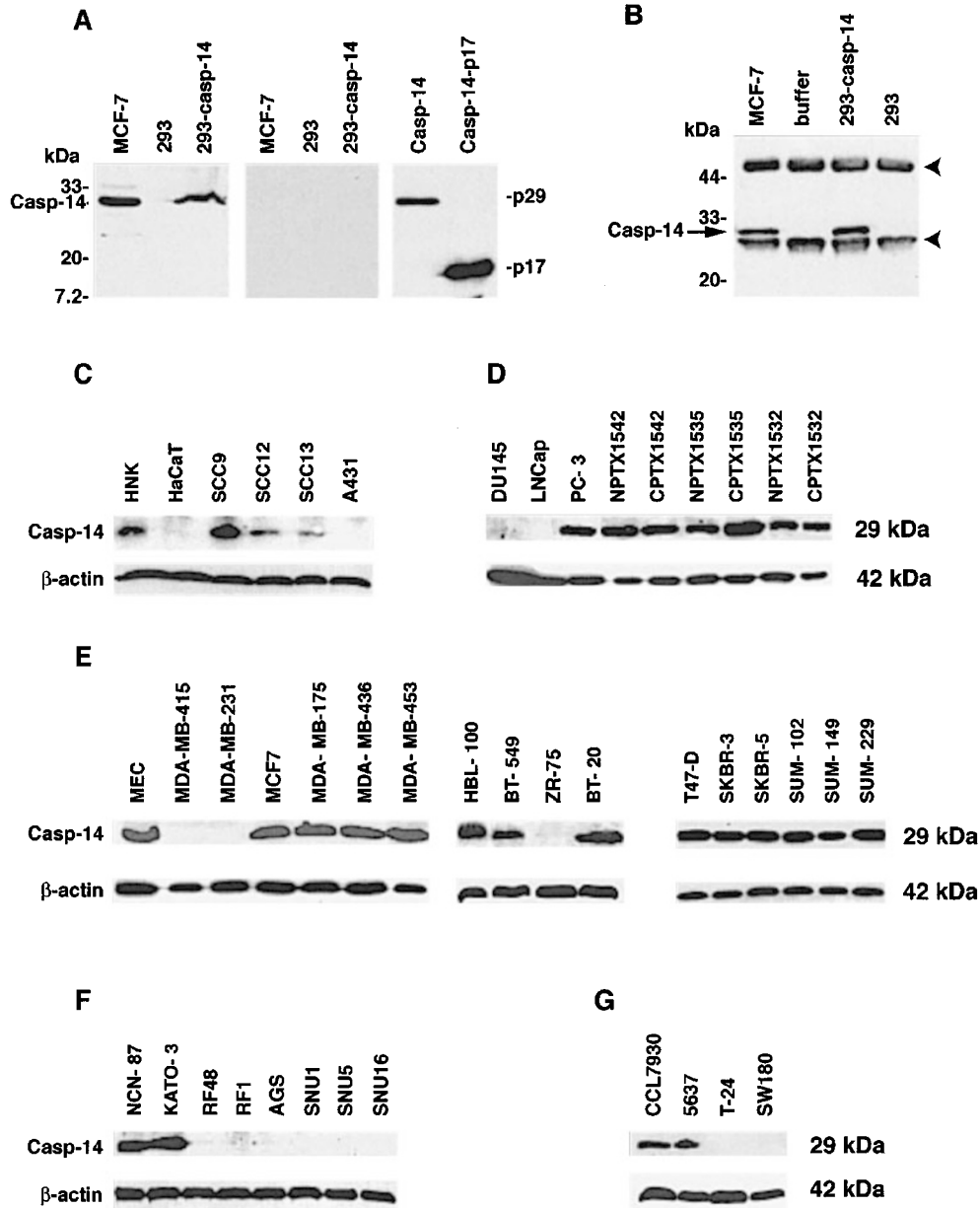
Figure 1 Specificity of the caspase-14 mAb, and analysis of the caspase-14 expression in human squamous and epithelial cancer cell lines. (**A**) Left panel, extracts from MCF-7, 293 and caspase-14-transfected 293 (293-casp-14) were fractionated by SDS–PAGE and then immunoblotted with caspase-14 mAb (clone # 1–71) as described in 'Materials and Methods'. No caspase-14 expression was observed in the non-transfected 293 cells (second lane). Middle panel, a duplicate blot, like in the left panel, was probed with the caspase-14 mAb after pre-incubation of the antibody with recombinant caspase-14 protein. Right panel, recombinant procaspases-14 (first lane) and caspase-14-p17 (residues 1–146, second lane) were fractionated by SDS–PAGE and then immunoblotted with caspase-14 mAb (clone # 1–71) as above. (**B**) Extracts from MCF-7, 293 and caspase-14-transfected 293 (293-casp-14) cells were immunoprecipitated with a second caspase-14 mAb (clone # 1–52). The immunoprecipitates were then immunoblotted with the caspase-14 mAb as in **A**. The IgG heavy and light chains are indicated by arrows. (**C–G**) Western blot analysis of caspase-14 in human squamous and epithelial cancer cell lines. (**C**) squamous carcinoma cell lines; (**D**) prostate cancer cell lines; (**E**) breast cancer cell lines; (**F**) stomach cancer cell lines; (**G**) bladder cancer cell lines. The molecular weight of caspase-14 is indicated on the right. Significant levels of caspase-14 were expressed in most of the cell lines tested. The blots were re-probed with an antibody to β-actin

keratinocytes underwent apoptosis as determined by cleavage of the caspase-3 target PARP (Figure 4A). As expected, UVB-induced PARP cleavage was markedly attenuated by pretreatment of keratinocytes with the caspase inhibitor Ac-DEVD-CHO. In contrast to PARP cleavage, procaspase-14 was not cleaved upon induction of keratinocyte apoptosis by UVB exposure as only the proenzyme was detected by

Western blot analysis in all experimental conditions. Next, we tested whether caspase-14 was processed in keratinocytes induced to undergo apoptosis (anoikis) by denying substrate attachment in forced suspension culture. This was accomplished by maintaining keratinocytes for up to 72 h in suspension on top of a 0.9% agarose gel. We have shown previously that this treatment induces extensive apoptosis of
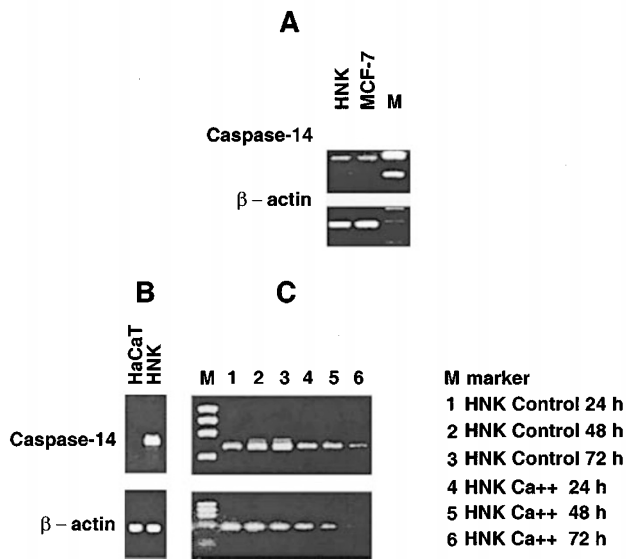
Figure 2 (**A**) RT–PCR analysis of caspase-14 expression in HNK and MCF-7 cells. (**B**) Expression of caspase-14 mRNA by human normal keratinocytes (HNK) but not immortalized keratinocytes (HaCaT). (**C**) Expression of caspase-14 mRNA over time in keratinocyte cultures in the presence and absence of high ambient Ca^{2+}. φX174 molecular weight was used as marker. β-actin mRNA was analyzed for all the samples to normalize for mRNA integrity and equivalent loading

primary human keratinocytes within 24 to 48 h.[25] This was confirmed by assessing PARP cleavage, which occurred within 48 h of forced suspension culture (Figure 4B). However, no cleavage of procaspase-14 was observed throughout the observation period. Similarly, MCF-7 cells in suspension culture underwent apoptosis as determined by PARP cleavage with no evidence of caspase-14 processing (Figure 4C). Finally, we generated HaCaT cells constitutively expressing high levels of caspase-14 and induced anoikis of these cells by forced suspension culture. Robust transgene expression was observed in attached HaCaT-caspase-14 cells and in cells held in forced suspension for up to 72 h (Figure 4E). However, forced suspension culture did not induce caspase-14 cleavage. Collectively, these observations confirm and extend recently published results, which indicated that induction of keratinocyte apoptosis by engagement of death receptors does not lead to caspase-14 cleavage.[26]

Induction of caspase-14 expression during forced suspension culture of human keratinocytes

Although forced suspension of primary keratinocytes did not induce caspase-14 cleavage it was associated with strong upregulation of caspase-14 protein levels relative to β-actin controls (Figure 4B). Western blot analysis of cells held in forced suspension revealed an increase of caspase-14 expression as early as 24 h in suspension. Further increases in caspase-14 expression were apparent at later time points (48 and 72 h) of forced suspension culture. Keratinocytes in forced suspension culture undergo not only apoptosis but also express markers of terminal differentiation, for example involucrin (data not shown). Therefore, upregulation of
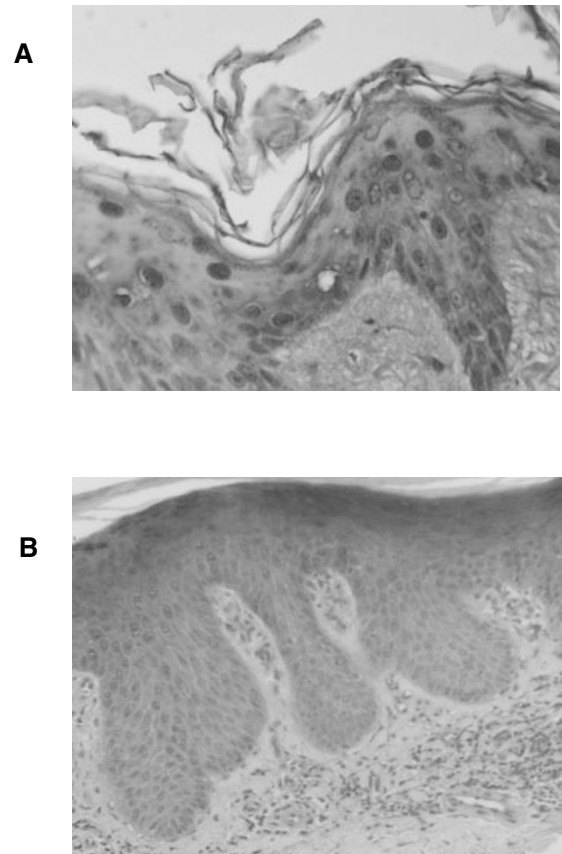

Figure 3 Immunohistochemical localization of human caspase-14 in normal and psoriatic skin. Immunoperoxidase staining was performed with a caspase-14 mAb as described in 'Materials and Methods'. (**A**) (x400) power image of a section of normal skin from human scalp. Highest level of caspase-14 expression was detected in the subcorneal epidermis. (**B**) (x200) power image of section of human psoriatic plaque. Increased expression of caspase-14 was evident when compared with normal skin

caspase-14 expression may reflect a role of caspase-14 in keratinocyte stratification and terminal differentiation as suggested by Lippens *et al.*[26] Expression of caspase-14 in epithelial cells derived from simple epithelia including breast, bladder, and prostate (Figure 1) allowed us to test this hypothesis further. If upregulation of caspase-14 expression in forced suspension were strictly associated with keratinocyte terminal differentiation it may be expected that the same treatment should not affect caspase-14 expression in non-stratifying epithelial cells. Thus, we tested whether forced suspension culture of MCF-7 breast carcinoma cells was associated with modulation of caspase-14 expression. Forced suspension culture of MCF-7 cells was accompanied by progressive PARP cleavage in a manner similar to normal human keratinocytes (Figure 4C). It also was associated with robust induction of caspase-14 expression in these cells. As in primary keratinocytes we observed no cleavage of procaspase-14 in MCF-7 cells in forced suspension cultures. Comparable results were obtained with normal breast epithelial cells placed in forced suspension culture (Figure 4D) demonstrating that lack of matrix adhesion is associated
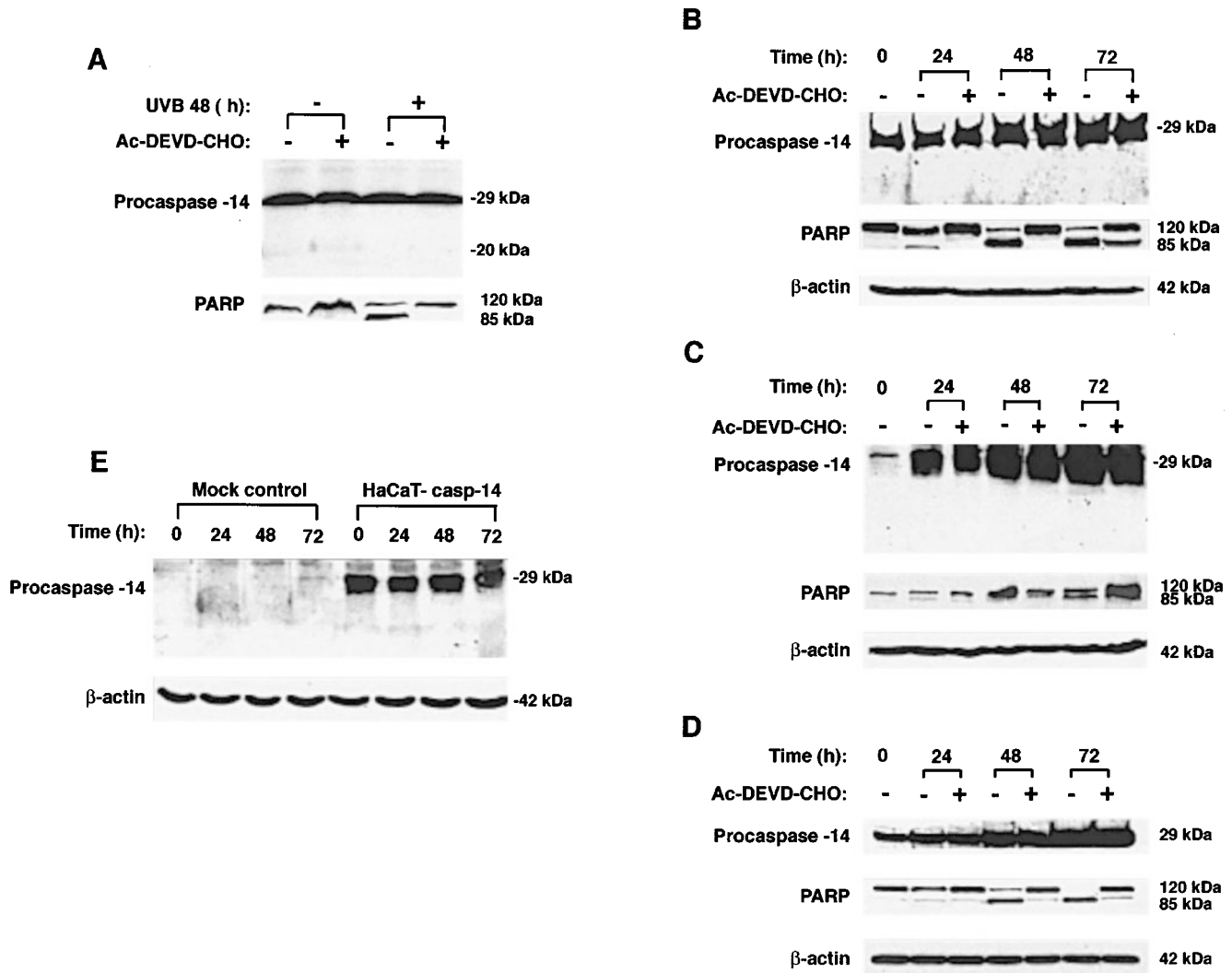
Figure 4 Caspase-14 expression in induced keratinocyte apoptosis by UVB radiation or forced suspension culture. (**A**) HNK were UVB irradiated in presence or absence of caspase inhibitor DEVD-CHO (120 μM). Caspase-14 expression was determined by Western blot analysis 48 h after radiation. No change in caspase-14 expression was observed after UVB exposure and Ac-DEVD-CHO pretreatment did not affect caspase-14 expression. PARP cleavage was determined by immunoblot analysis to account for UVB-induced apoptosis. Pretreatment with Ac-DEVD-CHO blocked PARP processing. (**B–D**) HNK (**B**), MCF-7 (**C**) and HMEC (**D**) were maintained in forced suspension culture as indicated in 'Materials and Methods' for the time periods indicated in presence or absence of DEVD-CHO (120 μM). Immunoblot analysis of caspase-14 protein using lysates prepared from all the cell suspension cultures revealed a time related increase in caspase-14 expression when compared with the control as early as 24 h suspension. p85-PARP cleavage was determined by Western blot as marker of apoptosis at all time tested and Ac-DEVD-CHO pretreatment markedly attenuated the PARP processing. In contrast, Ac-DEVD-CHO pretreatment did not influence the increase in caspase-14 protein. (**E**) no increase in caspase-14 protein level was observed in caspase-14 and mock-stable transfected HaCaT cells (see 'Materials and Methods') at the same experimental conditions mentioned above. β-actin expression was used as a normalizing control to confirm same amount of protein loading and transfer

with enhanced caspase-14 expression in normal and malignant cells derived from simple and complex epithelia. In all cell types tested induction of caspase-14 expression in forced suspension occurred in the presence and absence of the caspase inhibitor Ac-DEVD-CHO, which markedly attenuated PARP cleavage in this setting.

Caspase-14 expression and *in vitro* keratinocyte differentiation

These results were not consistent with a role of caspase-14 restricted to the differentiation program of stratifying epithelia.

We, therefore, tested whether caspase-14 expression was induced in other experimental settings commonly used to induce keratinocyte differentiation. First, we compared caspase-14 expression in monolayer cultures grown to low and high cell densities; high density confluent cultures commonly express higher levels of keratinocyte differentiation markers including involucrin as compared to low density cultures[27] and induction of caspase-14 expression in this setting has been described previously.[26] Caspase-14 expression was assessed by immunoblotting analysis of subconfluent and postconfluent parallel cultures (Figure 5A). Consistent with previous results,[26] postconfluent primary

keratinocytes cultures exhibited markedly increased levels of caspase-14 when compared with control subconfluent cultures. As expected postconfluent cultures also exhibited moderately higher involucrin expression levels as compared to low confluency cultures. Importantly, density-dependent regulation of caspase-14 expression was not restricted to primary keratinocytes but was also observed in MCF-7 breast carcinoma cells (Figure 5B) and occurred in these cells as well as in primary keratinocytes in the presence and absence of the caspase inhibitor Ac-DEVD-CHO. Thus, as in forced suspension, high density monolayer cultures of cells derived from simple and complex epithelia share molecular mechanisms that lead to enhanced caspase-14 expression.

Next, we used high concentrations of extracellular $CaCl_2$ (1.2 mM) to induce keratinocyte differentiation *in vitro*; this is a widely accepted method to induce differentiation of keratinocytes *in vitro* as it leads to the formation of cross-linked squames similar to those observed in late stage keratinocyte differentiation in skin.[28] Keratinocytes maintained in either low (0.05 mM) or high (1.2 mM) Ca^{2+} for 48 h expressed comparable levels of caspase-14 protein but increased involucrin levels in presence or absence of

the caspase inhibitor Ac-DEVD-CHO (Figure 5C). Thus, in contrast to forced suspension and high-density cultures, Ca^{2+} treatment did not induce caspase-14 expression in primary keratinocyte cultures.

Regulation of caspase-14 promoter activity by cell density and lack of matrix attachment

To assess molecular mechanisms of caspase-14 regulation in high density cultures we first performed RT–PCR analysis in primary keratinocytes grown in low (0.05 mM) and high (1.2 mM) Ca^{2+} for 24, 48 and 72 h. During the observation period control cultures maintained at low Ca^{2+} grew to confluency. As shown in Figure 2C, caspase-14 mRNA expression slightly increased with time in these control cultures whereas it was unchanged in parallel Ca^{2+}-treated cultures. These results were consistent with data obtained by immunoblotting analyses of caspase-14 expression in high and low-density keratinocyte cultures (Figure 5A). In these experiments we also included immortalized HaCaT keratinocytes to assess whether high density or Ca^{2+}-treatment induced caspase-14 expression in these cells. No caspase-
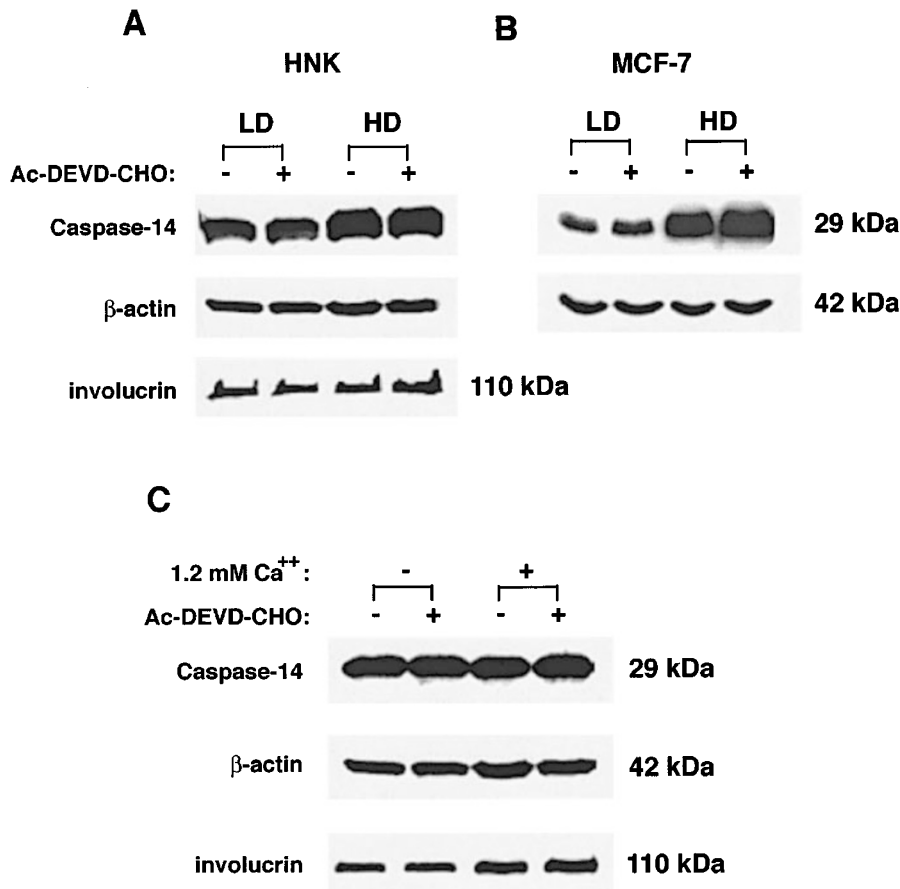

Figure 5 Caspase-14 expression in subconfluent and postconfluent HNK and MCF-7 cells and in high Ca^{2+} treated HNK. Caspase-14 immunoblots were performed from cell lysates obtained from cultures at low (LD) and high (HD) cell density of HNK and MCF-7 cells as described in 'Materials and Methods'. (**A,B**) upregulation of caspase-14 expression in high confluent cultures (**A**, HNK; **B**, MCF-7) occurred in presence or absence of Ac-DEVD-CHO (120 μM). (**C**) extracellular increase of calcium (1.2 mM) in the HNK medium for 48 h did not produce any change in caspase-14 protein level as compared to low calcium HNK cultures. Western blot of involucrin expression was also performed as a marker for keratinocyte differentiation. To account for differences in protein loading blots were re-probed using an antibody to β-actin

14 related amplimers were derived from HaCaT cells in any experimental condition (not shown).

To determine whether regulation of steady-state caspase-14 mRNA levels occurred through transcriptional or post-transcriptional mechanisms we first determined caspase-14 protein expression in HaCaT cells stably transfected with caspase-14 and held in forced suspension culture. This analysis revealed comparable high levels of caspase-14 protein expression at 24, 48 and 72 h of forced suspension culture consistent with the notion that caspase-14 expression in suspension culture was not regulated through either message or protein stabilization (Figure 4E). To address effects of suspension culture on caspase-14 transcription directly we cloned a 2 kb human caspase-14 promoter fragment and constructed reporter plasmids in which this promoter directed expression of luciferase. The reporter plasmid was transfected into primary keratinocytes followed by testing of promoter activity in attached keratinocytes at different cell densities, in suspended keratinocytes, and in keratinocytes maintained at low and high ambient Ca^{2+} concentrations (Figure 6B). The results of this analysis were very similar to the patterns of caspase-14 protein expression observed in these different experimental settings. Specifically, both high cell density culture and suspension culture were associated with markedly elevated luciferase activity as compared to subconfluent cultures and attached cells, respectively. By contrast and as expected, Ca^{2+} treatment did not affect activity of the reporter construct. Very similar results were obtained in MCF-7 cells transiently transfected with the reporter construct. Again, both high cell density and the absence of matrix attachment led to high luciferase activity in this cell line (Figure 6C). These results indicate that differential caspase-14 expression in the different experimental conditions tested here was due, in part, to regulation of transcription from the caspase-14 promoter.

Caspase-14 expression in keratinocytes is regulated in a cell cycle-independent and histone acetylation-dependent fashion

A common denominator of high density and forced suspension cultures is cell cycle arrest[29,30] raising the question whether caspase-14 expression is regulated in a cell cycle-dependent manner. To address this issue, we treated MCF-7 cells with mimosine, thymidine, and nocodazole which arrest cells at G1, S, G2/M, respectively (Figure 7A), followed by immunoblot analysis of caspase-14 expression. As shown in Figure 7B, none of the cell cycle inhibitors used affected caspase-14 expression to any appreciable degree. By contrast, treatment of keratinocytes and SCC 9 cells with trichostatin A, an inhibitor of histone acetylation, was associated with markedly reduced caspase-14 message levels as determined by RT–PCR (Figure 8).

Subcellular distribution of caspase-14

Lippens et al.[26] described that caspase-14 is present in both, the cytosol and nuclei of differentiating keratinocytes in situ.

We asked whether high cell density or forced suspension culture of primary keratinocytes affected the subcellular distribution of caspase-14 in primary keratinocytes. As shown in Figure 9, both functional states were associated not only with higher levels of caspase-14 expression but also differential subcellular distribution of caspase-14. As determined by immunocytochemistry and confocal microscopy, primary keratinocytes grown at subconfluent cell densities contained caspase-14 in both, the cytoplasmic and nuclear compartments (Figure 9A). By contrast, high cell density or forced suspension cultures (Figure 9B,C) revealed loss of nuclear caspase-14 and diffuse cytoplasmic staining with the caspase-14 specific antibody. Similarly, caspase-14 redistribution to the cytoplasm was seen in both high confluent and forced suspension culture MCF-7 cells (Figure 9E,F).
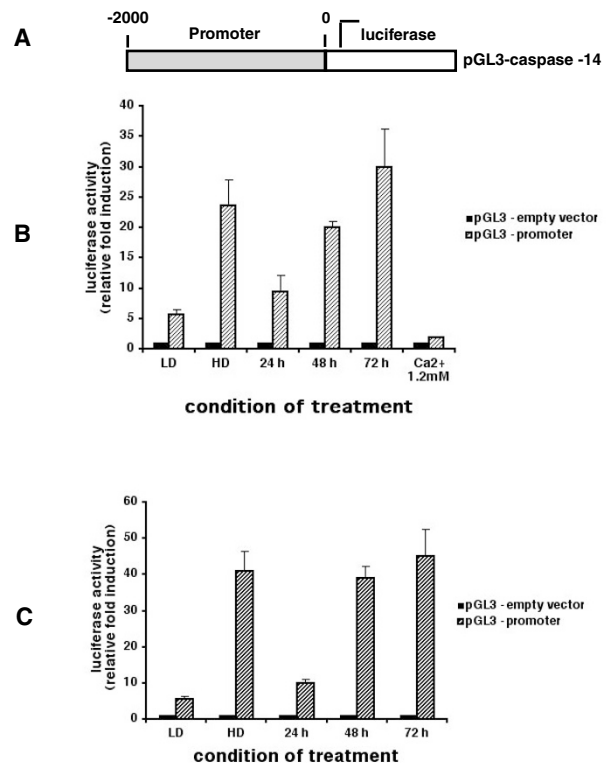

Figure 6 Transcription activation by 5′-promoter sequence of caspase-14 in HNK and MCF-7 cells. (A) schematic representation of the caspase-14 putative promoter indicating the 5′ end (−2000 bp) of the DNA segment upstream of the transcription start site (ATG) of caspase-14. This 2 kb segment was inserted into a pGL3-basic vector in front of luciferase gene reporter and transientely transfected into HNK and MCF-7 cells to test promoter activity (see 'Materials and Methods'). (B,C) promoter activity induced by pGL3-caspase-14 construct in HNK (B) and MCF-7 (C) cells before (LD) and after high density (HD) or in forced suspension cultures at different time points (see Materials and Methods). Promoter activity was also tested in HNK cultures after high-calcium induced differentiation. Luciferase activity was then determined on the same amounts of cell protein lysates and expressed as percentages relative to luciferase activity in promoterless pGL3-empty vector-transfected HNK and MCF-7 cells treated at the same experimental conditions. Results are means ± S.E. of three independent experiments, each performed in quadruplicate
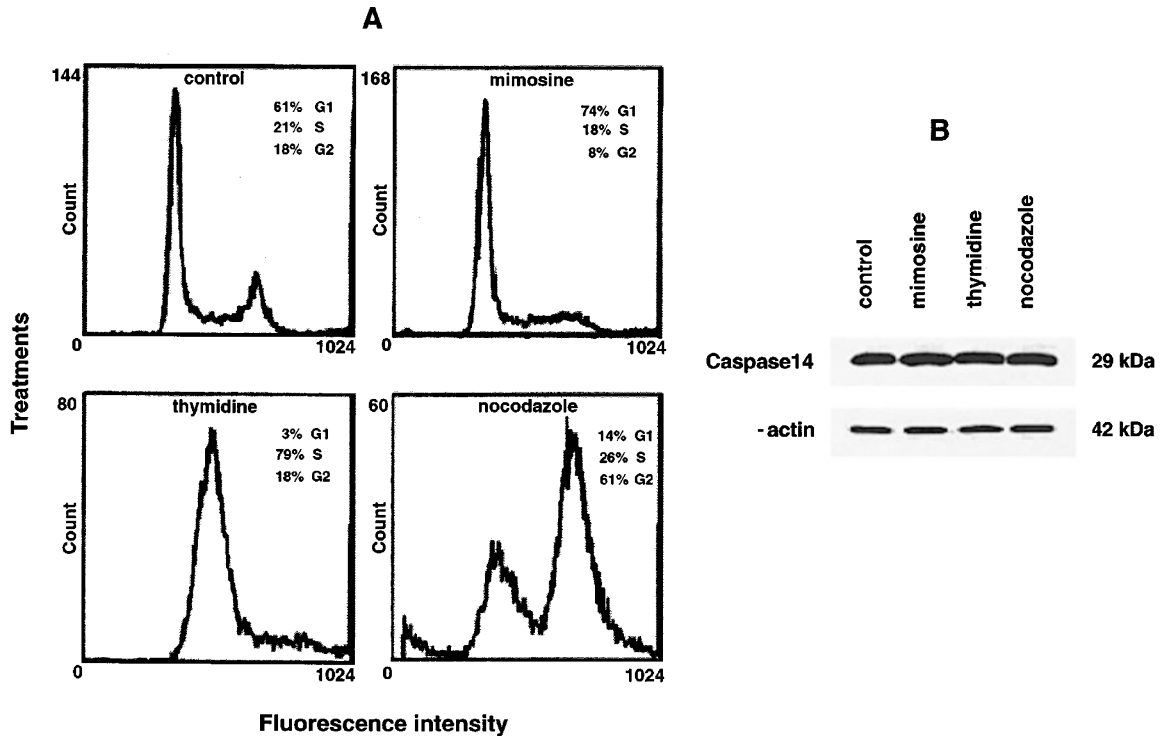
A

B

Figure 7 Cell cycle position following arrest with mimosine, thimidine, or nocodazole and lack of correlation with caspase-14-expression. (**A**) representative flow cytometric profiles of cell cycle position as determined by DNA content in untreated (control) or treated MCF-7 cells with 400 μM mimosine, 2 mM thimidine or 0.4 μg/ml nocodazole for 24 h. Cells were treated and fixed for FACS analysis as described in 'Materials and Methods'. The proportions of cells in G_1, S and G_2M phase are presented. B, immunoblot analysis of caspase-14 in lysates from synchronized MCF-7 cultures. Protein samples were prepared as described in 'Materials and Methods'. β-actin expression was used as a normalizing control to confirm same amount of protein loading and transfer
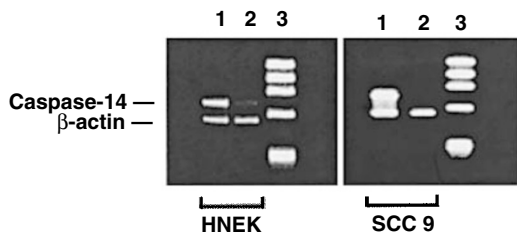

Figure 8 mRNA caspase-14 suppression by inhibitor of histone acetilation trichostatin-A. Lane 1, profile of caspase-14 mRNA in HNK and SSC cells. Lane 2, lack of caspase-14 mRNA after treatment with 500 μM trichostatin-A in both HNK and SSC cells. Lane 3, φX174 molecular weight. β-actin mRNA was analyzed for all the samples to normalize for mRNA integrity and equivalent loading

Discussion

This study establishes that caspase-14 is expressed in a broad range of epithelial cell types including human epidermal and breast epithelial cells derived from complex and simple epithelia, respectively. Consistent with earlier reports[26] we observed that high cell density in postconfluent cultures was associated with increased caspase-14 expression in human keratinocytes. We extended this observation to keratinocytes held in forced suspension culture. Importantly, upregulation of caspase-14 expression in these two experimental settings also occurred in normal and malignant breast epithelial cells.

Because breast epithelial cells represent a simple epithelium, induction of caspase-14 expression should not be considered to be unique to terminal differentiation of complex epithelia. This view is further supported by our observation that induction of terminal keratinocyte differentiation by treatment with 1.2 mM $CaCl_2$ did not affect caspase-14 expression in normal human keratinocytes. We confirm and extend an earlier study[26] by demonstrating that caspase-14 was not processed during keratinocyte apoptosis induced by either UVB radiation or forced suspension culture. These observations are consistent with our previous work demonstrated that caspase-14 is poorly if at all cleaved by other caspases.[16]

Regulation of caspase-14 expression in forced suspension cultures occurred independently of effector caspase activation as it was not affected by the caspase inhibitor Ac-DEVD-CHO. This inhibitor, however, was active as it attenuated PARP processing in forced supension of both, keratinocytes and mammary epithelial cells. Regulation of caspase-14 expression in high cell density and forced suspension cultures occurred in part through transcriptional mechanisms because transcriptional activity of a 2 kb caspase-14 promoter fragment was markedly induced in these conditions. Importantly, induction of caspase-14 promoter activity by high cell density or forced suspension culture was shared between breast and epidermal epithelial cells in further support of the argument that regulation of caspase-14 expression is not tightly linked to terminal differentiation of complex epithelia. It is remarkable that two
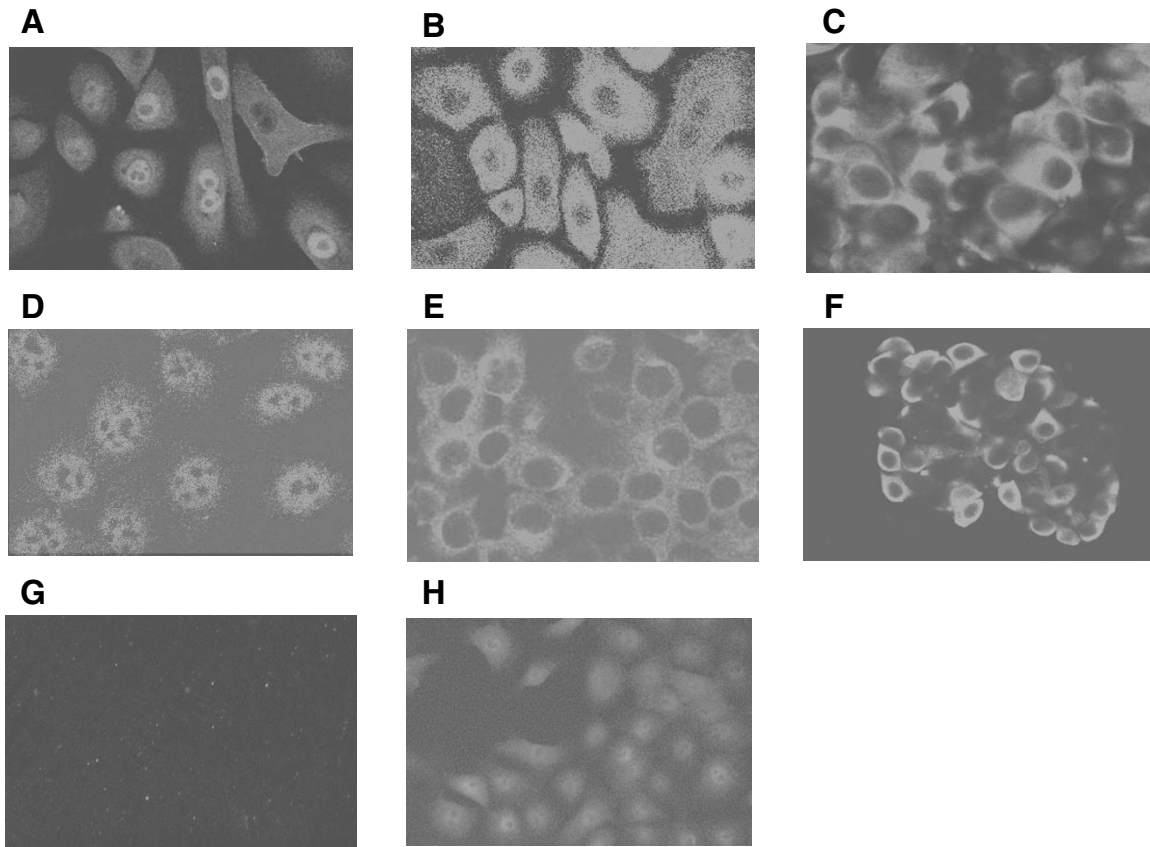
Figure 9 Caspase-14 confocal immunofluorescence analysis of HNK and MCF-7 cells. Immunofluorescent pattern of caspase-14 in HNK (**A – C**) or MCF-7 (**D – F**) cells. (**A** and **D**) sub-confluent cultures show endogenous subcellular nuclear-caspase-14 localization. (**B** and **E**) high confluent cultures, and (**C** and **F**) 72 h forced suspension cultures show cytoplasmic re-localization of caspase-14. Cells were immunostained as described in 'Materials and Methods'. Control non-transfected (**G**) and caspase-14 stably transfected (**H**) HaCaT cells were stained with the caspase-14 mAb to show the specificity of the caspase-14 staining

very different functional states of epithelial cells were associated with upregulated caspase-14 expression apparently through a shared mechanism. Whereas in forced suspension culture extracellular matrix-derived signals are absent such signals are maintained in high cell density cultures characterized by extensive cell-cell interactions. Of note, caspase-14 overexpression in forced suspension culture was not due to enhanced cell-cell contact as described previously in spheroid cultures.[31,32] This conclusion is based on the fact that in these assays we used a culture medium, which contained only 0.06 mM Ca^{2+}. At this Ca^{2+} concentration cadherin-based cell-cell interactions cannot be established or maintained. Thus, the suspended cells formed only loose aggregates, which were easily dispersed by gentle pipetting.

Cell cycle arrest is a common denominator of high density and forced suspension culture. Yet, regulation of caspase-14 expression was not linked to cell cycle progression as cell cycle arrest induced by pharmacological inhibitors did not detectably affect caspase-14 expression in immortalized keratinocytes. However, treatment of normal and malignant keratinocytes with the histone deacetylase inhibitor trichostatin-A led to markedly lower caspase-14 mRNA levels. It remains to be determined whether altered histone acetylation patterns are responsible

for the effects of high density and forced suspension culture on caspase-14 transcription.

Unexpectedly, we observed shuttling of caspase-14 between cytoplasmic and nuclear pools. Specifically, primary keratinocyte and MCF-7 cultures at high cell density or in forced suspension revealed relocalization of caspase-14 from cell nuclei to the cytoplasmic compartment when compared to low density attached cultures. Previously, caspase-14 expression has been reported in both the cytoplasmic and nuclear compartments of differentiating keratinocytes *in situ*[26] but has not been linked to specific functional states.

Nuclear import of proapoptotic caspases has recently been described upon induction of apoptosis in certain epithelial cells.[38,39] Of note, however, caspase-14 appears to be exported from the nucleus in forced suspension culture where apoptosis occurs. Furthermore, re-localization of caspase-14 from the nucleus to the cytoplasm also occurs in high density cultures in which apoptosis is negligible. In either setting, caspase-14 re-localization was not associated with cleavage of procaspase-14. These results suggest that shuttling of caspase-14 between cytoplasmic and nuclear pools is not linked to keratinocyte apoptosis. In aggregate, our findings underscore the notion that caspase-14 is an atypical caspase expressed in a wide

range of epithelial cells with no apparent role in executing caspase-dependent apoptotic processes.

Materials and Methods

Cell culture and cell lines

Human neonatal foreskin keratinocyte (HNK) cultures were initiated and propagated in MCDB153 complete medium as described earlier.[33] Spontaneously immortalized human keratinocytes (HaCaT) were kindly provided by Dr. N Fusenig.[34] These cells were maintained in W489 medium[35] consisting of 4 parts MCDB153 and 1 part L15 media supplemented with 2% fetal calf serum. MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were routinely maintained in DMEM medium supplemented with 10% fetal bovine serum (Gibco-BRL, Rockville, MD, USA). Human mammary epithelial cells (HMEC) were obtained from Clonetics/BioWhittaker (Walkersville, MD, USA) and were maintained in MEGM medium Clonetics/BioWhittaker (Walkersville, MD, USA).

Tissues

A total of six normal skin sections, six basal cell carcinomas (BCCs), six squamous cell carcinomas (SCCs) and six psoriatic plaques were obtained from the Department of Pathology, Jefferson Medical College of Thomas Jefferson University (Philadelphia, PA, USA). All samples were obtained from patients who gave informed consent to use excess pathological specimens for research purposes.

RT–PCR, constructs and plasmids

Total RNA from HNK, HaCaT, MCF-7 and SSC-9 cells was extracted using the RNAeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription (RT) was performed at 37°C for 1 h, using oligo-dT primer in a final reaction volume of 25 μl containing 200 U of MMLV reverse transcriptase, 1 $\times$ reverse transcriptase buffer, 40 U of RNAse inhibitor, and 0.5 mM dNTP mix. For each polymerase chain reaction (PCR), 10 μl of first-strand cDNA was added to 100 μl of PCR mix containing 100 ng each of 5′ and 3′ caspase-14 primers or human β-actin primers and 1 U Taq polymerase. Caspase-14 primers were as follows: forward, 5′-ATGAGCAATCCGCGGTCT-TTGG-3′; reverse, 5′-CTGCAGATACAGCCGGAG-3′. β-actin primers were as follows: forward, 5′-GTGGGGCGCCCCAG-GCACCA-3′and reverse, 5′-CTCCTTAATGTCACGCACGAT-TTC-3′. All PCR reagents were purchased from Life Technologies, Inc., (Gaithersburg, MD, USA). Caspase-14 cDNA obtained from MCF-7 cells by PCR was subcloned into the pcDNA3 (Invitrogen) and pMSCV-neo retroviral vector (Clontech, Palo Alto, CA, USA) for expression in mammalian cells. For promoter studies, a 2 kb sequence (nucleotides −2000 to 0) upstream of the 5′ start site of caspase-14 gene (GenBank accession # AC004699) was obtained by PCR amplification of total human genomic DNA (Clontech, Palo Alto, CA, USA) using the following primers: forward, 5′-CCCTCACAATCTTTGGCTCCTCTTTCGT-3′; reverse, 5′-TTCTCCTTTGGTTGTGAGTCCCGGCTC-3′. This segment was subcloned upstream of luciferase cDNA into the *Nhe*I and *Hind*III sites pGL3-basic (Promega, Madison, WI, USA).

Antibodies and generation of anti-human caspase-14 mAb

Mice were immunized with a recombinant purified human caspase-14 protein. Several hybridoma clones reactive with the immunogen were isolated and subcloned using standard techniques. Caspase-14 mAb (clone # 1–71) found to be reactive on Western blots was used in this study. A second caspase-14 mAb (clone # 1-52) found to be non-reactive on Western blots but reactive by ELISA assay was used for immunoprecipitation. Poly(ADP-ribose) polymerase (PARP) Ab was purchased from Biolabs, New England. Involucrin Ab was a generous gift of Dr. PJ Jensen (Dept of Dermatology, University of Pennsylvania). β-actin mAb was purchased from Pharmingen, USA.

Cell treatments and transfection

Suspension cultures were initiated by seeding primary keratinocytes or HaCaT cells onto 0.9% agarose gels prepared using MCDB base medium supplemented with 0.2% (vol/vol) BSA-FAF (bovine serum albumine-fatty-acid-free) and free of protein growth factors unless stated otherwise. After the indicated periods, cell aliquots were removed and processed for Western blot analysis. For UVB exposure subconfluent attached cell cultures were used. The UVB source and dosimetry is described elsewere.[24] Briefly, cells in PBS were exposed to UVB at 100 mJ per cm². After radiation, PBS was removed and fresh medium added. Control cells were subjected to the same treatment without exposure to UVB. Differentiation of keratinocytes was induced by raising the calcium concentration in the medium to 1.2 mM. Postconfluent keratinocyte cultures were obtained by maintaining confluent keratinocyte cultures for three additional days after reaching confluency. The caspase inhibitor Ac-DEVD-CHO (N-Acetyl-Asp-Glu-Val-Asp-CHO) was purchased from Biomol (Plymouth Meeting, PA, USA) and used at 120 μM. The histone acetylation inhibitor trichostatin A was purchased from Sigma (St. Louis, MO, USA) and used at 500 μM for 48 h. Mimosine, thymidine, and nocodazole were also purchased from Sigma and used respectively at 400 μM, 2 mM and 0.400 μg for 24 h for cell-cycle synchronization. Transfection of 293 cells with expression construct for caspase-14 was carried out using LipofectAMINE (Life Technologies, Inc.) as recommended by the manufacturer.

Reporter gene assays

For determination of promoter activity, 1.5 μg of the pGL3 caspase-14 putative promoter construct was transfected into 3×10^5 HNK or MCF-7 cells seeded in their appropriate medium in six-well plates one day prior to transfection. Transfections were performed using Fugene 6 transfection reagent (Roche Molecular Biochemicals, Germany) as recommended by the manufacturer. Twenty-four hour post-transfection, cell lysates from HNK and MCF-7 cells were extracted using the reporter lysis buffer (RLB) (Promega, Madison, WI, USA) to assay luciferase activity. Transfected HNK and MCF-7 cells were also placed on 0.9% agarose to determine the promoter activity at different time points of forced suspension culture. Furthermore, promoter activity was assessed in transfected cells, which were allowed to reach postconfluency within 5 days after transfection. Finally, the effect of Ca²⁺ on promoter activity was determined by exposing transfected cells at 1.2 mM Ca²⁺ for 48 h prior to reporter gene assays. Cell lysates were assayed for luciferase expression using the luciferase assay system (Promega, Madison, WI, USA). The protein concentration of cell lysate (10 μl) was determined using the Bio-rad protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Quantitative determination of luciferase activity (measured as relative light units) in the cell lysates was normalized to the protein concentration of cell lysate.[36]

Retroviral infection

Transient transfection of the packaging cell line Phoenix (GP Nolanís laboratory, Stanford University Medical Center, Stanford, CA, USA) was carried out with 30 μg of plasmid DNA by calcium posphate precipitation using the ProFection system (Promega, Madison, WI, USA). The empty pMSCV-neo plasmid was used to normalize for equal amount of transfected DNA. Supernatant was harvested 48 h after transfection, filtered (0.45 um pore diameter) and frozen at $-80°C$. For infection, 1×10^6 HaCaT cells were seeded in a six-well plate. After 24 h, cells were incubated with 2 ml of the virus supernatant stock, including polybrene at 4 μg/ml and centrifuged for 45 min at 1800 r.p.m. at 32°C. The cells were incubated at 32°C for 2 h in a CO^2 incubator and then subjected to another cycle of infection using fresh retrovirus enriched medium. After the infection the cells were washed and given fresh medium W489. On the fourth day after infection, cells were subjected to selection with 0.8 mg of G418/ml (Life Technologies, Inc., Gaithersburg, MD, USA). While in the control non-transfected cells extensive cell death occurred within two weeks, the infected HaCaT cells showed no signs of G418-induced death, indicating a high proportion of transduced cells. The expression of the transgene was verified by Western blotting of transfected cell lysates.

Determination of cell cycle distribution

2×10^6 MCF-7 cells were harvested and fixed in 70% ethanol. Fixed cells were washed with phosphate-buffered saline (PBS), incubated with 1 μg of RNase A per ml (Sigma, St. Louis, MO, USA) for 30 min at 37°C and stained with propidium iodide (Boehringer Mannheim, Indianapolis, IN, USA.). The stained cells were analyzed on a FACscan flow cytometer for relative DNA content and the percentage of cells in each cell cycle phase was determined by using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, CA, USA).

Western blot analysis and immunoprecipitation

For caspase-14 immunoblotting, cells were washed once in ice-cold PBS and lysed in non-reducing Laemmli buffer containing protease inhibitor (Complete cocktail, Roche Molecular Biochemicals, Indianapolis, IN, USA) followed by boiling for 5 min. Protein concentration of each sample was determined by Bio-rad protein assay reagent (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Sixty μg of proteins were loaded on 12.5% SDS-polyacrylamide gel, transferred to PVDF membrane and blocked in 1% nonfat powdered milk in PBS-Tween 20 overnight at 4°C. The membrane was incubated with human caspase-14 mAb (clone # 1–71)(1:10000) in 1% nonfat powdered milk in PBS-Twin 20 for 1 h. After washing, the membrane was incubated with 1:3000 diluted antimouse HRP Ab (Amersham Pharmacia Biotech). Following several washes, the blots were developed with ECL reagents (Amersham Pharmacia Biotech) according to manufacturer's protocol. For caspase-14 immunoprecipitation, MCF-7 or, vector- or caspase-14-transfected 293 cells were lysed 24 h after transfection in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl containing 0.5% Nonidet P-40, 10 μg/ml leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. The clarified lysates were preabsorbed on protein G-Sepharose (Amersham Pharmacia Biotech) and then incubated with human caspase-14 mAb (clone # 1–52) for 2 h, followed by protein G-Sepharose agarose IgG beads. Immune complexes were washed in the lysis buffer, eluted by boiling in SDS sample buffer, fractionated by SDS–PAGE and then immunoblotted with caspase-14 mAb (clone # 1–71).

Immunofluorescence and immunohistochemistry

HNK, MCF-7 cells were grown on coverslips and, where indicated, spun down in a swing-out rotor at $3000 \times g$. The cells were fixed and permealized with 3.7% of paraformaldeyde for 10 min at room temperature. Unspecific binding sites were blocked for 30 min with 10% NGS in 120 mM NaCl, 6 mM KCl, 1.2 mM MgCl2, 1 mM CaCl2 and 25 Mm HEPES, pH 7.4. The cells were stained with caspase-14 - mAb (clone # 1–71) diluted in this blocking solution (1:400) for 1 h at room temperature, washed three times with PBS before adding a streptavidin-FITC conjugate antimouse secondary Ab (1:200) (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The specifiticy of the staining was confirmed by performing the appropriate controls including incubations omitting the primary Ab and incubation of the secondary antibody with fivefold excess of the purified caspase-14 protein. The cells were washed, mounted and analyzed by confocal laser scanner microscopy. For immunochemistry, routine deparaffinization of all sections mounted on positive charge slides was carried out according to standard procedures,[37] followed by rehydration through an ethanol series. The slides were immersed in citrate buffer (0.01 mol/L sodium citrate, pH 6.0) and heated in a microwave oven at 600 W to enhance antigen retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase in methanol for 30 min. The sections were then incubated with anti-caspase-14 mAb (clone # 1–71), 1:7500 dilution overnight at room temperature. In the negative control, the mAb was omitted and replaced by PBS. After that, sections were treated with biotinylated antimouse antibody and streptavidin-biotin-peroxidase (His-tostain-SP Kit, Zymed Laboratories). Antibody localization was detected with diaminobenzidine as a chromogen substrate. Finally, sections were washed in distilled water and weakly counterstained with Harryís modified hematoxylin.

Acknowledgements

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