Autocrine Nerve Growth Factor Protects Human Keratinocytes from Apoptosis Through its High Affinity Receptor (TRK): A Role for BCL-2

Carlo Pincelli, Anne R. Haake,† Luisa Benassi, Emanuela Grassilli,* Cristina Magnoni, Daniela Ottani, Renata Polakowska,† Claudio Franceschi,* and Alberto Giannetti

From the Departments of Dermatology and *Biomedical Sciences, Section of General Pathology, University of Modena, Modena, Italy; and †Dermatology Department, University of Rochester, Rochester, New York, U.S.A.

Normal human keratinocytes synthesize and release nerve growth factor (NGF) and express both the low- and the high-affinity NGF receptor. Because NGF has been shown to rescue certain cell types from programmed cell death, we investigated the role of endogenous NGF in preventing keratinocyte apoptosis. We report here that apoptosis is induced in normal human keratinocytes in culture by blocking endogenous NGF signaling with either anti-NGF neutralizing antibody or K252, a specific inhibitor of the tyrosine kinase high-affinity NGF receptor. Apoptosis was assessed by DNA laddering, electron microscopy, and *in situ* nick end labeling technique. In anti-NGF-treated keratinocytes, the apoptotic process starts at 96 h, and is maximal at 120 h. After K252 treatment, apoptosis starts at 48 h and peaks at 120 h. Because the product of the bcl-2 proto-oncogene protects many cell types from apoptosis, we measured the levels of this protein in apoptotic keratinocytes. We found that both K252 and anti-NGF antibody strikingly downregulate bcl-2 expression, starting at 72 h. Furthermore, HaCat keratinocytes stably transfected with a plasmid containing bcl-2 cDNA fail to undergo apoptosis when treated with K252. These findings show that autocrine NGF acts as a survival factor for human keratinocytes *in vitro* through its high-affinity NGF receptor, possibly by maintaining constant levels of Bcl-2. ***Key words: HaCat cells/K252/programmed cell death. J Invest Dermatol 109:757–764, 1997***

Apoptosis or programmed cell death is an active, gene-dependent process of selective cell self-deletion that plays a fundamental role in the control of embryonic morphogenesis, tissue remodelling, and homeostasis as well as in many pathologic conditions such as progression of AIDS and cancer regression (Kerr *et al*, 1972; Cohen, 1993; Schwartz and Osborne, 1993; Wyllie, 1993; Ameisen *et al*, 1995). Apoptosis is morphologically and biochemically characterized by condensation of the cell, loss of plasma membrane microvilli, shrinking and segmentation of the nucleus, and degradation of chromosomal DNA (Hockenbery, 1995).

In human skin, apoptosis has been widely observed by morphologic criteria in pathologic conditions such as lichen planus, graft-versus-host disease, Bowen's disease, fixed drug eruption, squamous cell carcinoma, and alopecia areata (Weedon, 1990; Norris *et al*, 1995). Furthermore, apoptotic cells have been observed in the epidermis after ultraviolet (UV) exposure ("sunburn cells") (Young, 1987) and in the hair follicle during catagen (Weedon and Strutton, 1984). More recently, it has been proposed that apoptosis also plays a role in normal human skin. Apoptotic cells occur in developing human epidermis (Polakowska *et al*, 1994), and individual apoptotic nuclei have been detected in the suprabasal layer of normal adult epidermis by *in situ* nick-end labeling technique (Gavrieli *et al*, 1992). Moreover, Grubauer *et al* have shown the presence of apoptotic bodies in normal skin by electron microscopy (Grubauer *et al*, 1986). Despite this morphologic evidence, the role of apoptosis in the skin is not fully understood. Studies by Budtz on toad skin suggest that apoptosis is a crucial event in epidermal homeostasis: cell deletion by apoptosis removes the excess cells, thus maintaining the proper architecture (Budtz, 1994). It has been proposed that these mechanisms could also be operating in human skin and that some factors that regulate keratinocyte proliferation and differentiation may also be involved in the apoptotic processes in the epidermis (Polakowska and Haake, 1994).

Nerve growth factor (NGF) is a neurotrophic polypeptide that is necessary for survival and differentiation of various neuronal cell populations (Levi-Montalcini, 1987). NGF deprivation induces neuronal death through an apoptotic process that requires both mRNA and protein synthesis (Oppenheim *et al*, 1990). In addition, NGF recently has been shown to suppress apoptosis in murine neutrophils (Kannan *et al*, 1992). In the skin, keratinocytes synthesize and secrete biologically active NGF (Di Marco *et al*, 1991; Pincelli *et al*, 1994). Epidermal melanocytes undergo apoptosis after UV irradiation, but they can be rescued from this type of death by NGF (Zhai *et al*, 1996). NGF mediates its effects by binding two receptors, a low-affinity receptor of ≈75 kDa (p75) and a high-affinity receptor of ≈140 kDa (TRK) (Johnson *et al*, 1986; Kaplan *et al*, 1991a). There is a large body of evidence supporting the concept that TRK, but not p75, is the receptor mediating the

Manuscript received April 21, 1997; revised August 15, 1997; accepted for publication August 22, 1997.

Reprint requests to: Dr. Carlo Pincelli, Department of Dermatology, University of Modena, Via del Pozzo, 71, 41100 Modena, Italy.

Abbreviations: KGM, keratinocyte growth medium; NGF-R, nerve growth factor receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; TRK, high-affinity NGF-receptor.

This work was presented in part at the Society for Investigative Dermatology Meeting (Washington DC, May 1–5, 1996).

0022-202X/97/$10.50 · Copyright © 1997 by The Society for Investigative Dermatology, Inc.

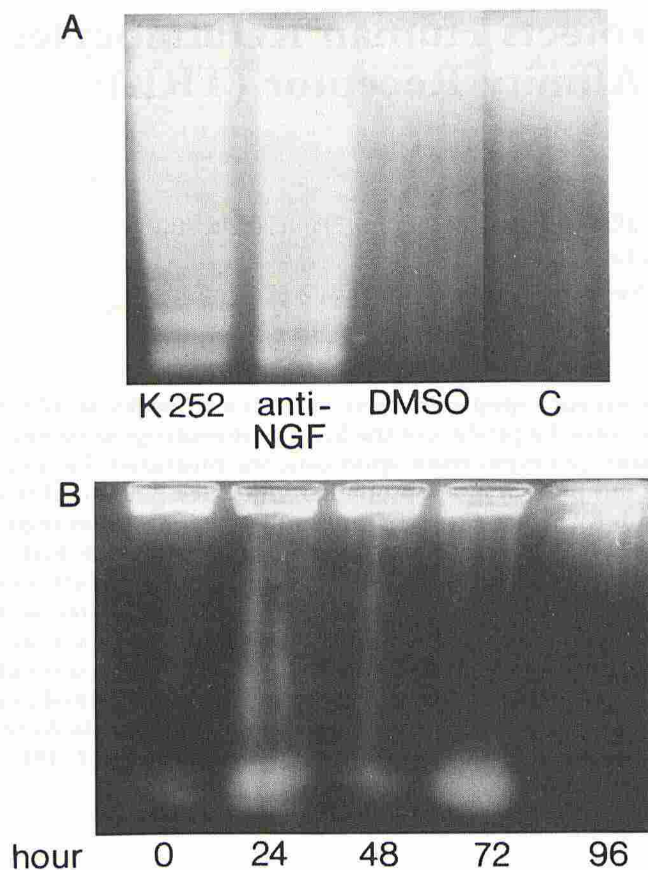

Figure 1. DNA fragmentation in keratinocytes. (*A*) Crude lysates were obtained from attached and detached cells (2×10^6) at different times after plating, as reported in *Material and Methods*. Cells were plated at 15×10^3 per cm^2 and lysates obtained from keratinocytes treated with K252 (200 nM), anti-NGF antibody (40 μg per ml), diluent (dimethylsulfoxide), or control preimmune IgG1 (C). Typical DNA ladder is shown 96 h after treatment with K252 and 120 h after treatment with anti-NGF antibody. (*B*) DNA fragmentation in fibroblasts. K252 was added to subconfluent human fibroblasts and crude lysates from 1.5×10^6 cells were analyzed at different times.

trophic activities of NGF (Barbacid, 1993). Human keratinocytes express the low- and the high-affinity NGF-receptor (NGF-R) both at the mRNA and at the protein level (Pincelli *et al*, 1994). Similarly to other cell types, in keratinocyte cultures it has been shown that addition of NGF induces tyrosine phosphorylation of TRK,[1] which is necessary for signal transduction (Kaplan *et al*, 1991b). K252 is an alcaloid-like compound isolated from Nocardiopsis that was originally characterized as an inhibitor of protein kinase C (Kase *et al*, 1987). Subsequently, K252 was shown to be a specific inhibitor of NGF-induced biochemical effects and neurite outgrowth in the pheochromocytoma cell line PC12 (Koizumi *et al*, 1988). In addition, Berg *et al* have reported that K252 inhibits NGF-induced tyrosine phosphorylation of TRK in PC12 cells, whereas this compound has no effect on the tyrosine phosphorylation of epidermal growth factor-receptor, demonstrating that K252 is a specific blocker of NGF-induced biologic responses mediated by TRK (Berg *et al*, 1992). We have recently demonstrated that K252 inhibits NGF-induced proliferation of cultured human keratinocytes (Pincelli *et al*, 1994).

The expression of bcl-2 proto-oncogene product blocks apoptosis in many cell types (Hockenbery *et al*, 1990, 1991). Immunohistochemical studies have revealed that Bcl-2 protein is expressed exclusively in the basal layer of the epidermis (Bianchi *et al*, 1994), the site where the functional high-affinity NGF-R is located (Di Marco *et al*, 1993; Pincelli *et al*, 1994).

In this paper, we report that both K252 and anti-NGF antibody induce apoptosis in human keratinocytes, indicating that endogenous NGF could be a survival factor for these cells in culture. Furthermore, both K252 and anti-NGF antibody strikingly downregulate the expression of Bcl-2 protein in human keratinocytes, whereas K252 fails to induce apoptosis in HaCat keratinocytes overexpressing bcl-2.

MATERIAL AND METHODS

Cell cultures Keratinocyte cultures were prepared as described (Pincelli *et al*, 1994). Briefly, keratinocytes for primary cultures were obtained from neonatal foreskin. Skin was minced and trypsinized (0.05% trypsin, 0.02% ethylenediamine tetraacetic acid) at 37°C for 3 h and keratinocytes were grown in 75-cm^2 culture flasks (Costar, Cambridge, MA) with mitomycin (10 mg per ml)-treated 3T3 cells (Sigma, St. Louis, MO) for 2 h at 37°C. Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12, 3:1) (Seromed-Biochrom, Berlin, Germany) containing insulin (5 μg per ml, Sigma), transferrin (5 μg per ml, Sigma), triiodothyronine (2 nM, Sigma), hydrocortisone (0.4 μg per ml, Sigma), adenine (180 mM, Sigma), mouse epidermal growth factor (10 ng per ml, Sigma), and 10% fetal calf serum (Seromed-Biochrom). Subconfluent primary cultures were passaged in secondary cultures and grown in serum-free medium containing bovine pituitary extract [keratinocyte growth medium (KGM), Clonetics, San Diego, CA] until subconfluency. Cells were plated at 15×10^3 per cm^2. For the experiments, keratinocytes were starved in medium deprived of growth factors and bovine pituitary extract (KBM, Clonetics) for 24 h and provided K252 (50, 100, 200 nM, Calbiochem, La Jolla, CA, USA), neutralizing goat anti-NGF antibody (R & D, 10, 20, 40 μg per ml; ND50 = 10 mg per ml in the IMR-32 neuroblastoma cell proliferation assay in response to 100 ng per ml NGF), preimmune IgG1, or diluents alone. Both attached and detached keratinocytes were collected 24, 48, 72, 96, 120, and 144 h after plating to evaluate apoptosis. Human fibroblasts (0.5×10^6) were seeded in T25 flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and cultured until subconfluency before K252 addition. Cultures were harvested 24, 48, 72, and 96 h after K252 addition (200 nM) and cellular pellets were frozen at −20°C to be further analyzed for DNA laddering. HaCat adult keratinocytes (originated in Dr. Norbert Fusenig's laboratory), an immortalized cell line, were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and were used for transfection studies, as described (Haake and Polakowska, 1995).

Stable transfection cDNA coding for bcl-2 was subcloned into the pcDNA/Neo eukaryotic expression vector and introduced by lipofection into HaCat keratinocytes. Control HaCat cells were transfected with the pcDNA/Neo vector minus bcl-2 cDNA insert. Parental HaCat cell line, HaCat cells stably transfected with bcl-2, and the control transfectant were cultured until they reached subconfluency. They were then treated with K252 alone or diluents, as for normal keratinocytes. Cultures were harvested at different times for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, and western blot.

DNA recovery and electrophoresis Frozen pellets were resuspended in lysis buffer containing 10 mM ethylenediamine tetraacetic acid, 50 mM Tris at pH 8, 0.5% Sarkosyl, 0.5 mg proteinase K (Boehringer, Mannheim, Germany) per ml. After 1 h incubation at 50°C, the suspension was supplemented with 0.25 mg heat-treated RNase A (Boehringer) per ml and further incubated in the water-bath at 50°C for 1 h. Crude extracts were then transferred to 70°C and added loading buffer (10 mM ethylenediamine tetraacetic acid at pH 8, containing 0.25% bromophenol blue, 1% low-melting agarose, and 40% sucrose). Electrophoresis was carried out overnight at 15 V on 2% agarose gel.

Morphology TUNEL keratinocytes were collected at different times (see above), cytospun onto slides precoated with 0.01% poly L-lysine and air dried. "In situ cell death detection kit" (Boehringer) was used as recommended by the manufacturer. Briefly, cells were fixed with a 4% buffered paraformaldehyde solution before permeabilization with Triton X (0.1%) and sodium citrate. Cells were incubated with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase for 1 h at 37°C. Keratinocytes were further incubated with anti-fluorescein antibody conjugated with alkaline phosphatase (AP). New Fuchsin was used as a substrate for developing the reaction. Approximately 100 cells were evaluated, in randomly selected high power fields, for each point and the percentage of TUNEL positive cells was counted. Each experiment was repeated three times. Negative control was obtained by replacing the

[1]Zhai S, Pincelli C, Yaar M, Gonsalves J, Gilchrest BA. The role of nerve growth factor in preventing keratinocyte apoptosis. *J Invest Dermatol* 104: 572a, 1995 (abstr.).

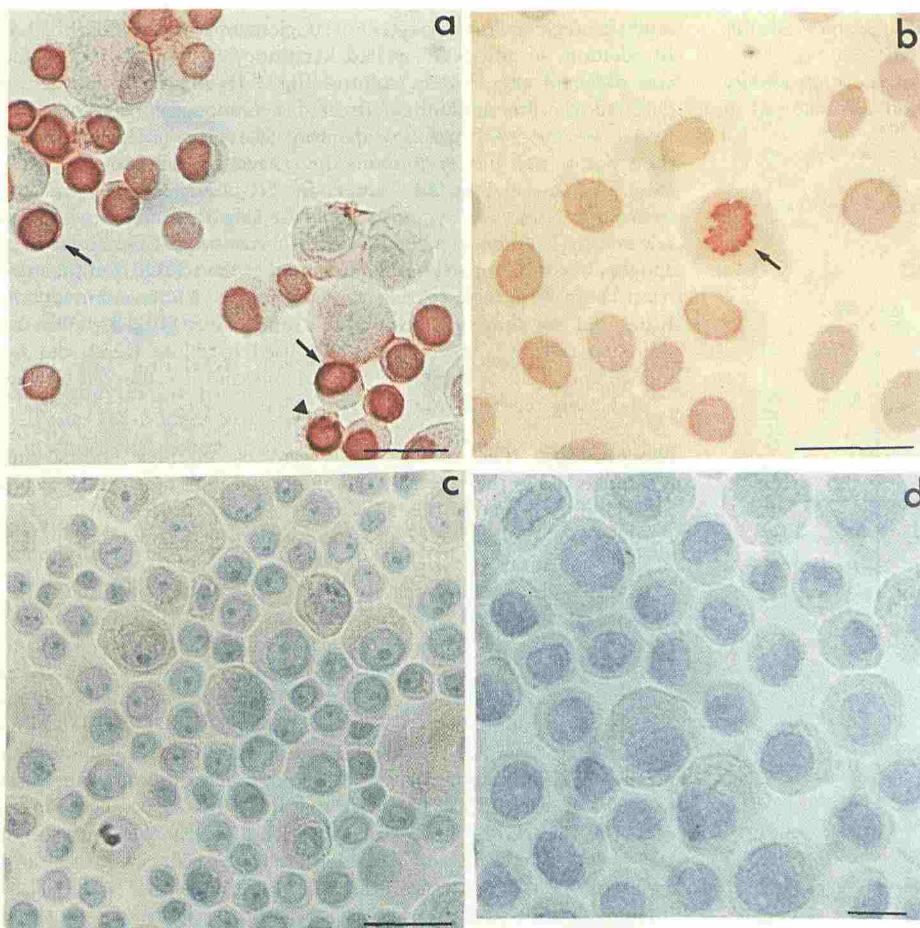

Figure 2. TUNEL staining. Keratinocytes were collected at 24, 48, 72, 96, 120, and 144 h, and cytospins stained for DNA breaks with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase followed by incubation with anti-fluorescein antibody conjugated with AP. Several apoptotic nuclei (→) are observed in keratinocytes 120 h after treatment with K252 (200 nM) (*a*) and with anti-NGF (40 μg per ml) (*b*), but not in keratinocytes treated with diluent alone at 120 h, respectively (*c* and *d*). *Arrowheads* point to the peripheral chromatin condensation. Staining was performed four times and pictures presented here are from a single representative experiment. *Scale bars*, 10 mm.

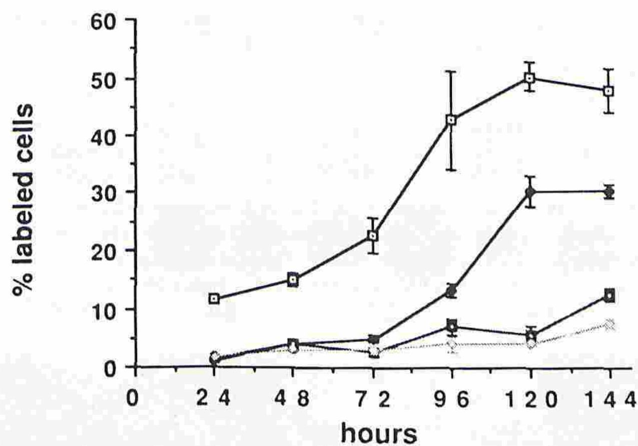

Figure 3. TUNEL staining. Kinetics of apoptosis induced in keratinocytes by K252 (□), anti-NGF (◆), diluent alone (■), or preimmune IgG1 (◇). Approximately 100 cells were counted, in randomly selected fields, for each point and percentages are expressed as the means ± SD of three experiments. Student t test was used for comparison of the means. Anti-NGF *versus* diluent at 72 h, $p < 0.002$; at 96 h, $p < 0.02$; at 120 h, $p < 0.0001$; at 144 h, $p < 0.003$. K252 *versus* diluent at 48, 72, and 120 h, $p < 0.0001$; at 96 h, $p < 0.02$; at 144 h, $p < 0.001$. Anti-NGF *versus* preimmune IgG at 96 h, $p < 0.02$; at 120 and 144 h, $p < 0.0001$.

primary incubation with a nucleotide mixture without terminal deoxynucleotidyl transferase.

Electron microscopy This technique was performed using standard protocols. Briefly, cells were grown and treated as described before. Keratinocytes were then washed gently in Tyrode, and fixed in 2.5% glutaraldehyde in Tyrode, pH 7.4. Postfixation was carried out in osmium tetroxide. Cells were dehydrated in graded alcohol solution and propylene oxide and embedded in Durcupan (Fluka, Buchs, Switzerland). Ultra-thin sections were stained with uranyl acetate and lead citrate before observation and photography with an electron microscopy Philips EM 400T/ST (Eindhoven, The Netherlands).

Western blot Monolayer cultures in 60-mm dishes were washed with phosphate-buffered saline and extracted with 1 ml of RIPA buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Na deoxycolate, 1% Triton × 100, 0.1% sodium dodecyl sulfate, 0.2% NaN_3, 10 mg phenylmethylsulfonyl fluoride per ml, aprotinin, leupeptin, pH 8.5, Sigma). Forty micrograms of protein per lane were loaded onto 10% polyacrylamide gel, and transferred to nitrocellulose filters. Filters were soaked overnight in 5% non-fat dry milk diluted in phosphate-buffered saline/Tween and a mouse monoclonal antibody specific for human bcl-2 (Dako, Glostrop, Denmark) was added at a 1:40 dilution in 1% non-fat dry milk for 2 h at room temperature. Blots were washed three times in phosphate-buffered saline/Tween and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA) at 1:500 dilution at room temperature for 1 h. Detection was performed with the ECL system (Amersham, IL).

RESULTS

DNA fragmentation The DNA ladder, caused by endonuclease cleavage of internucleosomal DNA, is one of the biochemical features of apoptosis and can be detected within a few hours or a few days depending on the apoptotic stimulus and the cell type. For instance, the entire apoptotic process leading to DNA fragmentation requires 1–3 h in dexamethasone-treated lymphocytes and thymocytes (Cohen and Duke, 1984). On the other hand, apoptosis seems to be longer in keratinocytes that may require up to 48–72 h to complete the apoptotic process (Young, 1987; Haake and Polakowska, 1993). Granular keratinocyte cultures exhibit a considerable nucleosomal ladder when cultured for 24 h in serum-free medium (McCall and Cohen, 1991), and in the mouse keratinocyte Pam 212 cell line, which

undergoes spontaneous apoptosis, DNA fragmentation is observed after 3 d in culture (Marthinuss *et al*, 1995).

Here we report that 96 h after the addition of the high-affinity NGF-R inhibitor K252, typical DNA fragmentation was detected in crude lysates from keratinocytes not supplemented with NGF (**Fig 1*A***). In addition, in anti-NGF-treated keratinocytes, typical DNA ladder was obtained after 5 d in culture (**Fig 1*A***). No DNA ladder was detected in dimethylsulfoxide-treated keratinocytes (**Fig 1*A***). No K252- or anti-NGF-induced apoptosis was observed at any earlier time point, nor did preimmune IgG have any apoptogenic effect (**Fig 1*A***). In the absence of exogenous NGF, K252- and anti-NGF-induced keratinocyte apoptosis could be due to inhibition of NGF released by keratinocytes themselves. Alternatively, K252 could cause apoptosis by inhibiting tyrosine kinases other than TRK. On the other hand, human fibroblasts, that do not express TRK, cultured with K252, did not show apoptosis at any time point (**Fig 1*B***). Dimethyl sulfoxide (DMSO) at concentrations used to dilute K252, did not induce apoptosis, as shown in **Fig 1*A***, nor did it cause cell toxicity, as previously reported (Pincelli *et al*, 1994).

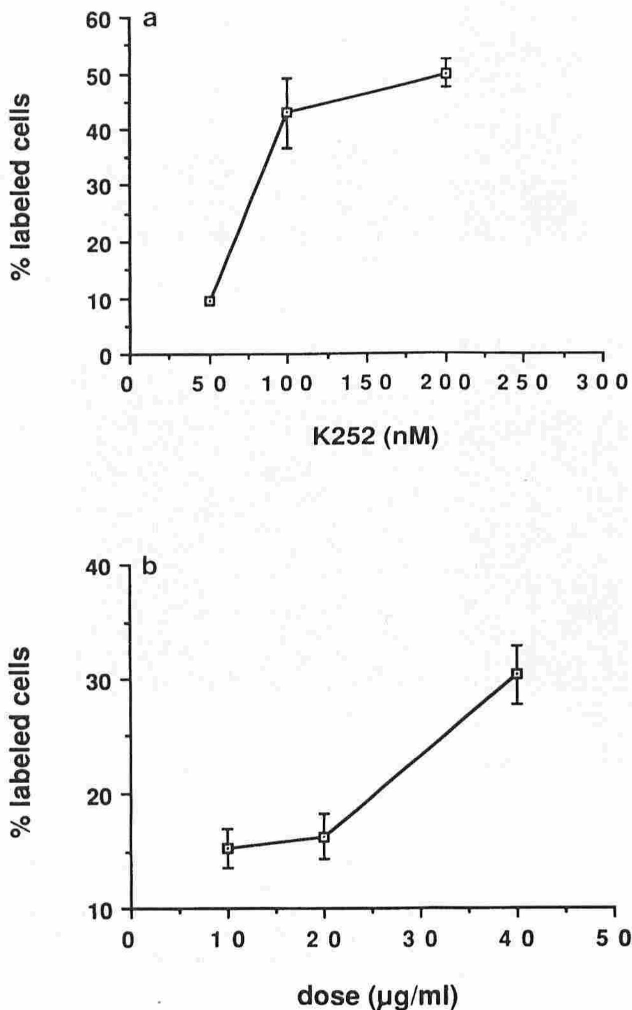

Figure 4. TUNEL staining. Dose–response curve of apoptosis induced in keratinocytes by K252 (*a*) and anti-NGF antibody (*b*) at 120 h. Around 100 cells were counted, in randomly selected fields, for each point and percentages are expressed as the means ± SD of three experiments.

Morphology The recently developed *in situ* nick end labeling technique allows the detection of apoptosis at the single cell level, and the staining precedes the appearance of the nucleosomal ladder. Furthermore, TUNEL enables a quantitation of the apoptotic process in cell populations (Gavrieli *et al*, 1992).

In this study, we show that 48 h after the addition of K252, ≈15% of keratinocytes were stained. The number of TUNEL positive cells progressively increased up to 120 h when roughly 50% of keratinocytes were apoptotic (**Figs 2*a*, 3**). On the other hand, TUNEL positive cell number did not change in anti-NGF-treated keratinocytes and untreated cells up to 48 h. At 72 h the percentage of TUNEL positive cells in anti-NGF-treated keratinocytes was twice the number of positive cells in the control. At 96 h, apoptotic cell number increased to 12% and reached 30% positivity at 120 h (**Figs 2*b*, 3**). The number of TUNEL positive cells appeared to reach a plateau after both stimuli at 144 h. Both K252 and anti-NGF antibody induced keratinocyte apoptosis in a concentration-dependent manner (**Fig 4*a*,*b***). These findings confirm that *in situ* nick end labeling technique allows an earlier detection of apoptosis (Gavrieli *et al*, 1992), which actually begins before the appearance of the DNA ladder. These results demonstrate that as early as 48 h after inhibition of endogenous NGF the apoptotic process begins in cultured keratinocytes. The delayed apoptosis observed in keratinocytes treated with anti-NGF antibody as compared with K252-treated cells is somehow expected. In fact, whereas K252, by specifically blocking the high-affinity NGF-R, completely knocks out NGF function in keratinocytes, it is possible that anti-NGF does not bind all NGF released into the medium and neutralizes only part of NGF activities, as also indicated by the manufacturer. The percentage of TUNEL positive cells in untreated keratinocytes did not exceed 5% up to 120 h, whereas at 144 h, apoptotic keratinocytes were ≈10%. These data are in agreement with previous reports of apoptotic keratinocytes being detected in normal

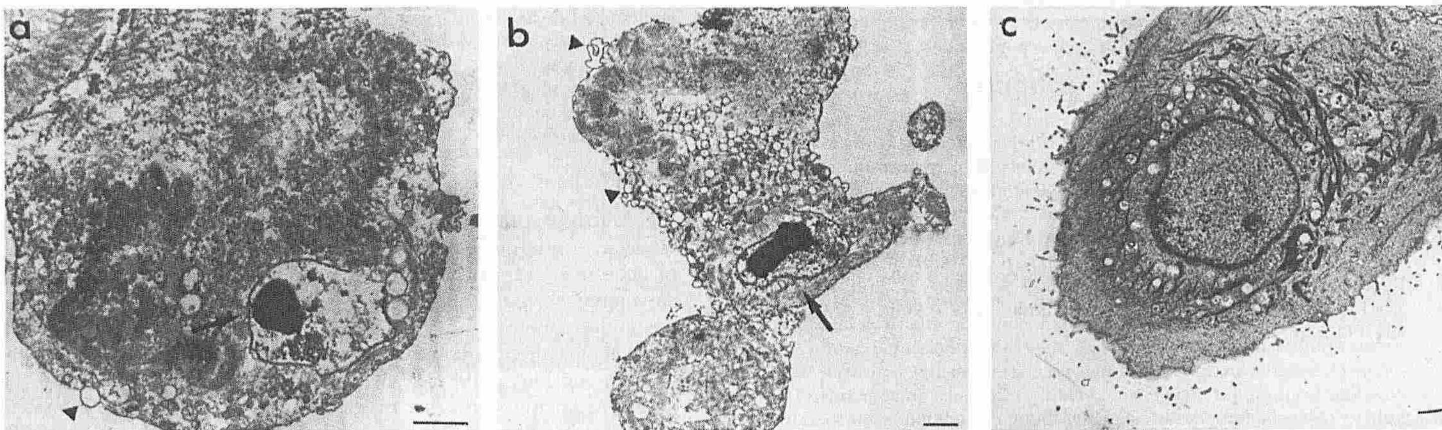

Figure 5. Electron microscopy. Examination of apoptosis induced in keratinocytes 120 h after treatment with 200 nM K252 (*a*), or anti-NGF antibody (40 µg per ml) (*b*). Untreated keratinocytes were examined at 120 h as controls (*c*). Note some of the typical features of apoptotic cells, including loss of microvilli on the plasma membrane, vacuolization of the cytoplasm and surface blebbing (*arrowheads*), and extensive condensation of nuclear chromatin (→). *Scale bars*, 2 mm.

human epidermis (Grubauer *et al*, 1986; Gavrieli *et al*, 1992; Polakowska *et al*, 1994).

Electron microscopy was employed to confirm that K252 or anti-NGF-treated keratinocytes exhibit the gross features of apoptosis, as described by Kerr *et al* (1972). Indeed, Fig 5 illustrates the morphologic changes induced in human keratinocytes after the inhibition of endogenous NGF. Keratinocytes treated with K252 or anti-NGF showed extensive vacuolization of the cytoplasm, loss of microvilli on the plasma membrane, margination, and condensation of the chromatin, whereas the nuclear membrane remained intact (**Fig 5*a,b***). On the contrary, no such morphologic changes were observed in untreated keratinocytes (**Fig 5*c***).

K252 and anti-NGF downregulate bcl-2 levels in human keratinocytes It has been recently reported that UV irradiation decreases Bcl-2 levels in keratinocytes and that overexpression of bcl-2 in transfected keratinocytes confers to these cells resistance to both spontaneous and UV-induced apoptosis (Haake and Polakowska, 1995). We have recently reported that NGF upregulates Bcl-2 levels in human keratinocytes (Zhai *et al*, 1996). *Bcl-2* is exclusively expressed in basal keratinocytes (Hockenbery *et al*, 1991; Bianchi *et al*, 1994), which also bear the high-affinity NGF-R (Di Marco *et al*, 1993; Pincelli *et al*, 1994). NGF released from keratinocytes, by operating in an autocrine mode, might act on basal cells and upregulate Bcl-2. To test this possibility, we cultured keratinocytes with or without the addition of K252 or anti-NGF neutralizing antibody. Bcl-2 proteins were still comparable in treated and untreated keratinocytes at 48 h. Bcl-2 levels significantly decreased in K252-treated keratinocytes at 72 h, and Bcl-2 proteins nearly disappeared at 96 h (**Fig 6*A***). Also, anti-NGF antibody decreased Bcl-2 expression in keratinocytes at 72 and 96 h, although to a lesser extent (**Fig 6*B***).

K252 does not induce apoptosis in HaCat keratinocytes overexpressing bcl-2 To establish the role of bcl-2 in the apoptotic process induced by blockade of NGF function, HaCat keratinocytes, an established cell line, were stably transfected with

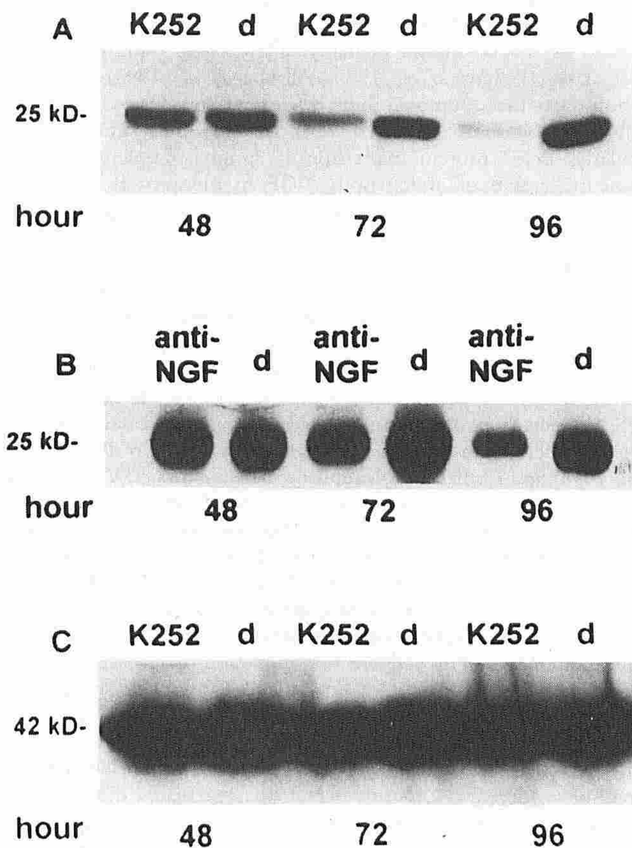

Figure 6. Kinetics of bcl-2 expression in keratinocytes. Western blot of proteins extracted from keratinocytes cultivated in serum-free medium. Proteins were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and visualized with an anti-bcl-2 monoclonal antibody. (*A*) Lysates from keratinocytes treated with K252 or diluent alone (d) at different times. (*B*) Lysates from keratinocytes treated with anti-NGF antibody or diluent alone (d) at different times. (*C*) Duplicate lysates from keratinocytes treated with K252 at different times and stained with an antibody against β-actin.

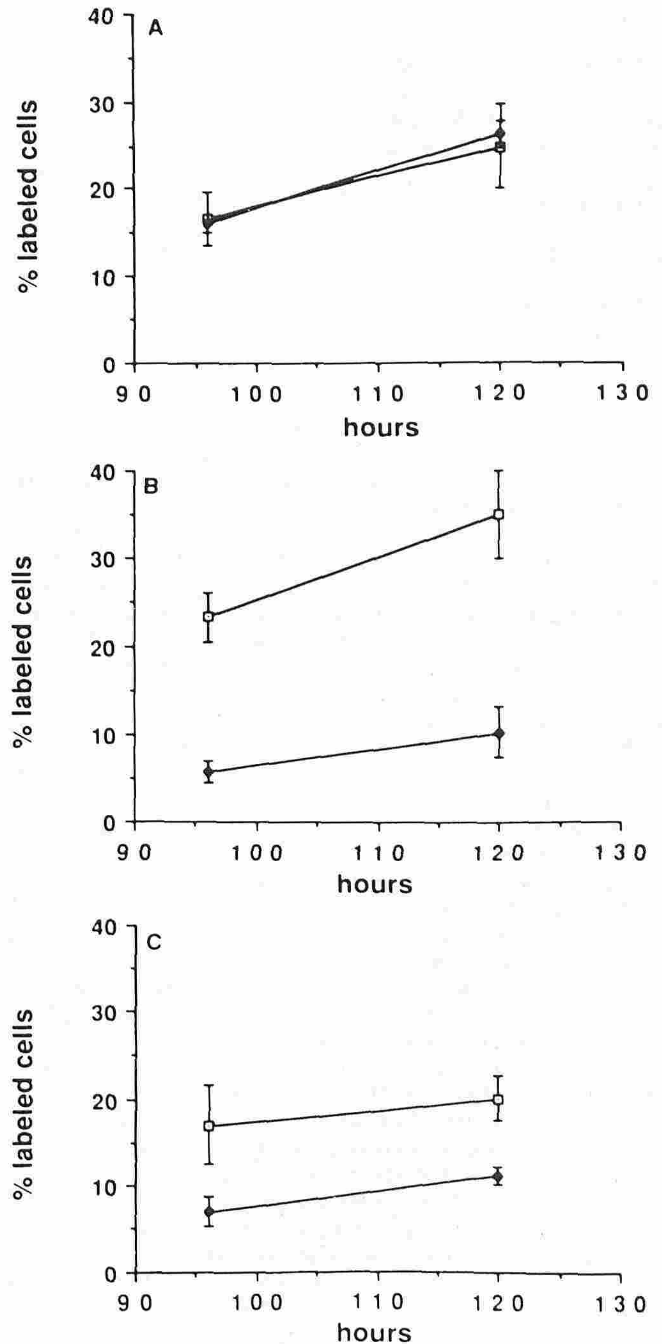

Figure 7. TUNEL staining of HaCat keratinocytes overexpressing bcl-2. Kinetics of apoptosis induced by K252 (□) or diluent (◆) in HaCat keratinocytes overexpressing bcl-2 (*A*), in control transfectant keratinocytes (*B*), and in parental HaCat cells (*C*). Approximately 100 cells were counted, in randomly selected fields, for each point and percentages are expressed as the means ± SD of three experiments. The Student's t test was used for comparison of the means. K252 *versus* diluent at 96 and 120 h in (*A*), not significant; K252 *versus* diluent at 96 and 120 h in (*B*), $p < 0.001$; K252 *versus* diluent at 96 h in (*C*), $p < 0.05$; K252 *versus* diluent at 120 h in (*C*), $p < 0.005$.

Figure 8. Bcl-2 protein expression in transfected HaCat keratinocytes. Western blot of proteins extracted from HaCat keratinocytes stably transfected with bcl-2 (*lanes 3* and *4*) and from control transfectant HaCat cells (*lanes 1* and *2*). Proteins were electrophoresed on a 10% polyacrylamide gel, transferred to nitrocellulose, and visualized with an anti-bcl-2 monoclonal antibody. Cells were treated with diluent alone (d) or with K252 for 72 h.

bcl-2 and treated with K252. Whereas both parental HaCat cells and the control transfectant HaCat cells underwent apoptosis after treatment with K252 starting at 96 h, the same treatment was ineffective in bcl-2 overexpressing cells. This finding was observed by the TUNEL staining that showed a significant increase of apoptotic cells at 96 and 120 h after treatment with K252 in parental HaCat cells and in control transfectants HaCat cells, but not in bcl-2 overexpressing HaCat keratinocytes (**Fig 7**). Furthermore, unlike in normal keratinocytes, Bcl-2 protein was only slightly affected by the treatment with K252 in bcl-2 overexpressing cells at 72 h (**Fig 8**).

DISCUSSION

In this study we have demonstrated that blocking endogenous NGF induces apoptosis in human keratinocytes, thus supporting the idea that NGF is a "survival factor" for these cells in culture. Recently, it has been proposed that cells carry a genetic death program and are destined to die unless they are rescued by "survival factors" from other cells (Kyprianou and Isaacs, 1988; Barde, 1989; Araki *et al*, 1990; Raff, 1992; Haake and Polakowska, 1993). Previous work from our and other laboratories has demonstrated that human keratinocytes synthesize and release NGF (Yaar *et al*, 1991; Di Marco *et al*, 1991; Pincelli *et al*, 1994). The present findings show that human keratinocytes, through this factor, protect themselves from apoptosis, thus providing evidence for an autocrine survival system in human epidermis.

Epidermal homeostasis is maintained by the balance between cell proliferation and cell death (Weinstein *et al*, 1984; Read and Watt, 1988). It appears that apoptosis is an important controlling mechanism of epidermal homeostasis (Polakowska and Haake, 1994). The important role played by apoptosis in establishing the epidermal architecture and maintaining the proper cell number has been convincingly demonstrated by Budtz in several studies on toad and human skin (Budtz, 1985, 1986). In normal epidermis, apoptosis appears to initiate in the basal cell layer (Budtz, 1991; Polakowska and Haake, 1994). Also in pathologic skin conditions, such as after UV irradiation, apoptotic cells are largely found in the proliferative basal cell layer (Haake and Polakowska, 1995). Particularly, S-phase cells of this compartment have been shown to contribute most to the formation of the so-called "sunburn cells" (Danno and Horio, 1982). It appears that dividing cells of the basal cell compartment are most sensitive to apoptosis, because they are undergoing a particular phase of the cell cycle (Polakowska and Haake, 1994) and an abortive entry into the cycle could lead to apoptotic cell death (Ucker, 1991; Rubin *et al*, 1993). NGF is released in increasing amounts by proliferating keratinocytes and the functional high-affinity NGF-R is expressed only in basal keratinocytes (Di Marco *et al*, 1993). Thus, the present study, which is based on the use of a medium (KGM) that selects proliferating keratinocytes, indicates that autocrine NGF could operate in the basal cell compartment to counteract the apoptotic program.

NGF has also been shown to be a potent mitogen for human keratinocytes (Di Marco *et al*, 1993; Pincelli *et al*, 1994). There is a growing body of evidence supporting the idea that mitogenic growth factors, before stimulating proliferation, act by promoting cell survival (Raff, 1992). In particular, it is now widely accepted that NGF exerts its neurotrophic effect by suppressing an apoptotic program rather than trophically stimulating anabolic processes (Martin and Johnson, 1991). It has been reported recently that NGF is an autocrine survival factor rather than a growth factor for memory B lymphocytes (Torcia *et al*, 1996)

The neurotrophic effects of NGF are mediated by the tyrosine kinase high-affinity receptor TRK (Barbacid, 1993). Also in human keratinocytes, TRK has been shown to be the functional NGF-R, because K252, which blocks tyrosine phosphorylation of TRK, inhibits NGF mitogenic activity (Pincelli *et al*, 1994) and induces apoptosis in human keratinocytes, as shown in the present study. Tyrosine kinase activity not only is associated with cell proliferation (Ulrich and Schlessinger, 1990), but is also involved in cell death. Indeed, natural killer cells, which constitutively express the tyrosine kinase receptor c-kit, are saved from apoptosis, in absence of serum or other growth factors, by the c-kit ligand. Furthermore, genistein, a tyrosine kinase inhibitor, dramatically reduces the protective effect of c-kit ligand on natural killer cells (Carson *et al*, 1994). In addition, c-kit ligand suppresses apoptosis in growth factor-deprived and gamma-irradiated mouse mast cells (Yee *et al*, 1994). Our study identifies a tyrosine kinase receptor, TRK, and its ligand, NGF, as mediators of human keratinocyte survival *in vitro*. Although the effect of K252 in inducing keratinocyte and not fibroblast apoptosis clearly demonstrates that NGF acts as a survival factor through its TRK receptor, the involvement of other tyrosine kinase receptors and their ligands in the mechanisms regulating keratinocyte apoptosis cannot be excluded. For instance, transforming growth factor-α, a polypeptide structurally related to epidermal growth factor, mediates its effects through a tyrosine kinase receptor that is constitutively expressed in the basal layer of the epidermis (Nanney *et al*, 1984; Carpenter and Cohen, 1990). Moreover, we have observed that, as expected, genistein induces apoptosis in human keratinocytes after 4 d in culture (data not shown).

Bcl-2 is the best known molecule involved in preventing cell death induced by several stimuli in many different cell types (Hockenbery *et al*, 1990; Nunez *et al*, 1990; Allsopp *et al*, 1993). This study demonstrates that apoptosis, induced in keratinocytes by blocking the activity of NGF, is associated with the downregulation of Bcl-2. Similarly, Bcl-2 protein expression is almost completely abolished by neutralization of endogenous NGF in memory B lymphocytes undergoing apoptosis (Torcia *et al*, 1996).

Bcl-2 downregulation by both K252 and anti-NGF antibody occurs earlier than DNA fragmentation, in agreement with the concept that Bcl-2 blocks a relatively early event associated with apoptosis (Reed, 1994). The inhibition of TRK by K252 results in downregulation of Bcl-2, thus indicating that tyrosine kinase signaling is involved in the regulation of this protein, as reported for other ligands and their tyrosine kinase receptors (Yee *et al*, 1994). The mechanisms by which bcl-2 protects from apoptosis are not clear yet. Bcl-2 has been shown to protect cells from oxidative damage (Hockenbery *et al*, 1993; Kane *et al*, 1993) and formation of sunburn cells following UV irradiation is accompanied by increased concentrations of oxygen intermediates (Danno *et al*, 1984; Punnonen *et al*, 1991). Interestingly, NGF protects UV-induced keratinocyte apoptosis by upregulating bcl-2 expression.[1] In the skin, bcl-2 overexpression inhibits UV-induced keratinocyte apoptosis (Haake and Polakowska, 1995). Similarly, this paper shows that HaCat keratinocytes transfected with bcl-2 are resistant to K252-induced apoptosis. This suggests that induction of apoptosis by inhibition of NGF activity is bcl-2 dependent. One could speculate that autocrine NGF, by maintaining constant levels of Bcl-2, protects keratinocytes from apoptosis.

In conclusion, this study represents the first evidence of a keratinocyte-derived autocrine factor that mediates the survival of human keratinocytes in culture. Endogenous NGF would thus appear to play a key role in epidermal homeostasis by preventing keratinocyte apoptosis. Although there is convincing morphologic evidence from this and other studies that apoptosis does occur in normal human keratinocytes, its role in epidermal homeostasis is not fully understood. According to some investigators keratinocyte terminal differentiation is a specialized form of apoptosis (Fesus *et al*, 1991; Alison and Sarraf, 1992; Polakowska and Haake, 1994). If this is the case, NGF could be regarded as a factor affecting keratinocyte differentiation, possibly counteracting the effect of other factors, such as TGFβ. To address

this question, the modulation of the expression of differentiation-specific genes, such as keratins, loricrin, and involucrin by NGF, should be evaluated. Furthermore, the role of apoptosis should be taken into account in relation to the mechanisms of certain hyperproliferative skin disorders, such as psoriasis (Goldsmith, 1986). Because apoptosis is regarded as the mechanism responsible for eliminating the excess cells in the epidermis (Budtz, 1994), NGF, by preventing it, could favor the epidermal thickness observed in psoriasis. It is interesting to note that NGF levels are increased in psoriatic skin (Fantini *et al*, 1995). Finally, keratinocyte apoptosis and its modulation by NGF could be the targets of future strategies for treating not only psoriasis, but also other hyperproliferative cutaneous conditions, such as skin neoplasia.

We gratefully thank Dr. Caterina Chiodino for helpful discussion and critical review of this manuscript, and Dr. Alessandra Marconi and Mrs Cristina Vaschieri for excellent technical assistance. This work was supported in part by a grant from the "Istituto Dermopatico dell'Immacolata (IDI), Roma."

REFERENCES

Alison M, Sarraf CE: Apoptosis: a gene-directed programme of cell death. *R Coll Phys Lond* 26:25–35, 1992

Allsopp TE, Wyatt S, Paterson HF, Davies AM: The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis. *Cell* 73:295–307, 1993

Ameisen JC, Estaquier J, Idziorek T, De Bels F: Programmed cell death and AIDS. significance, perspectives and unanswered questions. *Cell Death Differentiation* 2:9–22, 1995

Araki S, Shimada Y, Kaji K, Hayashi H: Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. *Biochem Biophys Res Commun* 168:1194–1200, 1990

Barbacid M: Nerve growth factor: a tale of two receptors. *Oncogene* 8:2033–2042, 1993

Barde YA: Trophic factors and neuronal survival. *Neuron* 2:1525–1534, 1989

Berg MM, Sternberg DW, Parada LF, Chao MV: K-252a inhibits nerve growth factor-induced TRK proto-oncogene tyrosine phosphorylation and kinase activity. *J Biol Chem* 267:13–16, 1992

Bianchi L, Farrace MG, Nini G, Piacentini M: Abnormal Bcl-2 and "tissue" transglutaminase expression in psoriatic skin. *J Invest Dermatol* 103:829–833, 1994

Budtz PE: Epidermal tissue homeostasis I. Cell pool size, cell birth rate and cell loss by moulting in the intact toad. Bufo Bufo. *Cell Tissue Kinet* 18:521–532, 1985

Budtz PE: Expectations of human epidermal kinetic homeostasis. *Br J Dermatol* 114:645–650, 1986

Budtz PE: *Tissue Kinetic Homeostasis: Studies on Toad Epidermis*. August Krogh Publications, Copenhagen, 1991

Budtz PE: Epidermal homeostasis: a new model that includes apoptosis. In: Tomei LD, Cope FO (eds.). *Apoptosis II. The Molecular Basis of Apoptosis in Disease.* Cold Spring Harbor Laboratory Press, New York., pp 165–185, 1994

Carpenter G, Cohen S: Epidermal growth factor. *J Biol Chem* 265:7709–7712, 1990

Carson WE, Haldar S, Baiocchi RA, Croce CM, Caligiuri MA: The c-kit ligand suppresses apoptosis of human natural killer cels through the upregulation of bcl-2. *Proc Natl Acad Sci USA* 91:7553–7557, 1994

Cohen JJ: Apoptosis. *Immunol Today* 14:126–130, 1993

Cohen JJ, Duke RC: Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol* 132:38–42, 1984

Danno K, Horio T: Formation of UV-induced apoptosis relates to the cell cycle. *Br J Dermatol* 107:423–428, 1982

Danno K, Horio T, Takigawa M, Imamura S: Role of oxygen intermediates in UV-induced epidermal cell injury. *J Invest Dermatol* 83:166–168, 1984

Di Marco E, Marchisio PC, Bondanza S, Franzi AT, Cancedda R, De Luca M: Growth-regulated synthesis and secretion of biologically active nerve growth factor by human keratinocytes. *J Biol Chem* 266:21718–21722, 1991

Di Marco E, Mathor M, Bondanza S, Cutuli N, Marchisio PC, Cancedda R, De Luca M: Nerve growth factor binds to normal human keratinocytes through high and low affinity receptors and stimulates their growth by a novel autocrine loop. *J Biol Chem* 268:22838–22846, 1993

Fantini F, Magnoni C, Bracci-Laudiero L, Pincelli C: Nerve growth factor is increased in psoriatic skin. *J Invest Dermatol* 105:854–855, 1995

Fesus LP, Davies JA, Piacentini M: Apoptosis: molecular mechanisms in programmed cells death. *Eur J Cell Biol* 56:170–177, 1991

Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501, 1992

Goldsmith LA: Toads and Psoriasis. *Arch Dermatol* 122:939–940, 1986

Grubauer G, Romani N, Kofler H, Stanzl U, Fritsch P, Hintner H: Apoptotic keratin bodies as autoantigens causing the production of IgM-anti-keratin intermediate filament autoantibodies. *J Invest Dermatol* 87:466–471, 1986

Haake AR, Polakowska RR: Cell death by apoptosis in epidermal cell biology. *J Invest Dermatol* 101:107–112, 1993

Haake AR, Polakowska RR: UV-induced apoptosis in skin equivalents: inhibition by phorbol ester and Bcl-2 overexpression. *Cell Death Differentiation* 2:183–193, 1995

Hockenbery DM: Defining apoptosis. *Am J Pathology* 146:16–19, 1995

Hockenbery DM, Nunez G, Milliman C, Schreiber RD, Korsemeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334–336, 1990

Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsemeyer SJ: BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* 88:6961–6965, 1991

Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsemeyer SJ: Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 75:241–251, 1993

Johnson D, Lanahan A, Randy Buck C, *et al*: Expression and structure of the human NGF receptor. *Cell* 47:545–554, 1986

Kane DJ, Sarafin TA, Auton S, *et al*: Bcl-2 inhibition of neural cell death: decreased generation of reactive oxygen species. *Science* 262:1274–1276, 1993

Kannan Y, Usami K, Okada M, Shimizu S, Matsuda H: Nerve growth factor suppresses apoptosis of murine neutrophils. *Biochem Biophys Res Commun* 186:1050–1056, 1992.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF: The TRK protooncogene product: a signal transducing receptor for nerve growth factor. *Science* 252:554–558, 1991a

Kaplan DR, Martin-Zanca D, Parada LF: Tyrosine phsphorylation and tyrosine kinase activity of the TRK protooncogene product induced by NGF. *Nature* 350:158–160, 1991b

Kase H, Iwahashi K, Nakanishi S, *et al*: K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* 142:436–440, 1987

Kerr JFR, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer* 26:239–257, 1972

Koizumi SM, Contreras L, Matsuda Y, Hama T, Lazarovici P, Guroff G: K-252: a specific inhibitor of the action of nerve growth factor on PC12 cells. *J Neurosci* 8:715–721, 1988

Kyprianou NJ, Isaacs T: Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology* 122:552–562, 1988

Levi-Montalcini R: The nerve growth factor 35 years later. *Science* 237:1154–1162, 1987

Marthinuss J, Lawrence L, Seuberg M: Apoptosis in Pam212, an epidermal keratinocyte cell line: a possible role for bcl-2 in epidermal differentiation. *Cell Growth Differentiation* 6:239–250, 1995

Martin DP, Johnson EM: Programmed cell death in the peripheral nervous system. In: Tomei LD, Cope FO (eds.). *Apoptosis: The Molecular Basis of Cell Death.* Cold Spring Harbor Laboratory Press, New York., pp 247–262, 1991

McCall CA, Cohen JJ: Programmed cell death in terminally differentiating keratynocytes: role of endogenous endonuclease. *J Invest Dermatol* 97:111–114, 1991

Nanney LB, Magid M, Stoscheck CM, King LE: Comparison of epidermal growth factor binding and recptor distribution in normal human epidermis and epidermal appendages. *J Invest Dermatol* 83:385–393, 1984

Norris DA, Duke R, Whang K, Middleton M: Immunologic cytotoxicity in alopecia areata: apoptosis of dermal papilla cells in alopecia areata. *J Invest Dermatol* 104:8S–9S, 1995

Nunez G, London L, Hockenbery DM, Alexander M, McKearn JP, Korsemeyer SJ: Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J Immunol* 144:3602–3610, 1990

Oppenheim RW, Prevette D, Tytell M, Homma S: Naturally occurring and induced neuronal death in the chick embryo in vitro requires protein and RNA synthesis: evidence for the role of cell death genes. *Dev Biol* 138:104–113, 1990

Pincelli C, Sevignani C, Manfredini R, *et al*: Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes. *J Invest Dermatol* 103:13–18, 1994

Polakowska RR, Haake AR: Apoptosis: the skin from a new perspective. *Cell Death Differentiation* 1:19–31, 1994

Polakowska RR, Piacentini M, Bartlett R, Goldsmith LA, Haake AR: Apoptosis in human skin development: morphogenesis, periderm, and human stem cells. *Dev Dynamics* 199:176–188, 1994

Punnonen K, Autio P, Kistala U, Ahotupa M: In-vivo effects of solar-simulated ultraviolet irradiation on antioxidant enzymes and lipid peroxidation in human epidermis. *Br J Dermatol* 125:18–20, 1991

Raff MC: Social controls on cell survival and cell death. *Nature* 356:397–400, 1992

Read J, Watt F: A model for in vitro studies of epidermal homeostasis: proliferation and involucrin synthesis by cultured human keratinocytes during recovery after stripping of the suprabasal layers. *J Invest Dermatol* 90:739–743, 1988

Reed JC: Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 124:1–6, 1994

Rubin LL, Philpott KL, Brooks SF: The cell cycle and cell death. *Curr Biol* 3:391–394, 1993

Schwartz LM, Osborne BA: Programmed cell death, apoptosis and killer genes. *Immunology Today* 14:582–590, 1993

Torcia M, Bracci-Laudiero L, Lucibelo M, *et al*: Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell* 85:345–356, 1996

Ucker DS: Death by suicide: one way to go in mammalian cellular development? *N Biol* 3:103–109, 1991

Ulrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203–212, 1990

Weedon D: Apoptosis. *Adv Dermatol* 5:243–256, 1990

Weedon D, Strutton G: Subtle clues to diagnosis by: The recognition of early stages catagen. *Am J Dermatopathol* 6:553–555, 1984

Weinstein GD, McCullough JL, Ross P: Cell proliferation in normal epidermis. *J Invest Dermatol* 82:623–628, 1984

Wyllie AH: Apoptosis. *Br J Cancer* 67:205–208, 1993

Yaar M, Grossman K, Eller M, Gilchrest BA: Evidence for nerve growth factor-mediated paracrine effects in human epidermis. *J Cell Biol* 115:821–828, 1991

Yee NS, Paek I, Besmer P: Role of kit-ligand in proliferation and suppression of apoptosis in mast cells: basis for radiosensitivity of White Spotting and Steel mutant mice. *J Exp Med* 179:1777–1787, 1994

Young AR: The sunburn cell. *Photodermatology* 4:127–134, 1987

Zhai S, Yaar M, Doyle SM, Gilchrest BA: Nerve growth factor rescues pigment cells from ultraviolet-induced apoptosis by upregulating BCL-2 levels. *Exp Cell Res* 224:335–343, 1996