Nerve Growth Factor Protects Human Keratinocytes from Ultraviolet-B-Induced Apoptosis

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Ultraviolet radiation is a potent inducer of apoptosis, whereas autocrine nerve growth factor protects human keratinocytes from programmed cell death. To evaluate the role of nerve growth factor in the mechanisms of ultraviolet B-induced apoptosis, cultured human keratinocytes were ultraviolet B irradiated following pretreatment with K252, a specific inhibitor of the tyrosine kinase high-affinity nerve growth factor receptor. Here we report that the addition of K252 significantly enhanced keratinocyte apoptosis. We then transfected normal human keratinocytes with pNUT-hNGF. Nerve growth factor overexpressing keratinocytes secreted the highest amounts of nerve growth factor in culture supernatants, were more viable, and had a higher rate of proliferation than mock-transfected cells. Whereas ultraviolet B radiation downregulated nerve growth factor mRNA and protein as well as the tyrosine kinase high-affinity nerve growth factor receptor in normal keratinocytes, it failed to do so in nerve growth factor-transfected cells. Moreover, nerve growth factor overexpressing keratinocytes were partially resistant to apoptosis induced by increasing doses of ultraviolet B at 24 and 48 h. These results indicate that downregulation of nerve growth factor function plays an important part in the mechanisms of ultraviolet B-induced apoptosis in human keratinocytes. In addition, ultraviolet B caused a decrease in BCL-2 and BCL-x_L expression in mock-transfected keratinocytes, but not in nerve growth factor overexpressing cells. Finally, nerve growth factor prevented the cleavage of the enzyme poly(ADP-ribose) polymerase induced in human keratinocytes by ultraviolet B. These results are consistent with a model whereby the autocrine nerve growth factor protects human keratinocytes from ultraviolet B-induced apoptosis by maintaining constant levels of BCL-2 and BCL-x_L, which in turn might block caspase activation. ***Key words: apoptosis/keratinocytes/nerve growth factor/ultraviolet B radiation. J Invest Dermatol 113:920–927, 1999***

Nerve growth factor (NGF) is a member of the family of proteins known as neurotrophins, which play a major part in the survival and differentiation of neuronal cells (Korsching, 1993). During development, increased NGF availability protects sensory and sympathetic neurons from cell death, whereas NGF deprivation leads to apoptosis (Kroemer, 1987; Oppenheim, 1991; Crowley *et al*, 1994). NGF also acts as a survival factor for cells of different lineage. Autocrine NGF rescues memory B lymphocytes from cell death (Torcia *et al*, 1996) and exogenous NGF protects skin melanocytes from ultraviolet (UV)-induced apoptosis (Zhai *et al*, 1996). Cellular responses to neurotrophins are mediated by two functionally interacting classes of receptors: a 75 kDa glycoprotein, named p75, which binds NGF and the other neurotrophins with low affinity (Dechant *et al*, 1994) and a tyrosine kinase receptor of 140 kDa belonging to the trk family which interacts with the individual neurotrophins in a specific manner and is responsible for the high-affinity binding (Barbacid, 1995). The trk receptor is directly responsible for neuronal cell survival (Barbacid, 1994), whereas the role of the p75 is still controversial.

Biologically active NGF is synthesized and secreted by keratinocytes (Di Marco *et al*, 1991; Yaar *et al*, 1991; Pincelli *et al*, 1994). In addition, normal human keratinocytes express both the low-affinity (p75) and the high-affinity (trk) NGF receptors (Di Marco *et al*, 1993; Pincelli *et al*, 1994). Trk, but not p75, mediates NGF signal also in human keratinocytes (Di Marco *et al*, 1993; Pincelli *et al*, 1994). We have shown recently that the natural alkaloid K252, which selectively inhibits the activity of trk, induces apoptosis in cultured human keratinocytes, indicating that autocrine NGF acts as a survival factor for these cells through its high-affinity receptor (Pincelli *et al*, 1997).

Apoptosis is an active gene-directed process of cell death which, among other functions, serves as a defense against environmental injuries, eliminating damaged cells that may be harmful to the organism (Kerr and Harmon, 1991; Thompson, 1995). UV radiation is the major source of cellular damage to the skin (Shea and Parrish, 1991) and keratinocytes are among the physiologically most relevant target of such injury. UV induces mutations in skin cells which are critical in tumorigenesis (Brash *et al*, 1991). UV also induces the formation of "sunburn cells" (Young, 1987), which have been shown to be apoptotic keratinocytes at the ultrastructural and at the biochemical level (Weedon, 1990; Haake and Polakowska, 1995). Because sunburn cells are damaged keratino-

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Abbreviations: PARP, poly(ADP-ribose) polymerase; Trk, tyrosine kinase high-affinity nerve growth factor receptor.

cytes which might have incurred mutations, UV-induced apoptosis appears to be a crucial mechanism for preventing skin cancer.

In this study we have investigated the role of NGF in UVB-induced apoptosis. We present evidence that UVB downregulates NGF and trk expression in human keratinocytes and that NGF protects these cells against UVB-induced apoptosis. We also show that NGF upregulates BCL-2 and BCL-x_L, while it prevents the cleavage of poly(ADP-ribose) polymerase (PARP) induced by UVB radiation in human keratinocytes.

MATERIALS AND METHODS

Keratinocyte cultures Normal human keratinocytes were obtained from foreskin and cultured as described (Pincelli *et al*, 1997). Briefly, keratinocytes were plated on mytomicin C-treated 3T3 cells (2.4×10^4 per cm^2; ATCC, Rockville, MD) and cultivated in Dulbecco's modified Eagle's medium and Ham's F12 media**.** Subconfluent secondary cultures were trypsinized with 0.05% trypsin/0.02% ethylenediamine tetraacetic acid and replated for the experiments in defined serum-free medium (KGM, Clonetics, San Diego, CA) without hydrocortisone for transfection experiments. Cell viability was evaluated by Trypan blue exclusion.

UVB radiation UVB radiation was delivered with a battery of lamps (TL 20W/12 RS UVB Philips Medical). Normal human keratinocytes were pretreated either with K252 (200 nM, Calbiochem, LaJolla, CA) or diluent 48 h before UVB (25, 50 mJ per cm^2) or sham irradiation. Cells were analyzed 24 h later. Cells were also irradiated 48 h after transfection and analyzed 24 and 48 h later. Before irradiation, keratinocytes were washed once with phosphate-buffered saline (PBS) and irradiated in the presence of PBS. Controls were sham irradiated for identical periods. We selected doses of 50, 75, and 100 mJ per cm^2, as measured by International Light Research Radiometer (Newburyport, MA).

Reverse transcription and polymerase chain reaction Total cellular RNA was extracted from cultures using TRI Reagent method performed as described by Sigma. One microgram of total cellular RNA extracted was reverse-transcribed and amplified as described (Pincelli *et al*, 1994). To evaluate the amount of RNA in each sample, the β-actin mRNA (Kupper *et al*, 1987; Imokawa *et al*, 1992) was amplified by reverse transcription–polymerase chain reaction. Nucleotide sequences of the oligomers used were as follows: NGF oligonucleotide sense 5′TCATCATCCCATCCC AT-CTT-3′; NGF oligonucleotide anti-sense 5′-CTTGACAAAGG TGTGAGTCG-3′; NGF probe 5′-ACTGTTTGAAT ACACTGTTG-TTAA TGTTCACCTCT CCCAA-3′; β-actin sense 5′-TGGAT GATGATATCG CCGCGCTCG-3′; β-actin anti-sense 5′CACATA GGAA-TCCTTCTGACCCA-3′; β-actin probe 5′-AGGGGAAGACG-GCCCGGGGGGCATCGTCG CCCG-3′; trk sense 5′GGCTC-CTCGGGACTG CGATG-3′; trk anti-sense 5′-CAGGAGAGAG-ACTCC AGAGCG-3′; trk probe 5′-GCCAC GAAACGGAGAC-CACTCTTCACGA TGGTG-3′. The relative intensity of bands on autoradiograms was quantitated by scanning laser densitometry. The linearity of the polymerase chain reaction assay was obtained by plotting values from densitometric analysis in each band *versus* the cDNA concentrations.

Enzyme-linked immunosorbent assay NGF quantitation was performed by a two-site enzyme immunoassay following a modification of the protocol described by Boehringer Mannheim (Mannheim, Germany). The sample concentration was determined by absorbance at 574 nm against recombinant human NGF standard protein.

Expression plasmid The cDNA for human NGF (hNGF) was originally subcloned into a dehydrofolate reductase-based pNUT expression vector downstream from the mouse metallothionein I promoter (mMT-I) and rat insulin II intron. The plasmid pNUT-hNGF (Winn *et al*, 1994) was kindly provided by Dr R.D. Palmiter (University of Washington, Seattle, WA).

Transfection Cells (1×10^6 cells per 10 cm^2 were transiently transfected using 10 μl of lipofectin (Life Technologies, Gibco/BRL) reagent and 15 μg of plasmid DNA, following directions of the manufacturer. In order to monitor transfection efficiency, keratinocytes were cotransfected with pSV-β-galactosidase control vector (Promega, Madison, WI). β-galactosidase activity was measured by staining cells *in situ* with the β-Gal staining set (Boehringer). The media were collected and the cells were lysed for quantitation of NGF. The synthesis of the NGF mRNA was analyzed by reverse transcription–polymerase chain reaction. The biologic activity of the NGF synthesized and secreted by transfected cells was studied using PC12, which elaborate neurites in response to NGF. The NGF protein level was measured by enzyme-linked immunosorbent assay.

Bioassay PC12 (ATCC) cells (Schweitzer and Kelly, 1985; Kelly, 1985) were plated on six-well plates at a density of 2×10^3 cells per well and grown in 1640 RPMI containing 4 mM glutamine, 10% horse serum, 5% fetal bovine serum (Sigma), and 50 IU of penicillin/streptomycin per ml (Biochrom). Forty-eight hours after transfection, conditioned medium (1:5 dilution) from control and transfected keratinocytes was centrifuged and added to PC12 cultures in the presence or absence of the anti-NGF monoclonal antibody.

Cell proliferation assay Control and transfected keratinocytes (6000 cells per well) were grown on 96 well plates in KGM. Media were changed 24 h after transfection. ^3H thymidine (1 mCi per well, Amersham-Pharmacia Biotech, Rainham, U.K.) incorporation was performed 12 h before harvesting of the cells and cells were collected at 48, 72, and 168 h after transfection. The incorporated radioactivity was determined by β-counter.

TdT-mediated dUTP nick end labeling Keratinocytes were directly stained on chamber slides by the "In situ cell death detection kit" (Boehringer), as described (Pincelli *et al*, 1997). The negative control was obtained by replacing the primary incubation with a nucleotide mixture without terminal deoxynucleotidyl transferase (TdT). Fluorescent specimens were analyzed by a confocal scanning laser microscopy (Leica TCS4D) in conjunction with a conventional optical microscope (Leica DM IRBE).

DNA recovery and electrophoresis Keratinocytes were collected at different times and lysed in lysis buffer (10 mM ethylenediamine tetraacetic acid, 50 mM Tris at pH 8, 0.5% Sarkosyl, 0.5 mg proteinase K per ml, Sigma). After 2 h incubation at 60°C, the suspension was precipitated and resuspended in Tris ethylenediamine tetraacetic acid supplemented with 0.25 μg per ml heat-treated RNase A (Boehringer). Extracts were further incubated in the water bath at 37°C for 4 h. Samples were then supplemented with loading buffer (10 mM ethylenediamine tetraacetic acid at pH 8, containing 0.25% bromophenol blue, FLUKA CHEMIE AG, Buchs, Switzerland, and 50% glycerol). Electrophoresis was carried out at 15 V on 2% agarose gel.

Flow cytometric analysis of keratinocyte apoptosis NGF and mock-transfected keratinocytes were cultivated as before, and, following UVB treatment, they were suspended in PBS. DNA content of nuclei was determined by staining nuclear DNA with propidium iodide (50 μg per ml) and measuring the relative DNA content using a fluorescence-activated cell sorter (FACS, Becton-Dickinson, San Jose, CA).

Western blot analysis Cells were washed with PBS and lysed on ice in RIPA buffer pH 8.5, as described (Pincelli *et al*, 1997). Thirty micrograms of total protein were analyzed under reducing conditions on 12% polyacrylamide gels and blotted on to nitrocellulose membrane. To verify equal loading of total protein in all lanes, the membrane was stained with Red Ponceau. The blot was incubated with 2.5 μg per ml anti-human BCL-2 monoclonal antibody (Dako, K/A, Glostrup, Denmark) or with 2.5 μg per ml anti-human BCL-x_L polyclonal antibody (Oncogene, Cambridge, MA) overnight at 4°C. Blots were also incubated with a monoclonal antibody against the human enzyme PARP (1:1000, Biomol, Plymouth Meeting, PA). Then membranes were washed in PBS/Tween 20, incubated with peroxidase-conjugated goat anti-mouse antibody for anti-BCL-2 and PARP, goat anti-rabbit antibody for BCL-x_L (BioRad, 1:800, Hercules, CA) for 45 min at room temperature, washed and developed using the ECL chemiluminescent detection system (Amersham). Relative intensity of bands on autoradiograms were quantitated by scanning laser densitometry, as described above.

RESULTS

UVB irradiation downregulates NGF and trk in human keratinocytes To investigate the role of NGF in the mechanisms of UV-induced apoptosis we first evaluated the effect of UV light on the expression of NGF and its high-affinity receptor. Subconfluent keratinocytes were irradiated with increasing doses of UVB, the biologically most relevant form of

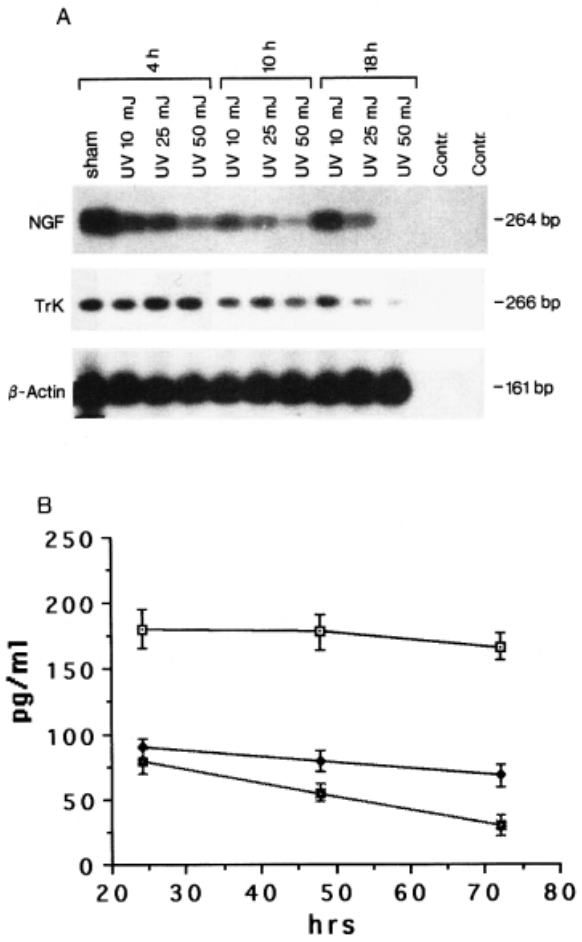


Figure 1. Effect of UVB irradiation on NGF expression and function in cultured keratinocytes. Subconfluent cells were UVB- or sham-irradiated with 10, 25, and 50 mJ per cm^2. Total cellular RNA was extracted from keratinocytes 4, 10, and 18 h post-irradiation and reverse-transcribed. NGF and trk mRNA expression was evaluated by reverse transcription–polymerase chain reaction and southern blot analysis using the appropriate primers and the radioactive oligodeoxynucleotide probes. β-actin transcript expression was obtained by amplification with specific primers to assess equivalent RNA amount. Control lanes represent amplification with no template and RNA without reverse transcription, respectively (*A*). Keratinocytes were cultivated in keratinocyte growth medium and NGF protein was measured using an enzyme-linked immunosorbent assay at 24, 48, and 72 h following 25 (*closed diamonds*) and 50 (*closed squares*) mJ per cm^2 UVB or sham irradiation (*open square*). NGF protein levels are given in pg per ml and results are expressed as mean ± SEM of triplicate from three different experiments (*B*). Sham *versus* UVB (25 and 50 mJ per cm^2) = $p < 0.01$.

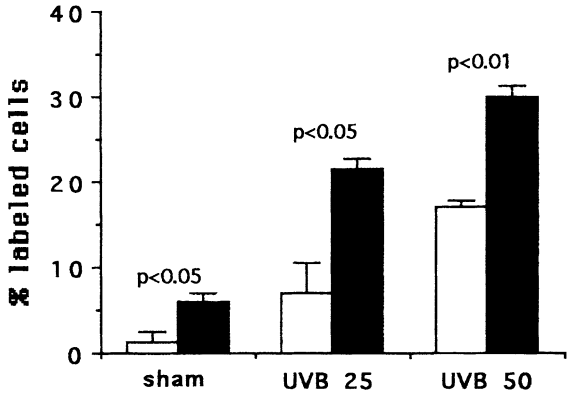


Figure 2. K252 enhances UVB-induced keratinocyte apoptosis. Cells were pretreated either with K252 (*black bars*) or diluent (*white bars*) 48 h before UVB or sham irradiation. Cells were directly stained at 24 h with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase. About 100 cells were counted in randomly selected fields for each point and percentages are expressed as mean ± SD of three experiments. Student's t test was used for comparison of the means.

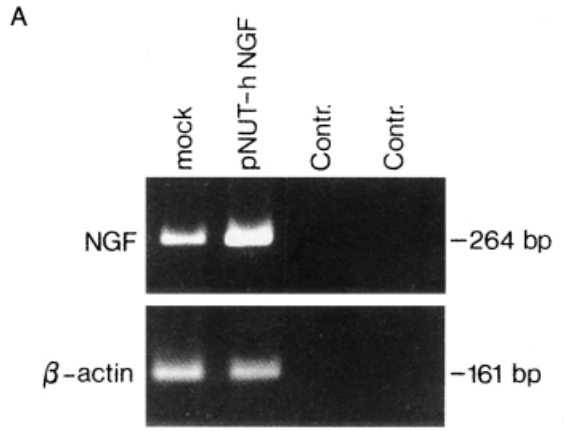
solar UV radiation which is absorbed by the epidermis. UVB downregulated the synthesis of NGF in human keratinocytes in a concentration-dependent manner. NGF mRNA appeared to be decreased as compared with control 4 h postirradiation with 10, 25, or 50 mJ per cm^2. The expression of NGF continued to diminish up to 18 h postirradiation when NGF synthesis was completely abolished by 50 mJ per cm^2 UVB (**Fig 1*A***). Similarly, UVB downregulated the secretion of NGF protein from keratinocytes in a concentration-dependent manner (**Fig 1*B***).

Because trk is the functional NGF receptor (Di Marco *et al*, 1993), we examined the effects of UVB irradiation on the expression of trk in human keratinocytes. Trk mRNA was slightly decreased 10 h following UVB irradiation. At 18 h, there was a clear concentration-dependent downregulation of trk synthesis which was almost abolished with 50 mJ per cm^2 (**Fig 1*A***). Amplification with specific oligomers for β-actin revealed equal amount of RNA in the samples (**Fig 1*A***).

K252 enhances UVB-induced keratinocyte apoptosis Because UVB irradiation strikingly affects NGF function and causes keratinocyte apoptosis, we asked whether endogenous NGF could protect keratinocytes from UVB-induced cell death. To this purpose, cultured keratinocytes were UVB irradiated with or without the addition of K252, a specific inhibitor of trk phosphorylation (Berg *et al*, 1992). As shown in **Fig 2**, percentage of apoptotic keratinocytes following UVB irradiation was significantly higher in cell cultures pretreated with K252 than in those provided with diluent alone.

Characterization of NGF-transfected keratinocytes To evaluate further the role of endogenous NGF in the mechanisms of UVB induced keratinocyte apoptosis, cells were transfected with a plasmid containing a full-length NGF cDNA. Twenty-four hours after transfection, NGF mRNA expression was markedly increased in transfected keratinocytes as compared with mock-transfected cells (**Fig 3*A***). To investigate whether NGF mRNA over-expression corresponds to increased NGF release from human keratinocytes, an enzyme-linked immunosorbent assay was performed on cell supernatants at different times. Increasing NGF protein levels were detected from 24 h up to 96 h in NGF-transfected cells. NGF protein amounts were significantly higher in NGF than in mock-transfected cells at all time points (**Fig 3*B***). In order to determine whether NGF produced by human keratinocytes was biologically active, a classic NGF bioassay was employed (Rukenstein and Greene, 1983). PC12 cells, a pheochromocytoma cell line which displays neurite outgrowth upon stimulation with NGF concentrations as small as 0.1 ng per ml (Green, 1977), were cultured in media conditioned by different keratinocyte populations. PC12 cells stimulated by the culture media derived from NGF-transfected cells displayed a marked neurite outgrowth which was partially blocked by the addition of an anti-NGF antibody (data not shown). These results demonstrated that human keratinocytes transfected with NGF cDNA produce increasing amounts of biologically active NGF.

Analysis of *in vitro* growth characteristics of NGF-transfected keratinocytes To investigate the behavior in culture of NGF overexpressing keratinocytes, cell viability was evaluated by Trypan blue dye exclusion. NGF-transfected keratinocytes were significantly more viable than controls starting at 72 h after transfection (**Fig 4*A***). In addition, DNA synthesis in



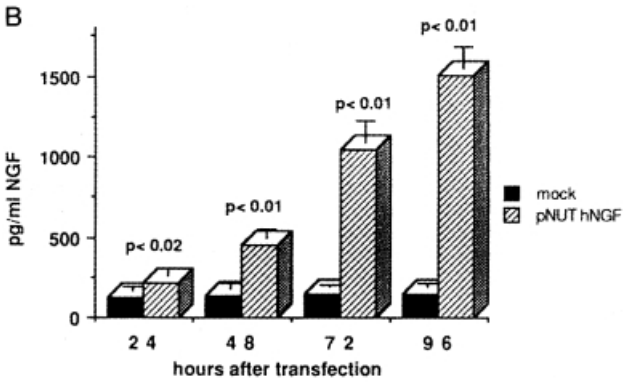


Figure 3. NGF mRNA and protein analysis in pNUT-hNGF transfected keratinocytes. 1×10^6 cells/10 cm^2 were transiently transfected with the plasmid pNUT-hNGF using 10 μl of lipofectin (percentage of transfected cells = 30–35%). Twenty-four hours after transfection cells were lysed for NGF mRNA quantification by reverse transcription–polymerase chain reaction and ethidium bromide staining. β-actin mRNA expression was used as a quantitative control of RNA. Control lanes represent no reverse-transcribed total cellular RNA and buffers without template (*A*). Keratinocytes were cultivated in KGM and NGF protein was measured at different times in mock (*closed bars*) and in NGF transfected cells (*striped bars*). NGF protein levels are given in pg per ml and results are expressed as mean ± SEM of triplicate from three different experiments (*B*).

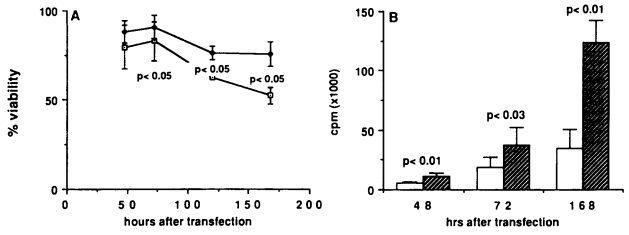


Figure 4. Viability and DNA synthesis in NGF-transfected keratinocytes. Keratinocytes were transfected with pNUT-hNGF and viability was evaluated by Trypan Blue dye exclusion at different times in mock (*open squares*) and NGF transfected cells (*closed circles*) (*A*). NGF (*striped bars*) and mock-transfected keratinocytes (*open bars*) were seeded at a density of 6000 per well and cultured up to 168 h in KGM [^{3}H]thymidine incorporation was determined at different times (*B*). Each point represents the mean cpm ± the SEM of separate determinations in six different wells from three different experiments. Student's t test was used for comparison of the means.

cultured human keratinocytes transfected with NGF was analyzed as compared with controls. There was a constant increase in thymidine incorporation in mock-transfected cells between 48 and 168 h, most likely due to the release of autocrine factors such as NGF (Di Marco *et al*, 1993). As expected, proliferation rate in NGF-transfected keratinocytes was significantly higher than in controls at 48 and 72 h. Interestingly, the DNA synthesis was increased by 3-fold in NGF overexpressing cells as compared with controls at 168 h (**Fig 4*B*).**

UVB do not affect NGF and trk in NGF-transfected keratinocytes NGF overexpressing keratinocytes were then used to assess the role of NGF in the mechanisms of UV-induced apoptosis. We first tested the effect of UVB irradiation on the expression of NGF and trk in NGF-transfected keratinocytes. NGF-transfected keratinocytes were treated with 50 and 75 mJ per cm^2 UVB. Whereas UVB irradiation caused a concentration-dependent decrease in NGF synthesis in mock-transfected cells at 24 h, it failed to affect the expression of NGF mRNA in NGF-transfected cells (**Fig 5*A*, *B***), possibly due to the presence of the metallothionein I promoter in the pNUT hNGF vector (Rimoldi et al, 1992). In addition, at 24 h trk mRNA in NGF-transfected keratinocytes was not downregulated by UVB irradiation (**Fig 5*C*, *D***). β-actin, used as an internal control, was not affected by UVB irradiation (**Fig 5*E*, *F***). Furthermore, the release of NGF protein was not significantly affected by UVB irradiation (50 and 75 mJ per cm^2) in transfected keratinocytes at 24 and 48 h (**Fig 6*A*, *B***). Whereas there was a 2–3-fold decrease in NGF protein levels in UVB-irradiated keratinocytes, as compared with sham-irradiated cells, in mock-transfected keratinocytes, the same difference in NGF-transfected keratinocytes was not statistically significant at any time point, except with 100 mJ per cm^2. This indicates that, despite UVB irradiation, NGF protein in supernatants of NGF-transfected cells remains at high levels.

Human keratinocytes overexpressing NGF do not undergo apoptosis Because an anti-apoptotic autocrine loop sustained by NGF exists in human keratinocytes (Pincelli *et al*, 1997), we reasoned that constant levels of NGF could protect these cells from UVB-induced apoptosis. After UVB irradiation, the number of viable cells was slightly but significantly higher in NGF overexpressing keratinocytes than in mock-transfected cells at 24 h (**Fig 7*A***). To investigate further the protective effect of NGF during UVB irradiation, the apoptotic process was evaluated in mock- and NGF-transfected keratinocytes. DNA fragmentation, as shown by the DNA ladder, was clearly observed in mock-transfected keratinocytes at 24 h following 75 mJ per cm^2 UVB. The DNA ladder, however, was less intense in NGF-transfected cells (**Fig 7*B***). TUNEL staining showed that the percentage of apoptotic cells 24 h after UVB irradiation (50, 75, and 100 mJ per cm^2 UVB) was slightly, but significantly, higher in mock-transfected than in NGF-transfected keratinocytes (**Fig 7*C***). At 48 h postirradiation (75 mJ per cm^2 UVB) the percentage of apoptotic cells was still significantly lower ($p < 0.02$) in NGF-transfected keratinocytes (data not shown). The decreased number of apoptotic cells in NGF-transfected keratinocytes was further confirmed by flow cytometric analysis with propidium iodide staining at 24 h with 75 mJ per cm^2 UVB. A sub-G_1 peak, indicating the amount of apoptotic nuclei, was higher in mock than in NGF-transfected keratinocytes after UVB irradiation (**Fig 7*D***). Caspases, the mammal homologs of the CED-3 gene in *Caenorabditis elegans*, are a family of at least 10 cysteine proteases that play a key part in apoptosis (Alnmeri *et al*, 1996). A number of proteins, including PARP, are specifically cleaved by caspase proteases in cells undergoing apoptosis (Fernandez-Alemneri *et al*, 1995; Tewari *et al*, 1995). To evaluate whether caspases are activated during the UVB-induced apoptotic process, we verified the cleavage of PARP in keratinocytes following 50 mJ per cm^2 UVB irradiation. Cleavage of PARP, as shown by the 85 kDa band, was observed at 24 h postirradiation in mock-transfected cells. By contrast,

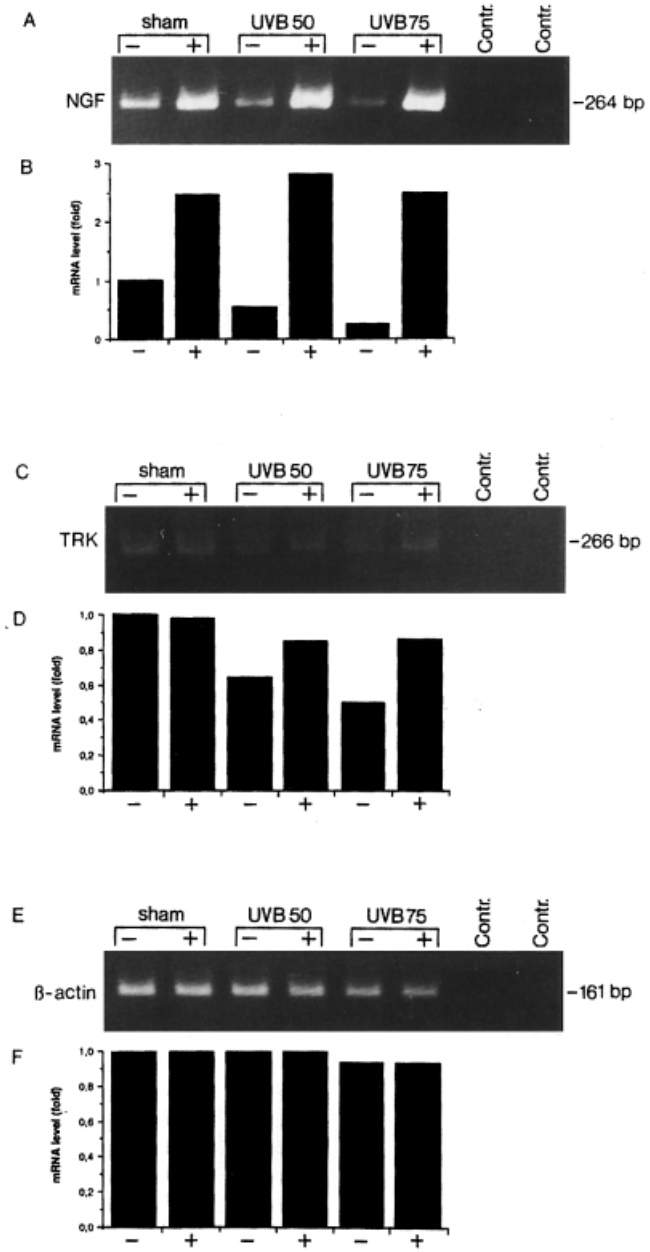


Figure 5. Effect of UV-B on NGF and trk mRNA expression in NGF-transfected keratinocytes. NGF (+) and mock transfected (–) keratinocytes were UVB (50 and 75 mJ per cm^2) or sham irradiated. NGF (*A*) and trk (*C*) mRNA were measured 24 h later by reverse transcription–PCR and ethidium bromide staining. Polymerase chain reaction controls are as in legend of Figs 1 and 2. β-actin (*E*) mRNA expression was used as a quantitative control of RNA. Relative intensity of bands was quantified by scanning laser densitometry (*B,D,F*). Values are expressed as fold variations compared to sham-irradiated, mock-transfected cells.

overexpression of NGF markedly diminished the generation of the 85 kDa PARP fragment in human keratinocytes (**Fig 7*E***).

NGF prevents UVB-induced downregulation of BCL-2 and BCL-$_{xL}$ in human keratinocytes *Bcl-2* and *bcl-xL* are members of the *bcl-2* family and protect many cell types from apoptosis (Boise *et al*, 1993; Yang and Korsmeyer, 1996; Kroemer, 1997). BCL-2 and BCL-x proteins are detected in normal human keratinocytes (Polakowska *et al*, 1994; Krajewski *et al*, 1994; Rodriguez-Villanova *et al*, 1995; Wrone-Smith *et al*, 1995). To investigate whether NGF protects human keratinocytes from UVB-induced apoptosis through the *bcl-2* pathway, western blot analysis was performed on extracts from NGF and mock-transfected

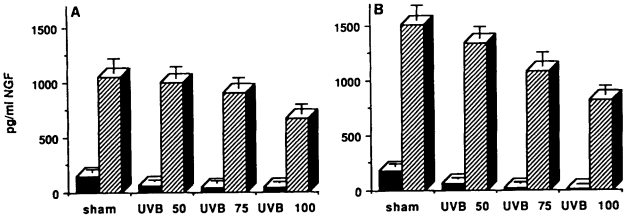


Figure 6. Effect of UVB on NGF protein expression in NGF-transfected keratinocytes. Keratinocytes were cultivated in KGM and NGF protein was measured at 24 h (*A*) and 48 h (*B*) after UVB irradiation in mock-transfected cells (*black bars*) and in NGF-transfected cells (*striped bars*). NGF protein levels are given in pg per ml and results are expressed as mean ± SEM of triplicate from three different experiments. At 24 and 48 h, mock vs. NGF-transfected keratinocytes = $p < 0.01$ at all time points. At 24 and 48 h: sham vs. UVB (50, 75, and 100 mJ per cm^2) in mock-transfected cells = $p < 0.02$. At 24 and 48 h: sham vs. UVB (50 and 75 mJ per cm^2) in NGF-transfected cells = not significant. At 24 and 48 h: sham vs. 100 mJ per cm^2 UVB in NGF-transfected keratinocytes = $p < 0.02$.

keratinocytes following UVB irradiation. At 24 h, BCL-2 protein levels were almost abolished in mock-transfected cells after 50 mJ per cm^2 UVB, as compared with those observed in sham-irradiated cells. On the other hand, after UVB irradiation, BCL-2 levels in NGF-transfected cells were comparable with those observed under baseline conditions. BCL-x_L levels were decreased at 24 h after UVB irradiation in mock-transfected cells. In NGF-transfected keratinocytes, BCL-x_L protein was slightly decreased following UVB irradiation, levels being still similar to sham-irradiated, mock-transfected cells. Interestingly, BCL-2 and BCL-x_L levels were increased in unirradiated, NGF-transfected cells as compared with mock-transfected keratinocytes (**Fig 8*A*, *B***).

DISCUSSION

UV irradiation has been shown to induce keratinocyte apoptosis both *in vivo* and *in vitro* (Haake and Polakowska, 1995; Schwarz *et al*, 1995; Norris *et al*, 1997). The mechanisms involved in this process, however, remain to be defined. This study provides evidence that NGF released from keratinocytes takes part in this process. UVB dramatically downregulated the synthesis and release of NGF in normal human keratinocytes, whereas in keratinocytes overexpressing NGF, NGF mRNA as well as the secretion of NGF in culture supernatants were not affected by UVB. In addition, we show that trk mRNA expression was markedly reduced by UVB irradiation in normal human keratinocytes, whereas it was not downregulated in NGF-transfected cells. These results indicate that UVB irradiation inhibits the expression and function of NGF in human keratinocytes.

Because NGF overexpressing keratinocytes are resistant to UVB-induced downregulation of NGF and its functional high-affinity receptor, it is conceivable that NGF exerts a protective effect on these cells. Indeed, here we demonstrate that NGF-transfected keratinocytes proliferate better and appear more viable than controls. It has been shown previously that exogenous NGF stimulates keratinocyte proliferation and its mitogenic effects are, under certain culture conditions, even greater than those exerted by epidermal growth factor (Pincelli and Yaar, 1997). In addition, anti-NGF antibodies and K252 inhibit keratinocyte proliferation in the absence of NGF, indicating that autocrine NGF in itself is a powerful mitogen for these cells (Di Marco *et al*, 1993). This study extends these observations, by showing that keratinocytes overexpressing NGF can release this growth factor to an extent that allows them to proliferate significantly better than controls even after 7 d in culture. Upon UVB irradiation, keratinocytes overexpressing NGF still appeared more viable than controls and showed little DNA fragmentation. Doses of UVB that normally induce apoptosis in cultured keratinocytes (Benassi *et al*, 1997; Baba *et al*, 1998) caused decrease of NGF and trk as well as apoptosis

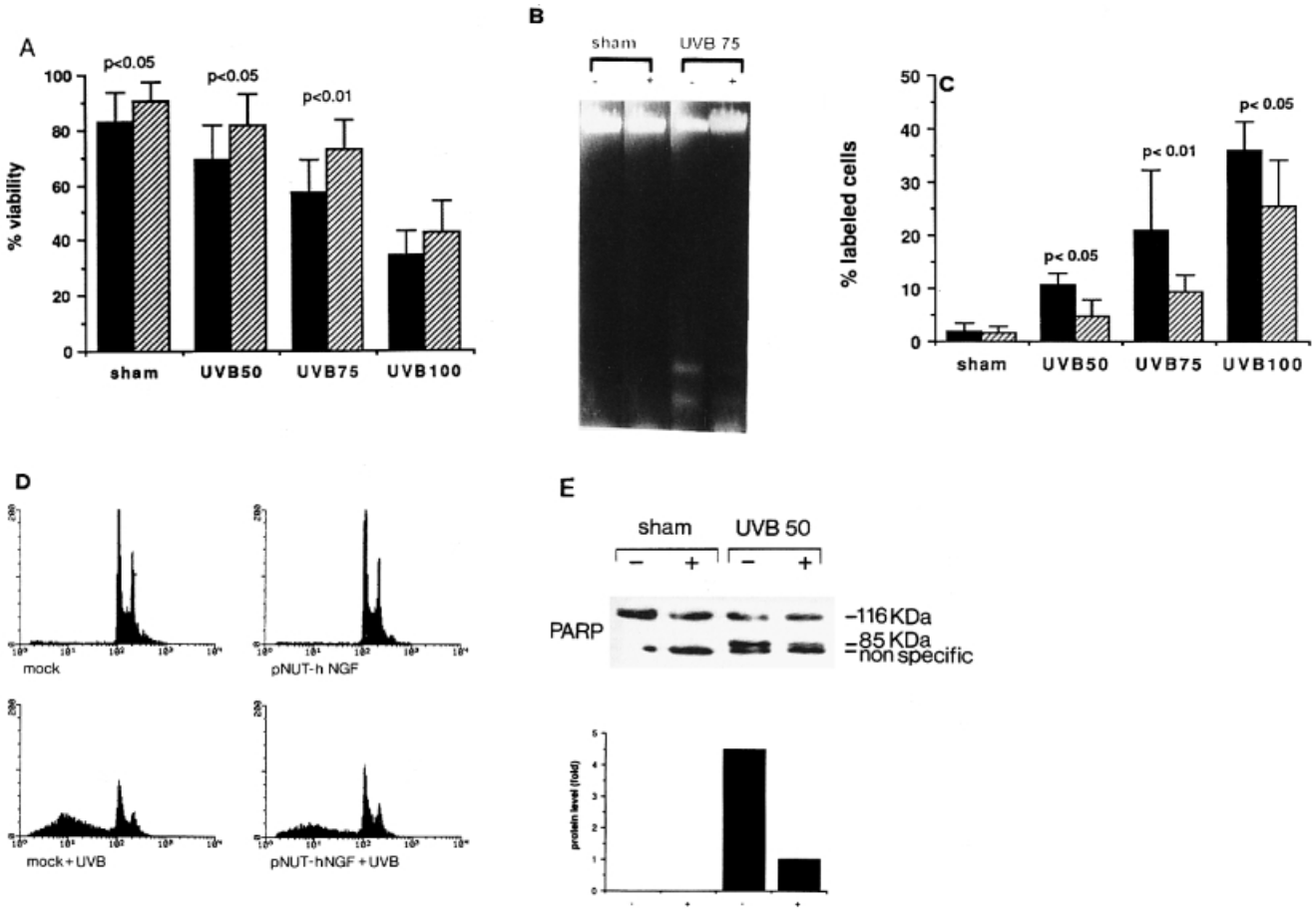


Figure 7. NGF-transfected keratinocytes are protected from UVB-induced apoptosis. Keratinocyte viability was evaluated by Trypan Blue dye exclusion at 24 h (*A*). DNA was extracted from mock-transfected (–) and NGF-transfected keratinocytes (+) 24 h after sham or UVB irradiation. DNA was electrophoresed on a 1.5% agarose gel (*B*). NGF- (*striped bars*) and mock-transfected (*black bars*) cells were cultured on slide flasks and irradiated with different doses of UVB or sham-irradiated. Cells were directly stained at 24 h with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase. About 100 cells were counted in randomly selected fields for each point, and percentages are expressed as the mean ± SD of three experiments. Student's t test was used for comparison of the means (*C*). DNA histograms from mock-transfected (49% apoptotic cells) and NGF-transfected keratinocytes (34% apoptotic cells) after UVB (75 mJ per cm^2, *lower panels*) or sham-irradiation (*upper panels*) at 24 h. DNA content was stained with propidium iodide (50 mg per ml) and analyzed by flow cytometry (*D*). NGF-transfected keratinocytes (+) and mock-transfected cells (–) were cultured in KGM and were either UVB (50 mJ per cm^2) or sham irradiated. At 24 h cells were lysed and protein analyzed by western blotting using anti-PARP monoclonal antibody, as described in *Materials and Methods*. Relative intensity of bands on autoradiograms was quantified by scanning laser densitometry. Values of 84 kDa bands are expressed as fold variations compared with UVB-irradiated NGF-transfected cells. A nonspecific band is present immediately below the 85 kDa band (*E*).

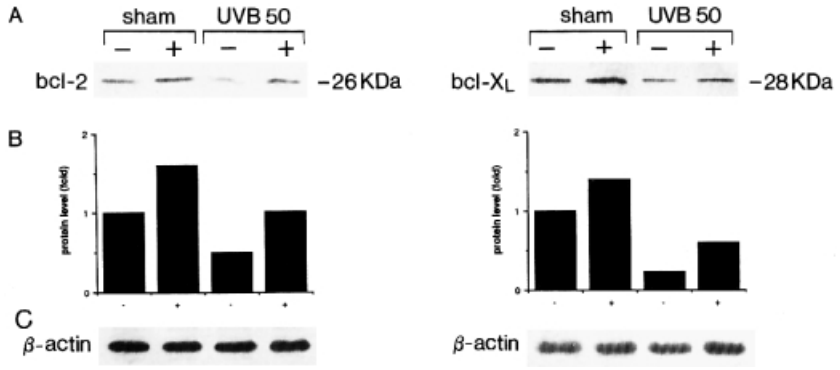


Figure 8. NGF protects human keratinocytes from UVB-induced apoptosis through *bcl-2* and *bcl-xL*. NGF-transfected keratinocytes (+) and mock-transfected cells (–) were cultured in KGM and were either UVB (50 mJ per cm^2) or sham-irradiated. Twenty-four hours later cells were lysed and protein analyzed by western blotting using anti-BCL-2 and anti-BCL-xL antibodies (*A*) as described in *Materials and Methods*. Relative intensity of bands on autoradiograms was quantified by scanning laser densitometry (*B*). Values are expressed as fold variations compared with sham-irradiated, mock-transfected cells. Anti-β-actin antibody was used to assess equal loading of the protein (*C*).

in mock-transfected cells. On the contrary, NGF-transfected keratinocytes were protected from UVB-induced cell death. We therefore conclude that downregulation of NGF function plays an important part in the mechanisms of UVB-induced apoptosis in human keratinocytes. Other growth factors have been shown to prevent apoptosis in the skin. Indeed, inhibitors of epidermal growth factor receptor induce cell death in normal and human papilloma virus infected keratinocytes (Ben-Bassat *et al*, 1997; Rodeck *et al*, 1997). This report is in good agreement with the recent observation by Norris *et al* (1997) showing that human keratinocytes maintain strong anti-apoptotic defenses which can be altered by growth factor withdrawal. One would expect that high levels of NGF proteins in NGF-transfected keratinocytes allow a more pronounced anti-apoptotic effect. Although the

role of NGF as a survival factor appears to be very clear, it should be taken into account that the average UVB doses employed in this study were quite high. Secondly, other factors could certainly take part in the mechanisms involved in the protection of keratinocytes against UV light. Third, these factors might function through a pathway independent from BCL-2 (Adida *et al*, 1998; Tamm *et al*, 1998; Chiodino *et al*, 1999).

This study demonstrates that UVB downregulates trk expression in normal human keratinocytes, but not in NGF overexpressing cells, which in turn are protected from UVB-induced apoptosis. Moreover, inhibition of trk function by K252 enhances keratinocyte apoptosis following UVB irradiation. This indicates that trk is the receptor mediating the NGF survival signal in keratinocytes, in accordance with previous work (Pincelli *et al*, 1997). Whereas trk mediates the NGF rescuing effects also in neuronal cells (Fagan *et al*, 1996; Belliveau *et al*, 1997), recent literature point to p75 as a pro-apoptotic receptor in certain neuronal populations and in human melanocytes (Casaccia-Bonefil *et al*, 1996; Frade *et al*, 1996; Yaar *et al*, 1997). On the other hand, little is known on the signaling pathway mediated by p75 in human keratinocytes (Di Marco *et al*, 1993; Pincelli *et al*, 1994). Because these cells secrete NGF, the role of p75 as a ''death receptor'', in the absence of its ligand, is difficult to assess and awaits further investigations. It has been shown recently that UV light induces keratinocyte apoptosis via direct activation of another member of the tumor necrosis factor receptor superfamily, the CD95 (Fas/APO-1) (Aragane *et al*, 1998) which share the so-called ''death domain'' with p75 and tumor necrosis factor receptor (Chapman, 1995). It was not the purpose of this study to evaluate whether NGF exerts any protection from CD95-mediated apoptosis. Whereas NGF signaling pathways through p75 receptor in terms of apoptosis are well established at least in certain cell types (Dobrowsky *et al*, 1994), nothing is known on NGF interfering with CD95-mediated apoptosis.

Bcl-2 and *bcl-x_L* inhibit multiple forms of cell death (Reed, 1994; Boise *et al*, 1995) and their modulation seems to play a crucial part in UV-induced apoptosis (Martin *et al*, 1995; Gillardon *et al*, 1994; Pena *et al*, 1997). Furthermore, UVB radiation downregulates BCL-2 protein levels in human keratinocytes and HaCat keratinocytes stably transfected with *bcl-2* are protected from UV-induced apoptosis (Haake and Polakowska, 1995). We have previously shown that HaCat keratinocytes transfected with *bcl-2* are protected from apoptosis induced by suppression of autocrine NGF function, indicating that NGF rescues human keratinocytes through a *bcl-2*-dependent mechanism (Pincelli *et al*, 1997).

In addition, NGF upregulates the expression of BCL-2 in human keratinocytes[1] and melanocytes (Pincelli and Yaar, 1997), whereas downregulation of BCL-2 abrogates the NGF protective effect from UV-induced apoptosis in melanocytes (Zhai *et al*, 1996).

In this study, we demonstrate that overexpression of NGF overcomes the UVB-induced downregulation of BCL-2 and BCL-x_L in human keratinocytes. This seems to indicate that NGF protects these cells from UVB-induced apoptosis by maintaining constant levels of two potent anti-apoptotic members of the *bcl-2* family.

In the execution stage of apoptosis, the activation of caspases occurs, which is controlled by *bcl-2* (Golstein, 1997). UV radiation has been shown to activate caspases (Caulin *et al*, 1997), whereas caspase inhibitors can prevent UV-induced apoptosis (Manji *et al*, 1997). NGF withdrawal induces caspase activity and apoptosis in neuronal cells which is blocked by *bcl-2* (Schulz *et al*, 1997).

Moreover, *bcl-x_L* can protect Jurkat cells from apoptosis by inhibiting PARP cleavage (Boise and Thompson, 1997). Little is known, however, on the caspase activation in human keratinocytes (Takahashi *et al*, 1997). Our study shows that UV radiation induces PARP cleavage in these cells. Most importantly, overexpression of NGF inhibited UV-induced PARP cleavage, suggesting that this growth factor protects human keratinocytes from UVB-induced apoptosis by preventing caspase activation.

Sun exposure is the major environmental agent implicated in the induction of nonmelanoma skin cancers (Shea and Parrish, 1991; Kripke, 1993; Jonason *et al*, 1996; Kraemer, 1997). The apoptotic effects of UV, however, may as well be important in inhibiting the development of skin cancer (Kemp *et al*, 1993). NGF, by preventing UV-induced keratinocyte apoptosis, would then appear to be involved in skin tumorigenesis. NGF is exclusively secreted by proliferating basal keratinocytes (Di Marco *et al*, 1991; Pincelli *et al*, 1994), whereas keratinocytes that are in the S-phase of the cell cycle contribute most to the formation of sunburn cells (Danno and Horio, 1982). In addition, it has been shown recently that the proliferative basal cell compartment of the epidermis contains clones of cells with characteristic UV-type *p53* mutations, which are more frequent and larger in sun-exposed areas than in sun-protected areas (Jonason *et al*, 1996). Therefore, an autocrine survival system sustained by NGF could be operational in the basal layer of the epidermis leading to the expansion of mutated keratinocytes and to the initiation of skin cancer.

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