Differentiation and Apoptosis in Human Immortalized Sebocytes

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Increased cell volume, accumulation of lipid droplets in the cytoplasm, and nuclear degeneration are phenomena indicating terminal differentiation of human sebocytes followed by holocrine secretion and cell death. The molecular pathways of natural and induced sebocyte elimination are still unknown, however. In this study, SZ95 sebocytes were found to exhibit DNA fragmentation after a 6 h culture followed by increased lactate dehydrogenase release after 24 h, indicating cell damage. With the help of morphologic studies and using Oil Red detection of cellular lipids, cell enlargement, accumulation of lipid droplets in the cytoplasm, and nuclear fragmentation could be observed under treatment with arachidonic acid. Staurosporine, a potent inhibitor of phospholipid Ca^{2+}-dependent protein kinase, increased externalized phosphatidylserine levels on SZ95 sebocytes, detected by annexin V/propidium iodide flow cytometry, as early as after 1 h, whereas dose-dependent reduction of *bcl-2* mRNA and protein expression, enhanced DNA fragmentation, and increased caspase 3 levels, detected by caspase 3 inhibitor/propidium iodide flow cytometry, were found after 6 h of treatment. SZ95 sebocyte death was detected as early as after 6 h of SZ95 sebocyte treatment with high staurosporine concentrations (10^{-6}–10^{-5} M). 5α-Dihydrotestosterone (10^{-8}–10^{-5} M) did not affect externalized phosphatidylserine levels and DNA fragmentation in SZ95 sebocytes but slightly decreased lactate dehydrogenase cell release. Neither acitretin nor 13-*cis* retinoic acid (10^{-8}–10^{-5} M) affected externalized phosphatidylserine levels, DNA fragmentation, and lactate dehydrogenase cell release, despite the increased caspase 3 levels under treatment with 13-*cis* retinoic acid. The combined staurosporine and 13-*cis* retinoic acid treatment enhanced DNA fragmentation in SZ95 sebocytes to the same magnitude as in cells only treated with staurosporine. In conclusion, SZ95 sebocytes *in vitro* undergo apoptosis, which can be enhanced by the terminal differentiation inductor arachidonic acid or by staurosporine and leads to cell death. 5α-Dihydrotestosterone inhibits SZ95 sebocyte death without involving apoptotic pathways, and retinoids did not affect the programmed death of human sebocytes. The latter result fits well with the currently reported inability of normal skin cells to undergo apoptosis after treatment with retinoids, in contrast to their malignant counterparts. ***Key words: apoptosis/cell line/differentiation/retinoids/sebaceous gland. J Invest Dermatol 120:175–181, 2003***

Increased cell volume, accumulation of lipid droplets in the cytoplasm, and nuclear degradation are phenomena indicating terminal differentiation of human sebocytes *in vitro* and *in vivo* (Zouboulis *et al*, 1994). These events are followed by holocrine secretion and cell death. 13-*cis* Retinoic acid (13cRA) and 5α-dihydrotestosterone (5α-DHT) are antipodal regulators of sebocyte activity. 13cRA inhibits proliferation, lipid synthesis, and differentiation of human sebocytes *in vivo* and *in vitro* (Landthaler *et al*, 1980; Strauss *et al*, 1980; Zouboulis *et al*, 1991; 1994). In contrast, 5α-DHT stimulates proliferation of human sebocytes *in vitro* (Akamatsu *et al*, 1992; Zouboulis *et al*, 1999) and the 5α-DHT precursors dehydroepiandrosterone, androstenedione, and testosterone enhance sebum secretion in humans (Pochi and Strauss, 1969; Diamond *et al*, 1996).

The molecular pathways of natural and induced sebocyte elimination are still unknown. Therefore, this study was conducted to investigate the mode of elimination after terminal differentiation of human sebocytes *in vitro*. In addition, we assessed the modifying effects of 13cRA and 5α-DHT on natural elimination of human sebocytes *in vitro*.

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Abbreviations: 5α-DHT, 5α-dihydrotestosterone; cDNA, first strand DNA; DMSD, dimethyl sulfoxide; FBS, Fetal bovine serum; LDH, lactate dehydrogenase; 13cRA, 13-*cis* retinoic acid; RT-PCR, reverse transcription polymerase chain reaction.

MATERIALS AND METHODS

Cell cultures Immortalized human sebocytes (SZ95 sebocytes) (Zouboulis *et al*, 1999) at passages 60–70 were grown in Sebomed® culture medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 50 μg per ml gentamicin, and 5 ng per ml epidermal growth factor (all from Biochrom) in a humidified atmosphere

containing 5% CO_2 at 37°C. The medium was changed every 3 d. Subconfluent cell cultures were treated with 0.1% trypsin/0.02% ethylenediamine tetraacetic acid solution and were then propagated in culture medium as described above.

Chemicals 13CRA, acitretin (both gifts from Hoffmann-La Roche, Basel, Switzerland), 5α-DHT, and staurosporine (both purchased from Sigma-Aldrich, Deisenhofen, Germany) were dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) to give final concentrations of 10^{-8}–10^{-5} M. The final concentration of DMSO in medium without and with retinoids, 5α-DHT, or staurosporine was 0.2%. Retinoids and staurosporine were handled under dimmed yellow light. Arachidonic acid (Sigma-Aldrich) was dissolved in ethanol to give a final concentration of 10^{-4} M. The final concentration of ethanol in medium without and with arachidonic acid was 0.1%.

Oil Red staining of intracellular lipids SZ95 sebocytes, seeded onto eight-well multitest glass slides (ICN, Aurora, OH), were treated with arachidonic acid (10^{-4} M) for 48 h. Cells treated with vehicle solution (0.1% ethanol) served as controls. After treatment SZ95 sebocytes were washed with phosphate-buffered saline without Ca^{2+} and Mg^{2+}, fixed in Baker's formol for 5 min, washed twice in aqua bidest for 2 min each, incubated in 60% isopropanol for 5 min, stained in freshly prepared Oil Red solution [0.5% Oil Red (Kodak, Rochester, NY) in isopropanol and then diluted in aqua bidest 6 : 4 vol/vol] for 5 min, and washed in aqua bidest for 1 min. Nuclei were counterstained with hematoxylin for 20 s. Stained cells were mounted in Dako mounting medium (Dako, Glostrup, Denmark).

DNA fragmentation assay SZ95 sebocyte cultures seeded at a concentration of 3×10^4 cells per well in 24-well culture plates (Nunc, Wiesbaden, Germany) were maintained upon subconfluency and were then treated with the chemicals tested in medium containing 3% FBS for 1–24 h. DNA fragmentation was detected using a photometric enzyme immunoassay for the qualitative and quantitative evaluation of cytoplasmatic histone-associated DNA fragments (Cell death detection ELISA^{Plus}; Boehringer-Mannheim, Mannheim, Germany). This sandwich enzyme immunoassay principle determines mononucleosomes and oligonucleosomes in the cytoplasm of apoptotic cells that are characteristic for DNA degradation *in vitro*. After staurosporine treatment for 1, 6, 12, and 24 h, 13cRA or 5α-DHT treatment for 6 and 24 h, and acitretin treatment for 24 h at 37°C in a 5% CO_2 atmosphere, the culture plates were centrifuged for 10 min at $200 \times g$. Subsequently, the supernatants were removed, the cell pellets were resuspended in lysis buffer, and the culture plates were incubated for 30 min on a plate shaker at 250 rpm at room temperature. The lysates were then centrifuged for 10 min at $200 \times g$. Twenty microliters of each lysate were transferred into streptavidine-coated microtiter plates, 80 µl of the immunoreagent mix were added, and the microtiter plates were incubated for 2 h. The plates were then rinsed thrice with 250 µl per well incubation buffer, 100 µl substrate solution were added, the plates were incubated on a plate shaker at 250 rpm for 20 min, and absorbance of the solutions was measured at 405 nm.

Lactate dehydrogenase (LDH) assay SZ95 sebocyte cultures seeded at a concentration of 3×10^4 cells per well in 24-well culture plates (Nunc) were maintained upon subconfluency and were then treated with the chemicals tested in medium containing 3% FBS for 1–24 h. Cell necrosis was determined using a colorimetric assay for quantification of cell death and cell lysis (Cytotoxicity detection kit; Boehringer-Mannheim) based on LDH released from the cytoplasm of damaged cells into the supernatant. After incubation with the chemicals tested cell culture plates were centrifuged for 10 min at $200 \times g$. One hundred microliters of the culture supernatant were then transferred into optically clear 96-well flat bottom microtiter plates and 100 µl of the incubation mixture were added according to the manufacturer's protocol. Absorbance was measured after 20 min at 490 nm.

Detection of apoptosis-related molecules by fluorescence-activated cell sorting analysis For flow cytometry, cell cultures were dissociated from culture dishes with accutase (PAA, Cölbe, Germany). The expression of the apoptosis-related externalized phosphatidylserine and of the intracellular proteins caspase 3, bcl-2, and bax was analyzed by two-color fluorescence-activated cell sorting on a Becton-Dickinson flow cytometer FACS-Calibur. For detection of externalized phosphatidylserine, subconfluent SZ95 sebocytes were maintained in medium containing 3% FBS. After stimulation, the harvested cells were stained with annexin V fluorescein and propidium iodide (Bender, Vienna, Austria). The method is based on the interaction of the anticoagulant annexin V with externalized phosphatidylserine (Takahashi *et al*, 2000; Shvedova *et al*, 2002). For detection of active caspase 3, after stimulation the harvested cells were stained with a specific carboxyfluorescein-labeled caspase inhibitor and with propidium iodide (CaspaTag Caspase-3 DEVD Activity Kit; Intergen, New York, NY) (Ekert *et al*, 1999). The method is based on the covalent binding of the active effector caspase 3 to the caspase inhibitor. For bcl-2 and bax detection, after stimulation the harvested cells were fixed with paraformaldehyde, permeabilized with saponin (Cytofix/Cytoperm Kit; PharMingen, San Diego, CA) (Sander *et al*, 1991), and then stained with phycoerythrin-conjugated hamster antihuman bcl-2 monoclonal antibody (PharMingen) and fluorescein-isothiocyanate-labeled rabbit antihuman bax monoclonal antibody (Dako). All fluoresceins were excited at 488 nm with an argon laser. The fluorescein emission of annexin V fluorescein and carboxyfluorescein-labeled caspase inhibitor was detected at 530 nm, of propidium iodide at 585 nm, of isothiocyanate at 525 nm, and of phycoerythrin at 575 nm. The overlapping emission of annexin V fluorescein and of propidium iodide as well as of isothiocyanate and phycoerythrin was corrected by electronic compensation. Ten thousand cells were assessed in each sample. CELLQuest software (Becton-Dickinson) was used to establish four regions (two for single-positive cells, one for double-positive, and one for double-negative cells) according to the intensity of fluorescence of the nonspecific isotypic antibody controls.

Quantification of caspase-3-bound caspase inhibitor in SZ95 sebocytes by fluorescence spectroscopy SZ95 sebocytes without or with 13cRA (10^{-7} M) were directly labeled in 96-well plates with carboxyfluorescein-labeled caspase inhibitor and after 24 h of treatment were analyzed on a Molecular Devices SPEKTRAmax GEMINI fluorescence plate reader at 485 nm excitation and 535 nm emission wavelengths.

RNA isolation For RNA isolation, SZ95 sebocytes were seeded at a concentration of 3×10^6 cells in 75 cm^2 culture bottles and treated with 13cRA and staurosporine (10^{-7} M) added to the cells in a culture medium containing 3% FBS for 6, 12, 24, and 48 h (13cRA) and 30 min, 1, 2, 3, 4, 5, 6, and 12 h (staurosporine). RNA extraction was carried out using the RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was photometrically measured in a Pharmacia GeneQuant II spectrophotometer and stored at –20°C until use.

Oligonucleotide sequences for reverse transcription polymerase chain reaction (RT-PCR) The following oligonucleotides were used for mRNA detection by RT-PCR. The appropriate sequences of mRNA, cDNA, or exons of the genomic DNA were found in the GenBank data bank via Internet. The annealing temperature used and the length of the expected products are given in parentheses. *Bcl-2*-specific oligonucleotides were synthesized by Metabion (Martinsried, Germany), and *bax*- and β-*actin*-specific oligonucleotides by TIB MOLBIOL (Berlin, Germany) as follows: *bcl-2* forward primer 5′-ACG GTG GTG GAG GAG CTC TT, reverse primer 5′-TGG CTC AGA TAG GCA CCC AG (annealing temperature 65°C for 30 s, 320 bp); *bax* forward primer 5′-CGA GTG GCA GCT GAC ATG TTT, reverse primer 5′-TTC TTC CAG ATG GTG AGC GAG (annealing temperature 60°C for 30 s, 290 bp); β-*actin* forward primer 5′-AGC CTC GCC TTT GCC GA, reverse primer 5′-CTG GTG CCT GGG GCG (annealing temperature 67°C for 60 s, 167 bp).

RT-PCR First strand DNA (cDNA) was reverse transcribed from 5 µg of total RNA using a T-Primed First-Strand Ready to Go kit (Pharmacia Erlangen, Germany). A 33 µl aliquot of the final product was dissolved in 67 µl H_2O. 5 µl (*bcl-2* and *bax*) or 2 µl (β-*actin*) cDNA template was amplified with specific primers of each gene tested in 25 µl final PCR mixture, which contained 1 × PCR buffer, 1.5 mM $MgCl_2$ (both from Perkin Elmer, Branchburg, NJ), 0.2 mM dNTP mix (Boehringer-Mannheim), 0.03 U per µl (*bcl-2*), 0.02 U per µl (*bax*), or 0.05 U per µl (β-*actin*) Taq polymerase (Perkin Elmer), and 0.8 µM (*bcl-2*), 0.4 µM (*bax*), or 0.5 µM (β-*actin*) of each of the forward and reverse primers. cDNA amplification was carried out for 40 cycles (*bcl-2*), 30 cycles (*bax*), or 35 cycles (β-*actin*) in a hot-top Stratagene robocycler using step-cycle programs that consisted of 30 s denaturation at 94°C, annealing times and annealing temperatures as described above, and 30 s (*bcl-2*), 15 s (*bax*), or 60 s (β-*actin*) polymerization at 72°C. The final extension was allowed to proceed for 10 min at 72°C. Ten microliters of PCR products were subjected to electrophoresis on 1% agarose gels that contained 0.25 µg per ml ethidium bromide in 1 × triacetate electrophoresis buffer. The gels were photographed under ultraviolet light and quantitated by video densitometry using the computer program Tina Version 2.09, Copyright 1993.

Presentation of the data and statistical analysis All data are presented as mean values ± standard deviation. Three or more consecutive

experiments were performed with each assay. Statistical significance of the data was evaluated by the two-sided Student *t* test. Mean differences were considered to be significant for $p < 0.05$.

RESULTS

Spontaneous apoptosis and lysis of SZ95 sebocytes Significant DNA fragmentation in SZ95 sebocytes was already detectable as early as after 6 h in culture, whereas significant rates of cell lysis, detected by LDH release, could only be assessed at 24 h ($n = 3$; **Fig 1**). These findings indicate that SZ95 sebocytes undergo spontaneous apoptosis *in vitro*, which may be responsible for their natural elimination. An increase of SZ95 sebocyte volume with time in culture was observed in as many as approximately 25% SZ95 sebocytes in control cultures. Only a few Oil-Red-stained intracellular lipid droplets could be identified, however (**Fig 2*A*, *B***).

Enhancement of SZ95 sebocyte differentiation and apoptosis by arachidonic acid To determine whether cytoplasmic lipid accumulation, a major marker of sebocyte differentiation, is enhanceable, and whether sebocyte differentiation is associated with apoptosis, arachidonic acid was added to the cultures for 48 h. Arachidonic acid and its metabolites have been suggested to play a role in the physiology of epithelial cell differentiation (Henke *et al*, 1986; Keeney *et al*, 1998), including human sebocytes (Zouboulis, 2000). SZ95 sebocytes treated with arachidonic acid were found to produce marked amounts of lipids, which could be visualized by Oil Red staining as cytoplasmic droplets (**Fig 2*C*, *D***), whereas most control cells were Oil Red negative (**Fig 2*A*, *B***). Moreover, fragmented nuclei, characteristic for apoptotic cells, were observed in large SZ95 sebocytes that exhibited abundant Oil Red positive droplets in their cytoplasm after treatment with arachidonic acid (**Fig 2*D***).

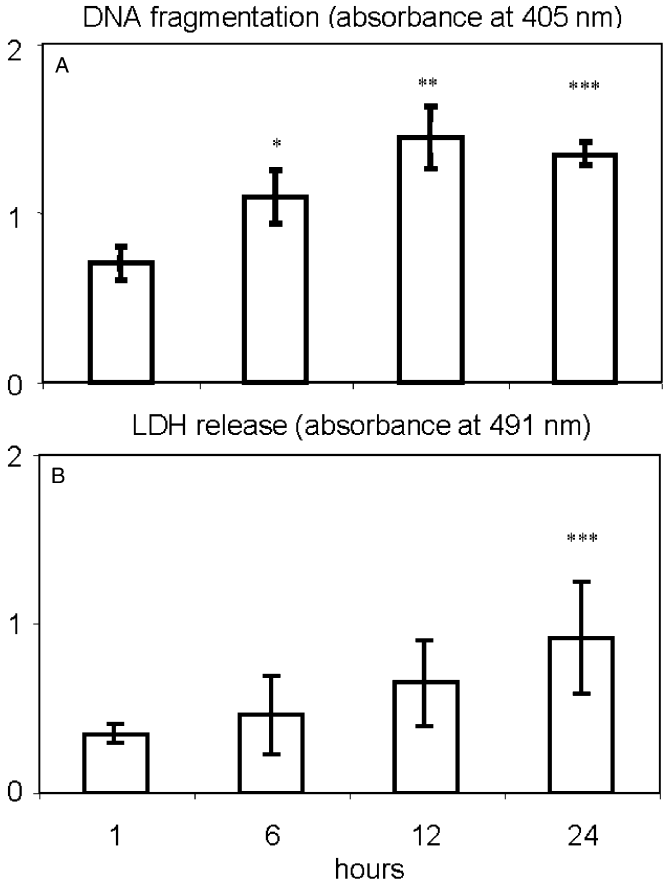

Figure 1. Spontaneous apoptosis and necrosis in SZ95 sebocytes. (*A*) Significant, time-dependent DNA fragmentation in SZ95 sebocytes. (*B*) Increased LDH activity released from the cytoplasm of damaged SZ95 sebocytes into the supernatant after 24 h in culture. The values represent mean ± standard deviation of three consecutive experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

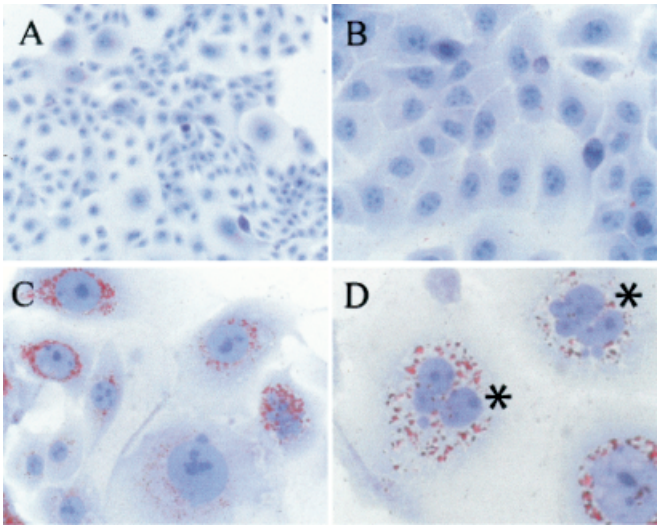

Figure 2. Lipid synthesis and fragmentation of nuclei in SZ95 sebocytes after treatment with arachidonic acid. Visualization of lipids in SZ95 sebocytes by Oil Red staining. Cells were treated with vehicle solution as control (*A*, *B*) or with 100 μM arachidonic acid for 48 h (*C*, *D*). Nuclei were counterstained with hematoxylin. Note markedly Oil Red positive cells as well as cells with fragmented nuclei in arachidonic-acid-treated cultures (*). *Magnifications*: (*A*) 160 ×; (*B*, *C*) 630 ×; (*D*) 1260 ×.

Enhancement of apoptosis by staurosporine To further determine whether apoptosis is enhanceable in cultured SZ95 sebocytes, the cells were treated with staurosporine, a potent inhibitor of phospholipid Ca^{2+}-dependent protein kinase C. Staurosporine was administered to SZ95 sebocyte cultures at concentrations of 10^{-8}–10^{-5} M for 1, 6, 12, and 24 h and the results were compared with those of untreated controls ($n = 3$). DNA fragmentation already increased at a concentration of 10^{-7} M staurosporine after 1 h of treatment compared to the control, indicating an enhancement of apoptosis by staurosporine. The highest level of DNA fragmentation was observed after 6 h of incubation (**Fig 3*A***). Increased levels of lyzed cells, as detected by increased LDH release, were found first at 6 h at high staurosporine concentrations (10^{-6} and 10^{-5} M) (**Fig 3*B***), indicating a time interval between detection of apoptotic signals and cell lysis (**Figs 1, 2**).

Effects of 5α-DHT on DNA fragmentation and LDH release of SZ95 sebocytes 5α-DHT (10^{-8}–10^{-5} M) did not affect DNA fragmentation of SZ95 sebocytes compared to controls after 6 and 24 h of treatment ($n = 3$) (**Fig 4*A***). In contrast, it was able to slightly decrease LDH release levels and therefore cell death (**Fig 4*B***).

Effects of retinoids on DNA fragmentation and LDH release of SZ95 sebocytes 13cRA ($n = 11$) and acitretin ($n = 7$), a monoaromatic retinoid, which is barely active on the proliferation and lipid synthesis of primary human sebocytes and SZ95 sebocytes *in vitro* (Zouboulis *et al*, 1991; 1999), neither affected DNA fragmentation nor LDH release from SZ95 sebocytes after 6 and 24 h (13cRA) and 24 h (acitretin) of treatment at concentrations of 10^{-8}–10^{-5} M.

To further analyze the negative results with 13cRA, SZ95 sebocytes were treated with combined 13cRA (10^{-8} M or 10^{-7} M) and staurosporine (10^{-7} M) for 6 h. The combined treatment

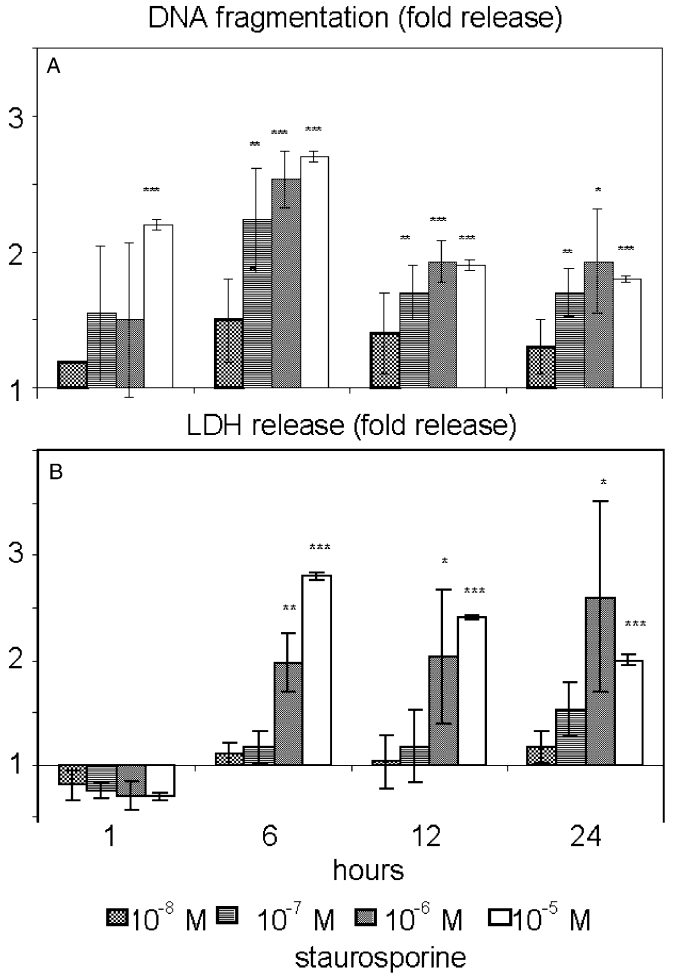

Figure 3. Enhancement of sebocyte apoptosis by staurosporine in a time- and dose-dependent manner. (*A*) The highest levels of DNA fragmentation under staurosporine treatment in SZ95 sebocytes were detected after 6 h of incubation. (*B*) Staurosporine exhibited a cytotoxic effect on SZ95 sebocytes at concentrations of 10^{-6} and 10^{-5} M after 6–24 h of incubation as detected by the LDH assay. The values represent the y-fold increase of the mean ± standard deviation (where y=the value of the y axis) of three consecutive experiments, whereas control = 1. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

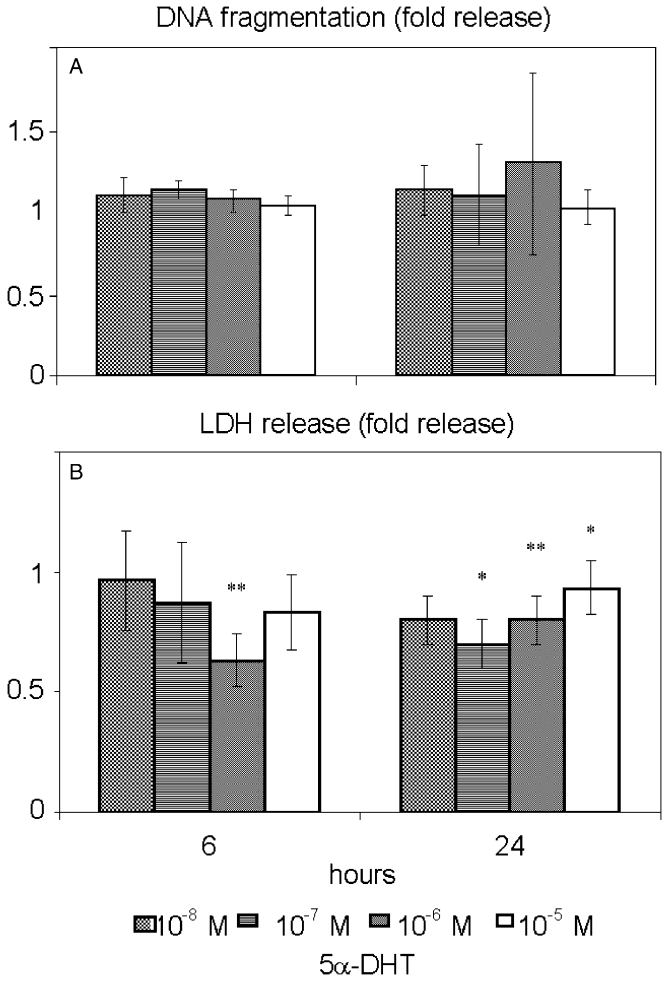

Figure 4. Effects of 5α-DHT on DNA fragmentation and LDH release by SZ95 sebocytes. 5α-DHT did not affect DNA fragmentation (*A*) but significantly inhibited LDH release of SZ95 sebocytes compared to controls after 6 h and especially after 24 h of treatment (*B*). The values represent the y-fold increase of the mean ± standard deviation (where y=the value of the y axis) of three consecutive experiments, whereas control = 1. $^{*}p < 0.05$, $^{**}p < 0.01$.

induced a DNA fragmentation in SZ95 sebocytes, which was of the same magnitude as that of SZ95 sebocytes treated with staurosporine alone ($n = 3$) (**Fig 5*A***). In addition, as with staurosporine alone, the combined 13cRA/staurosporine regimen did not increase LDH release from SZ95 sebocytes at the concentrations tested in this experiment (**Fig 5*B***).

Effects of 5α-DHT, retinoids, and staurosporine on phosphatidylserine externalization in SZ95 sebocytes To corroborate the results obtained using the DNA fragmentation assay, annexin V/propidium iodide flow cytometry of SZ95 sebocytes was performed in cultures treated with 5α-DHT (10^{-7} M), acitretin (10^{-7} M), 13cRA (10^{-7} M), and staurosporine (10^{-7} M and 3×10^{-7} M) for 1–24 h. In normal viable cells, phosphatidylserine is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids in cells occur leading to exposure of phosphatidylserine on the cell surface. *In vitro* detection of externalized phosphatidylserine can be achieved through interaction with the anticoagulant annexin V, whereas propidium iodide identifies dead cells (Takahashi *et al*, 2000; Shvedova *et al*, 2002). Rates of 15 ± 3% to 19 ± 4% SZ95 sebocytes were annexin V positive at all time points tested (**Fig 6**). Whereas staurosporine significantly increased the rate of annexin V positive SZ95 sebocytes in a dose-dependent manner ($p < 0.001$), in 5α-DHT-, acitretin-, and 13cRA-treated cultures the rates of annexin V positive cells were similar to those of the controls.

Effect of 13cRA and staurosporine on caspase 3 expression in SZ95 sebocytes Caspase 3 may exhibit an important role in propagating the caspase cascade, in addition to its role as an effector caspase within the cell death program (Slee *et al*, 1999). Therefore, we performed caspase 3/propidium iodide flow cytometry of SZ95 sebocytes under treatment with 13cRA (10^{-7} M) or staurosporine (10^{-7} M) for 6 or 24 h. As also found by the combination of DNA fragmentation/LDH release assays, in untreated control SZ95 sebocyte cultures both caspase 3 positive/propidium iodide negative viable cell rates as well as caspase 3 positive/propidium iodide positive dead cell rates increased between 6 and 24 h from 6% to 23% and from 6% to 10%, respectively (**Fig 7**). Interestingly, 13cRA increased the caspase 3 positive cell rate in comparison to the control (35 ± 5%) as detected by fluorospectrometric analysis but only slightly affected the rate of dead cells. Staurosporine exhibited a marked induction of caspase 3 positive viable cells (**Fig 7**).

Influence of 13cRA and staurosporine on *bax* and *bcl-2* mRNA levels and protein expression To further elucidate

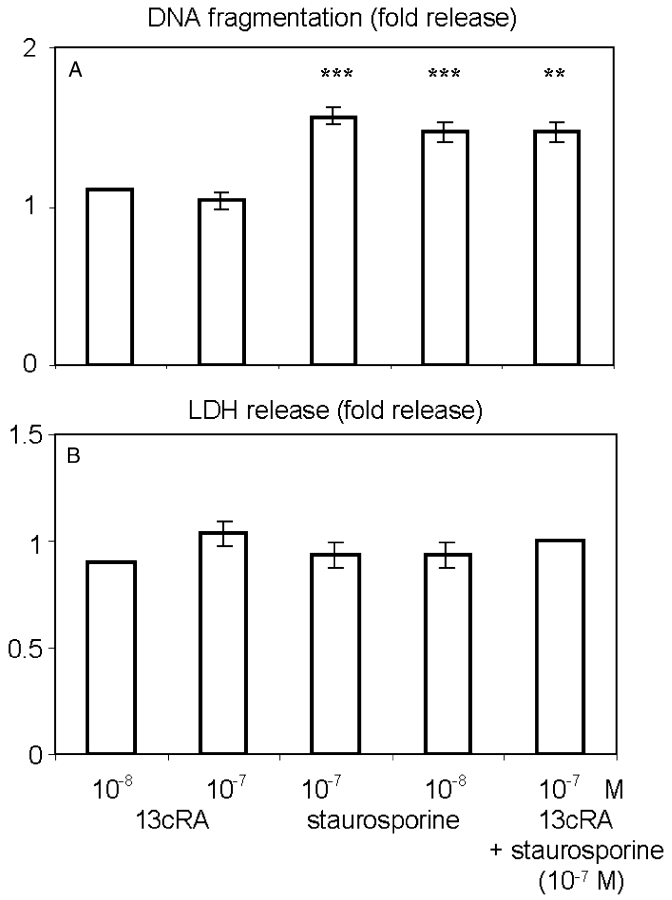

Figure 5. Effects of combined 13cRA/staurosporine treatment on DNA fragmentation and LDH release by SZ95 sebocytes. 13cRA (10^{-8} M and 10^{-7} M) and staurosporine (10^{-7} M) induced DNA fragmentation in SZ95 sebocytes to the same magnitude as SZ95 sebocytes treated with staurosporine alone ($n=3$) (*A*) but did not affect LDH release (*B*). The values represent the y-fold increase of the mean ± standard deviation (where y=the value of the y axis) of three consecutive experiments, whereas control = 1. $^{**}p<0.01$, $^{***}p<0.001$.

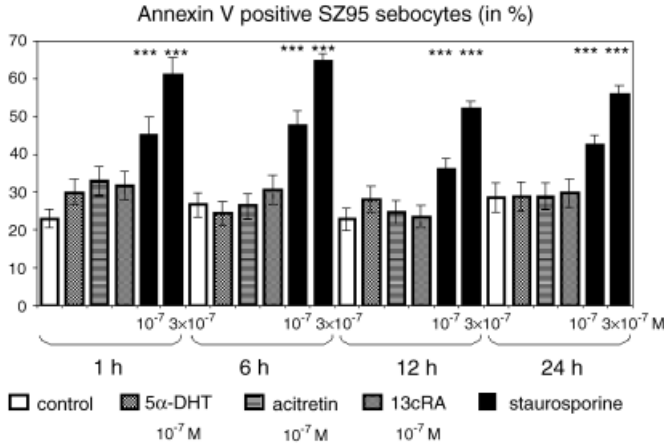

Figure 6. Effects of retinoids, 5α-DHT, and staurosporine on phosphatidylserine externalization in SZ95 sebocytes. Staurosporine (10^{-7} and 3×10^{-7} M) enhanced phosphatidylserine externalization, and consequently apoptosis, in SZ95 sebocytes as assessed by annexin V detection using flow cytometry. In contrast, 13cRA (10^{-7} M), acitretin (10^{-7} M), and 5α-DHT (10^{-7} M) induced detectable annexin V rates were similar to those in control cultures. The values represent mean ± standard deviation of three consecutive experiments. $^{***}p<0.001$.

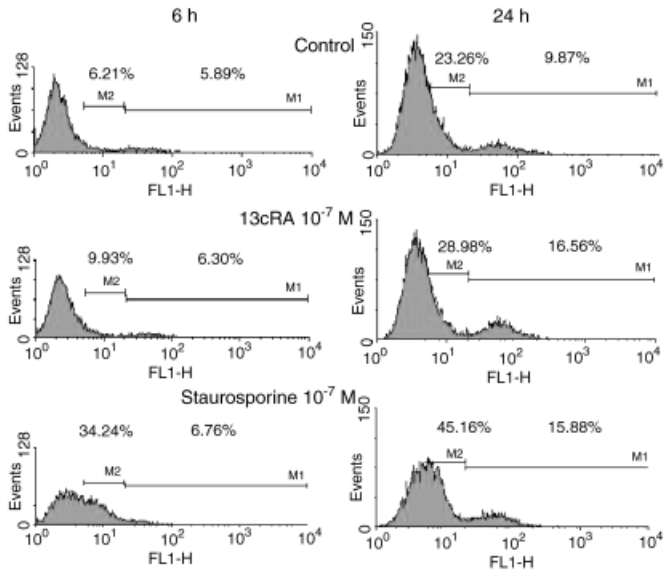

Figure 7. Effects of 13cRA and staurosporine on caspase 3 expression in SZ95 sebocytes. Caspase 3 expression in SZ95 sebocytes was analyzed by flow cytometry; marker 1 (M1) represents caspase 3 positive dead cells and marker 2 (M2) caspase 3 positive vital cells [100 − (M1+M2): caspase negative cells]. Dead cells were detected by staining with propidium iodide. Data are representative of three experiments. FL1, excitation 488 nm, emission 530 nm.

the mechanism of apoptosis induced in SZ95 sebocytes by staurosporine but not by 13cRA, *bcl-2* and *bax* mRNA levels were evaluated by RT-PCR and their protein expression was assessed by flow cytometry ($n=3$). Decreased *bcl-2* mRNA expression at 4–6 h of staurosporine treatment and no significant change of *bax* mRNA levels at the same time points were detected (**Fig 8**). The ineffectiveness of 13cRA in enhancing apoptosis in SZ95 sebocytes was confirmed by the finding that neither gene was affected at the mRNA level by 13cRA at any time point tested. In addition, as with staurosporine alone, decreased *bcl-2* mRNA expression at 4–6 h of 13cRA/staurosporine treatment was found.

Decreased bcl-2 expression at the protein level was also found under 6 h staurosporine treatment (10^{-7} M; 0.7 ± 0.3 compared to control), whereas bax expression was not affected. 13cRA (10^{-7} M) exhibited no effect on the expression of both proteins tested. The bax/bcl-2 ratio was increased by staurosporine (10^{-7} M; $p<0.01$) and the combined staurosporine (10^{-7} M)/13cRA (10^{-7} M) treatment ($n=2$; $p<0.05$) at similar levels, whereas it was not affected by 13cRA (**Fig 9**).

DISCUSSION

Terminal differentiation and apoptosis are different genetically programmed cell events both followed by cell death as an essential feature of eukaryotic cell life. Terminal differentiation of human sebocytes is associated with lipid synthesis, accumulation of lipid droplets in the cytoplasm, increase of cell volume, and signs of nuclear degeneration and is followed by cell burst and death. Up to the present time, it was unclear whether terminal sebocyte differentiation is followed by apoptosis (Zouboulis *et al.*, 1998). This study provides an explanation of the pattern of natural sebocyte death by demonstrating that SZ95 sebocytes in culture undergo apoptosis. Furthermore, apoptosis of SZ95 sebocytes *in vitro* can be enhanced in association with increased rates of terminal sebocyte differentiation, as detected under treatment with arachidonic acid.

The capacity of the cells to undergo apoptosis is controlled by several factors, including phospholipid signaling (Takahashi *et al*, 2000; Shvedova *et al*, 2002), activation of the caspase family (Ekert

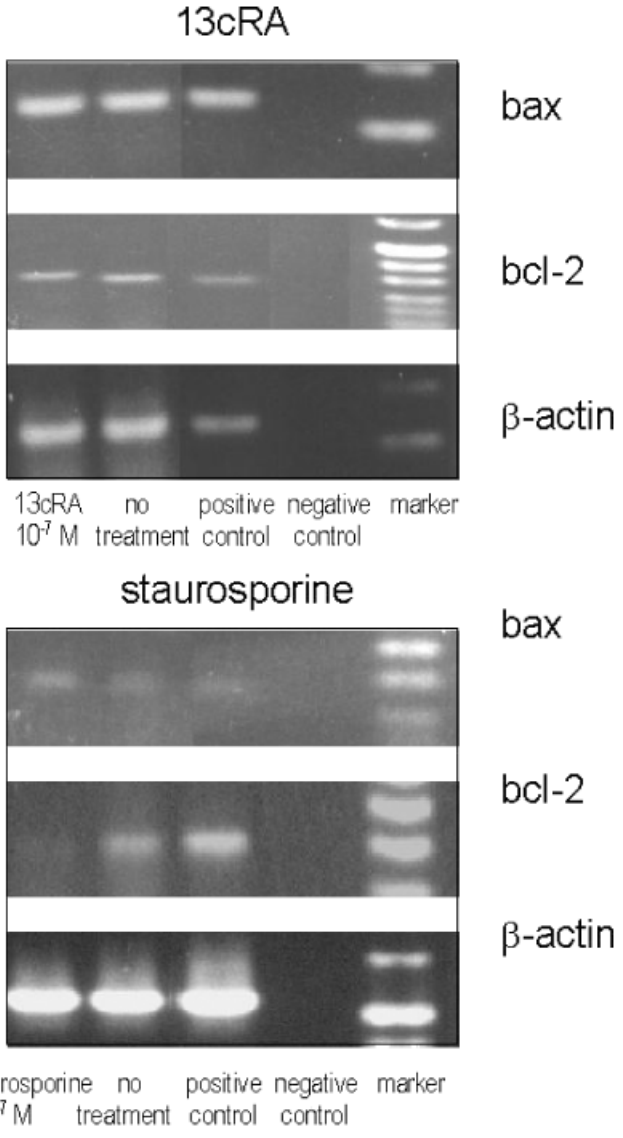

Figure 8. *bax* and *bcl-2* mRNA expression under 13cRA and staurosporine treatment. *bax* and *bcl*-2 mRNA expression in SZ95 sebocytes under 13cRA (10^{-7} M) or staurosporine (10^{-7} M) treatment for 6 h was detected by RT-PCR and evaluated in comparison to β-actin. *bcl*-2 mRNA expression was downregulated by staurosporine but not by 13cRA.

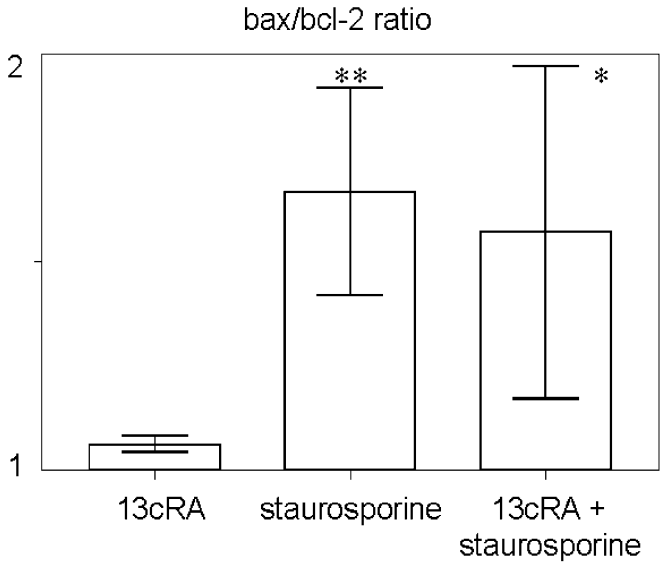

Figure 9. bax/bcl-2 ratio after flow cytometry of bax and bcl-2 protein expression under 13cRA and staurosporine treatment. The bcl-2/bax ratio was increased by staurosporine and the combined staurosporine (10^{-7} M) ($n = 3$) and 13cRA (10^{-7} M) treatment ($n = 2$) at similar levels, whereas it was not affected by 13cRA ($n = 3$). The values represent the y-fold increase of the mean ± standard deviation (where y=the value of the y axis) of the subsequent experiments, whereas control = 1. $^{*}p < 0.05$, $^{**}p < 0.01$.

et al, 1999; Slee *et al*, 1999), and the mitochondrial Ca^{2+}-associated protein bcl-2, which increases cell resistance to apoptosis by preventing the release of apoptogenic factors, such as apoptosis-inducing factor (Kroemer *et al*, 1997) and cytochrome C (Liu *et al*, 1996). Bcl-2 overexpression leads to increased mitochondrial Ca^{2+} content, increased mitochondrial membrane potential, and inhibition of staurosporine-induced apoptosis. Staurosporine is a global inductor of apoptosis in several types of cells including human keratinocytes and dermal papilla fibroblasts (Falcieri *et al*, 1993; Ferraris *et al*, 1997; Sung *et al*, 1997). The decrease of mitochondrial Ca^{2+} content is the earliest event during staurosporine-induced apoptosis preceding activation of the caspase cascade and DNA fragmentation (Zhu *et al*, 1999). The ratio of the pro-apoptotic protein bax, which promotes cell death by competing with bcl-2 (Basu and Haldar, 1998), to bcl-2 determines the inherent susceptibility of cells to respond to an apoptotic signal. Both bcl-2 and bax are transcriptional targets for the tumor suppressor protein p53, which induces cell cycle arrest or apoptosis in response to DNA damage.

Recently, phospholipid signaling was recognized as one of the early and essential components of apoptosis (Martin *et al*, 1995; Verhoven *et al*, 1995). Like the decrease of mitochondrial Ca^{2+} content, phosphatidylserine translocation from the inner to the outer plasma membrane surface occurs much earlier than any detectable changes in other apoptotic markers such as caspase 3 activation, DNA fragmentation, or changes in nuclear morphology. On the other hand, caspases represent a family of intracellular cysteine proteases, the actions of which are linked to both the initial and final stages of apoptosis in virtually all types of vertebrate cells. They interact with the bax/bcl-2 and phospholipid signaling pathways (Ekert *et al*, 1999). In particular, caspase 3, a downstream member of the family, is an ortholog from the family of mammalian caspases that acts as an effector of the cell death pathway.

In our studies, staurosporine-induced apoptosis in SZ95 sebocytes *in vitro* in a dose-dependent and time-related manner was associated with enhanced externalization of phosphatidylserine, increased caspase 3 expression, and reduced bcl-2 levels. The latter mechanism has also been reported in dermal papilla fibroblasts (Ferraris *et al*, 1997). Bcl-2 is also expressed in sebaceous gland cells *in vivo* (Hisa *et al*, 1995). The combined treatment of staurosporine and 13cRA induced apoptosis to the same magnitude as staurosporine alone, thus indicating a sole staurosporine-induced effect.

On the other hand, 5α-DHT, as expected, exhibited a protective effect against sebocyte death. 5α-DHT stimulates sebocyte proliferation (Akamatsu *et al*, 1992) and, in contrast to the *in vivo* knowledge, is unable to induce lipid synthesis and terminal differentiation of human sebocytes *in vitro*.[2]

Retinoids did not affect apoptosis or cell death, although 13cRA upregulated caspase 3 expression and has been shown to inhibit sebocyte proliferation *in vitro* (Zouboulis *et al*, 1991). Neither acitretin nor 13cRA, which significantly isomerizes to the retinoic acid receptor ligand all-*trans* retinoic acid in SZ95

[2]Chen W, Yang C-C, Sheu H-M, Seltmann H, Zouboulis ChC: Expression of PPAR and c/EBP transcription factors in cultured human sebocytes. Submitted for publication.

sebocytes (Tsukada *et al*, 2000), changed the natural cell death process in these cells. This result fits well with the currently reported inability of normal skin cells to undergo apoptosis after treatment with retinoids, in contrast to their malignant counterparts (Toma *et al*, 1997; Hail and Lotan, 2001; Ulukaya *et al*, 2001). In tumor cells, bcl-2 was found to be decreased under treatment with retinoids (Piattelli *et al*, 1999; Tosi *et al*, 1999), and ligand activation of retinoic acid receptor in HL-60 cells resulted in a global suppression of bcl-2 expression (Nagy *et al*, 1996). Mitochondrial swelling and rapid decrease in mitochondrial membrane potential was induced by 13cRA by release of cytochrome C from the mitochondria to cytoplasm in cells obtained from rat liver (Rigobello *et al*, 1999). In contrast to the apoptosis-associated antiproliferative effect of retinoids on tumor cells, the antiproliferative effect on normal skin cells is probably due to retinoid-induced G1 arrest (Hail and Lotan, 2001). Interestingly, the induction of caspase 3 through 13cRA is apparently not essential for the induction of apoptosis (Matikainen *et al*, 2001).

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