Expression and Function of Nerve Growth Factor and Nerve Growth Factor Receptor on Cultured Keratinocytes

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Keratinocytes, a key cellular component both for homeostasis and pathophysiologic processes of the skin, secrete a number of cytokines and are stimulated by several growth factors. Nerve growth factor (NGF) is synthesized in the skin and basal keratinocytes express the low-affinity nerve growth factor receptor (NGF-R). We present evidence that normal human keratinocytes in culture express the low- and the high-affinity NGF-R both at the mRNA level, as determined by reverse-transcription polymerase chain reaction and at the protein level, as shown by cytofluorimetric analysis. NGF significantly stimulates the proliferation of normal human keratinocytes in culture in a dose-dependent manner. This effect can be prevented by the addition of both an anti-NGF neutralizing antibody and a high-affinity NGF-R (trk) specific inhibitor, the natural alkaloid K252a. By contrast, keratinocyte proliferation is not inhibited by an anti-low-affinity NGF-R monoclonal antibody, thus suggesting that NGF effect on human keratinocytes is mediated by the high-affinity NGF-R. Moreover, NGF mRNA is expressed in normal human keratinocytes and NGF is secreted by keratinocytes in increasing amounts during growth, as detected by enzyme-linked immunosorbent assay. These results suggest that NGF could act as a cytokine in human skin and take part in disorders of keratinocyte proliferation. *Key words: trk/polymerase chain reaction/proliferation/ELISA/secretion. J Invest Dermatol 103:13–18, 1994*

Nerve growth factor (NGF) is a neurotrophic protein that plays a crucial role in the development and maintenance of sensory and sympathetic neurons [1]. NGF is retrogradely transported from target tissues and nerve terminals to neuronal cell bodies and also exerts neurotropic properties for some primary neural crest–derived sensory neurons, peripheral adrenergic, and central cholinergic neurons [2].

There is increasing evidence that NGF, in addition to its actions within the nervous system, possesses a number of biologic effects on cells of the immune-inflammatory compartment. Specifically, NGF increases the number of mast cells in rats [3], causes a massive degranulation of rat peritoneal mast cells [4,5], promotes myeloid progenitor cell growth [6], induces proliferation and differentiation of human B lymphocytes [7], and profoundly modulates human basophil mediator release [8]. Furthermore, NGF induces interleukin 1 (IL-1) expression in PC12 pheochromocytoma cells [9], as it suppresses leukotriene C4 production by human eosinophils [10].

Keratinocytes, the most numerous cells in the epidermis, are thought to be crucial to cutaneous inflammatory responses [11]. Indeed, they produce a variety of mediators such as eicosanoids [12] and cytokines [13], and proliferate in response to different factors [14]. Among others, hormonal and neural substances have been shown to control cell growth. Bovine pituitary extract (BPE) is usually added to culture to increase the proliferation of keratinocytes [15] and prolactin, a hormone of the anterior pituitary gland, has been recently demonstrated to stimulate keratinocyte growth [16]. In addition, the neuropeptide vasoactive intestinal polypeptide is mitogenic for human keratinocytes *in vitro* [17,18] and substance P stimulates the proliferation of Pam 212 cells [19].

NGF synthesis has been shown in developing mouse skin, beginning with sensory innervation [20], and NGF messenger RNA (mRNA) is expressed by human keratinocytes *in vitro* [21,22]. Moreover, NGF-receptor (NGF-R) has recently been demonstrated by immunohistochemistry in normal human skin and NGF-R immunoreactive nerve terminals have been shown in contact with basal keratinocytes [23]. Two classes of NGF-R have been identified, a low-affinity receptor of ~75 kd (p75) and a high-affinity receptor of ~140 kd (p140) [24,25]. This latter has been recently found to be the proto-oncogene trk (p140 trk). Human trk proto-oncogene encodes a 140-kd tyrosine kinase with the structural characteristics of a growth factor receptor [26]. Trk gene is expressed prominently in sensory neurons of spinal ganglia and in the portion of sensory neurons of cranial ganglia that originates from neural crest [27]. NGF specifically stimulates tyrosine autophosphorylation of p140 trk in PC12 cells, in spinal ganglia sensory neurons, and in NIH 3T3 cells transfected with trk cDNA [28,29].

In the present study, we report that normal human keratinocytes express both the low- and the high-affinity NGF-R. Furthermore, NGF stimulates the proliferation of human keratinocytes in culture,

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Abbreviations: BPE, bovine pituitary extract; NGF, nerve growth factor; NGF-R, nerve growth factor receptor; p75, low-affinity NGF-R; p140 trk, high-affinity NGF-R.

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Table I. Oligodeoxynucleotides Used in the RT-PCR Reaction

Oligomer[a]	Nucleotide sequence (5′-3′)	Region	Reference
NGF-R DP	TGAGTGCTGCAAAGCCTGCAA	236–256	
NGF-R RP	TCTCATCCTGGTAGTAGCCGT	445–465	[24]
NGF-R rev pr	TCACCACGTCGGAGAACGTCACGCTGTCCAGGCAG	311–345	
trk DP	GGCTCCTCGGGACTGCGATG	214–233	
trk RP	CAGGAGAGAGACTCCAGAGCG	459–479	[26]
trk rev pr	GCCACGAAACGGAGACCACTCTTCACGATGGTG	372–404	
NGF DP	TCATCATCCCATCCCATCTT	9496–9515	
NGF RP	CTTGACAAAGGTGTGAGTCG	9740–9759	[32]
NGF rev pr	ACTGTTTGAATACACTGTTGTTAATGTTCACCTCTCCCAA	9610–9649	
β-Actin DP	TGGATGATGATATCGCCGCGCTCG	75–98	
β-Actin RP	CACATAGGAATCCTTCTGACCCA	213–235	[33]
β-Actin rev pr	AGGGGAAGACGGCCCGGGGGGCATCGTCGCCCG	138–170	

[a] DP, direct primer; RP, reverse primer; Rev pr, reverse probe.

whereas the trk specific inhibitor K252a prevents this effect. Finally, we show that keratinocytes synthesize and secrete NGF.

MATERIALS AND METHODS

Keratinocyte Cultivation Keratinocyte cultures were prepared as described [18]. Briefly, keratinocytes for primary cultures were obtained from skin plastic surgery. Skin was minced and trypsinized (0.05% trypsin, 0.02% ethylenediaminetetraacetic acid [EDTA]) at 37°C for 3 h and keratinocytes were grown in 75-cm^2 culture flasks (Costar, Cambridge, MA) with mitomycin-treated (10 mg/ml for 2 h at 37°C [Sigma, St. Louis, MO]) 3T3 cells. Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12, 3:1) (Seromed-Biochrom KG, Berlin, Germany) containing insulin (5 μg/ml, Sigma), transferrin (5 μg/ml, Sigma), triiodothyronine (2 nM, Sigma), hydrocortisone (0.4 μg/ml, Sigma), adenine (180 mM, Sigma), mouse epidermal growth factor (EGF, 10 ng/ml, Sigma), and 10% fetal bovine serum (FBS) (Seromed-Biochrom). Subconfluent primary cultures were passaged in secondary cultures as described [18]. FBS was removed from the medium in all the experiments. The K562 cell line has been grown in RPMI 1640 medium supplemented with 20% FBS, 2 mML glutamine, and 40% Iscove's. PC12 cell line was a kind gift from FIDIA S.p.a. (Abano Terme, Italy).

RNA Purification and RQ1 Digestion RNA was purified using a modified guanidinium/cesium chloride centrifugation method as described [30]. Briefly, keratinocytes from secondary cultures were subcultivated in DMEM/F12, as described above, until near confluence. Cells were then lysed in 4 M guanidinium thiocyanate, and the lysates were extracted by ultracentrifugation through a cesium chloride cushion (5.7 M CsCl, 0.1 EDTA, pH 7). The RNAs were then recovered, extracted once with phenol-chloroform, precipitated with ethanol, and resuspended in sterile bidistilled water. To further purify the RNAs, to avoid genomic DNA contamination, samples were digested with RQ1 (Promega, Madison, WI) as described [31]. The RNAs were then extracted, precipitated, and resuspended in sterile bidistilled water containing 200 U/ml RNAse inhibitor (RNAsin, Promega, Madison, WI).

Oligonucletides Primers and Probes Oligonucletides primers and probes were synthesized on an automatic solid-phase synthesizer (Applied Biosystems, Inc., Mod 381 A, Foster City, CA) by standard phosphoramidite chemistry and purified by several extractions with NH_4OH, followed by incubation at 56°C for at least 16 h, and concentrated by ethanol precipitation or polyacrylamide gel electrophoresis [31]. Table I lists the sequences of the synthesized oligomers and their relationship to the organization of the cDNAs regarding the low-affinity NGF-R, the high-affinity NGF-R, NGFβ, and β actin [24,26,32,33]. Computer analysis, performed to compare the synthesized oligomers to the human sequences in the gene bank data base (Hitachi Software Engineering America Ltd., San Bruno, CA), revealed no more than 70% homology among all the other genes. Oligodeoxynucleotides (200 ng) were labeled using standard procedures [34]. Specific radioactivities ranged from 1 to 3×10^8 cpm/μg DNA.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) and Southern Blotting The reactions were carried out using a modification of the technique described [35]. Briefly, 1 μg of total cellular RNA extracted from 30×10^6 cells was reverse-transcribed using 400 U of M-MuLV reverse-transcriptase (GIBCO BRL, Life Technologies Inc., Gaithersburg, MD) and 1 μg of OligodT 15 primer (Boehringer, Mannheim, Germany) for 1 h and 30 min at 37°C in 1 × cDNA buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 10 mM dithiothreitol, and 200 μM dATP, dCPT, dGPT, and dTTP). The resulting specific cDNA fragments were amplified with 2.5 U of Taq polymerase (Promega) in the presence of 0.5 μg direct primer (DP), 0.5 μg reverse primer (RP), both specific for the RNAs examined, and 1 × cDNA buffer. DNA fragments corresponding to the different genes studied were generated during 40 cycles of PCR (denaturation at 94°C for 1 min, annealing for 2 min at temperatures ranging from 40°C to 55°C, depending on the base composition of the different primers, and extension at 72°C for 4 min). Twenty-five microliters of the PCR reaction mixture (50 ml total volume reaction) were separated on a 2% agarose gel, denatured in 0.2 N NaOH, 0.6 M NaCl for 45 min, neutralized in 25 mM phosphate buffer (pH 6.5) for 45 min, and electroblotted onto a Gene-Screen membrane (NEN, Boston, MA) fixed by UV cross-linking (0.6 Joules/cm^2, 312 nm). Blots were hybridized with labeled oligodeoxynucleotide probes as described [36] and autoradiography performed under standard conditions. To evaluate the amount of RNA in each sample, the β actin mRNA was amplified by RT-PCR with an optimized number of 25 cycles. For each sample studied several negative controls were performed, as detailed in the Fig 1 legend. Each experiment was carried out at least three times.

Indirect Immunofluorescence Subconfluent keratinocytes from secondary cultures, cultivated as described above, were harvested using a trypsin/EDTA solution for 15 min and washed. Cell suspensions were fixed with a 2% paraformaldehyde solution w/wo 0.5% Triton X100 (Sigma) for 10 min and rinsed in RPMI plus 10% FBS. Cells were incubated with a monoclonal antibody (MoAb) directed against p75 NGF-R (1:50) (Amersham, Buckinghamshire, England) and a polyclonal antibody against p140 trk NGF-R (epitope corresponding to amino acids 777–790 within the carboxy terminus, 1:50, Santa Cruz Biotechnology, CA) for 1 h at 4°C. Cells were then incubated with biotinylated horse anti-mouse IgG (Vector, CA, diluted 1:30) or with biotinylated goat anti-rabbit IgG (Vector, diluted 1:30) for 45 min at 4°C. Finally, cells were incubated with streptavidin-fluorescein (Amersham, 1:50) for 30 min at 4°C and rinsed in RPMI plus 10% FBS. Cells were then washed again, resuspended in cold phosphate-buffered saline, and analyzed by an argon-equipped flow cytometer (FACSCAN, Becton Dickinson, San Jose, CA). A minimum of 10,000 cells per sample was acquired in list mode by electronically gating the region of cells with physical characteristic of keratinocytes. Controls were performed by replacing the primary antibody with irrelevant mouse or rabbit sera. Cells were also incubated with anti-S-100 antibody to rule out the possibility of melanocyte contamination.

Cell Proliferation Assay Keratinocytes (1×10^4 per well) were cultivated on 3T3 cells (3×10^4 per well) in triplicate on 24-well plates (2 cm^2 per well, Costar) in DMEM/F12 serum-free medium containing 0.1% bovine serum albumin (BSA) (Boehringer), EGF, and various concentrations of NGFβ (Boehringer) (10, 100, 500 ng/ml). In control experiments, keratinocyte cultures were established as above, w/wo the addition of an excess of anti-NGF neutralizing MoAb (Boehringer), or equal concentrations of an isotype-matched control antibody. Keratinocytes were also pretreated with the natural alcaloid K252a (100 nM, Sigma) or with an anti-p75 MoAb. Medium containing EGF, without the addition of NGF, has been referred to as control medium. Media were changed every second day. Keratinocytes were harvested by using a trypsin/EDTA solution for 15 min and counted in an automatic cell counter (Coulter Electronics Ltd., Luton, England). Results are expressed as means ± SEM and Student t test was used for compari-

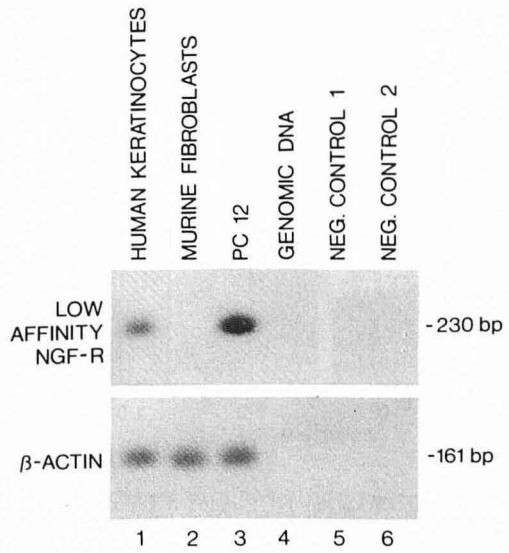

Figure 1. Detection of the low-affinity NGF-R mRNA expression by RT-PCR and Southern blot analysis. *Upper panel:* the autoradiogram of the NGF-R mRNA obtained after Southern analysis is illustrated. Total cellular RNA extraction from the different cell populations as well as RT-PCR experimental conditions are detailed in *Materials and Methods* and the oligomers used are reported in Table I. Cell populations and controls are as follows: *lane 1,* human keratinocytes; *lane 2,* murine fibroblasts; *lane 3,* PC12 cell line (positive control); *lane 4,* genomic DNA derived from healthy donor leukocytes, amplified under the same experimental conditions to exclude possible DNA contamination; *lane 5,* PCR amplification performed on total cellular RNA without reverse transcription; *lane 6,* negative control performed under the same experimental conditions without any template to exclude cross-contamination. *Lower panel:* β actin mRNA expression has been used as a quantitative control of RNA.

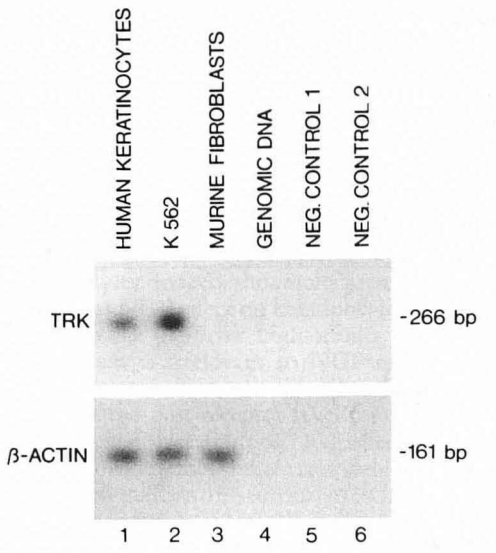

Figure 2. Autoradiogram of the trk (high-affinity NGF-R) mRNA expression by RT-PCR and Southern blot analysis. The size of the amplified fragment and the cell populations studied are illustrated. Cell populations and controls are as follows: *lane 1,* human keratinocytes; *lane 2,* K562 cell line (positive control); *lane 3,* murine fibroblasts; *lanes 4–6* represent the same controls as described in Fig 1. In the *lower panel,* the β-actin mRNA expression, used as a quantitative control, is reported.

son of the means. Results were obtained from three independent experiments.

Enzyme-Linked Immunosorbent Assay (ELISA) Assay for NGF Keratinocytes were subcultivated in serum-free DMEM/F12 with EGF and 0.1% BSA. Culture-conditioned media were collected at different times of incubation and, after centrifugation, stored at −20°C until used. The NGF content was measured using a two-site ELISA specific for human NGF, which has been previously described [37]. Culture-conditioned media from feeder layer fibroblasts (3T3) were collected as a control. Results are expressed as the mean from three independent experiments.

RESULTS

Detection of NGF-R on Normal Human Keratinocytes The RT-PCR data obtained from the study of the low-affinity NGF-R mRNA are shown in Fig 1. A single band of expected size (230 bp) is observed in lane 1 corresponding to human keratinocytes, demonstrating that these cells express the low-affinity NGF-R at the mRNA level. PC12 cells expressing the low-affinity NGF-R mRNA were used as a positive control (Fig 1, *lane 3*). In addition, RT-PCR analysis for trk mRNA expression shows a single band of the expected size (266 bp), which hybridizes with the specific probe after Southern blot analysis (Fig 2, *lane 1*). The same amplified fragment of 266 bp is clearly detectable in K562 cell line that has been used as a positive control (Fig 2, *lane 2*). Negative controls are listed in the legends of Figs 1 and 2. Particularly, the RT-PCR of an equivalent quantity of total RNA from murine fibroblasts (3T3) fails to show any amplified fragment both for the low-affinity NGF-R and trk (Fig 1, *lane 2* and Fig 2, *lane 3*), demonstrating that the signal observed in human keratinocytes is not due to contaminating feeder layer cells. As a control for the amount of RNA in the samples, specific oligomers for the β actin mRNA [33] were used in a parallel amplification reaction generating an amplified fragment of 161 bp. Negative controls, performed as detailed in the legends of Figs 1 and 2 *(lanes 4–6),* show that neither the specific gene studied nor the β actin amplified fragments are detectable. These results clearly demonstrate that human keratinocytes express both the low- and the high-affinity NGF-R at the mRNA level.

Indirect Immunofluorescence Cytofluorimetric analysis on subconfluent keratinocytes was performed to demonstrate the presence of the low- and the high-affinity NGF-R at the protein level. Figure 3 shows that nearly all keratinocytes express both p75 and p140 NGF-R. As illustrated in *A,* with anti-p75 MoAb, most cells display high fluorescence intensity. On the other hand, p140 *(B),* although expressed on most of the cells, shows a much weaker intensity. No staining was observed with irrelevant mouse or rabbit sera and no immunoreactivity was detected using anti-S-100 antibody (data not shown).

Effect of NGF on Keratinocytes To evaluate whether NGF-Rs on keratinocyte are functional, the effect of NGF on keratinocyte proliferation was examined. Keratinocytes from secondary cultures were grown in DMEM serum-free medium containing 0.1% BSA, EGF, and NGFβ (10, 100, and 500 ng/ml). NGFβ significantly stimulated the proliferation of keratinocytes in a dose-dependent fashion (Figs 4 and 5). The specificity of NGF effect on human keratinocytes was assessed by the addition of an excess of anti-NGF neutralizing MoAb that caused a significant reduction of NGF-induced proliferation (Fig 4). The addition of an isotype-matched control antibody did not affect keratinocyte growth (data not shown). To examine the role of p75 and p140 trk NGF-R, the anti-p75 MoAb or the natural alcaloid K252a were added to culture medium. K252a selectively inhibits NGF-induced trk proto-oncogene tyrosine phosphorilation [38], thus blocking the high-affinity NGF-R, whereas anti-p75 inhibits NGF binding to the low-affinity NGF-R. Figure 5 shows that K252a dramatically inhibits NGF-induced keratinocyte proliferation at all concentrations used. The addition of K252a in absence of NGF did not exert any effect on keratinocyte proliferation (Fig 5), indicating that inhibition is not due to cytotoxicity. On the other hand, anti-p75 MoAb could not affect NGF activity on keratinocytes (data not shown).

Detection of NGF in Normal Human Keratinocytes Using sequence-specific NGFβ primers (Table I), samples of reverse-transcribed keratinocyte RNA show a single band of the predicted size

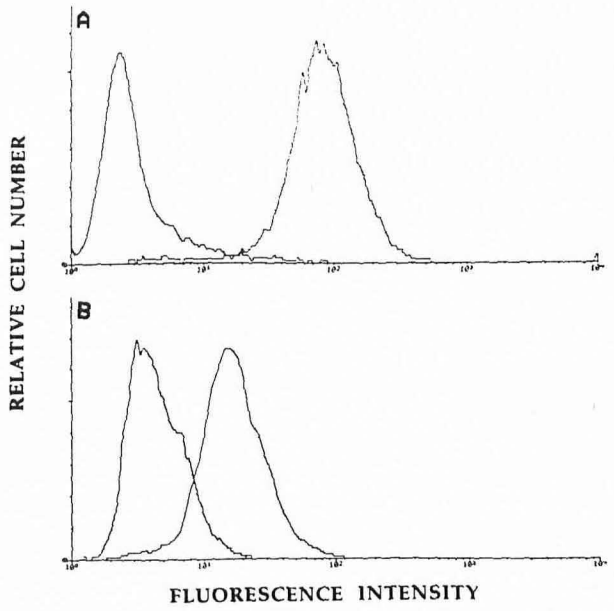

Figure 3. Cytofluorimetric analysis of the expression of the low-affinity NGF-R *(A)* and the high-affinity NGF-R *(B)* on keratinocytes. Subconfluent keratinocytes were harvested using a trypsin/EDTA solution and stained as detailed in *Materials and Methods*. A minimum of 10,000 cells per sample was acquired in list mode by electronically gating the region of cells with physical characteristic of keratinocytes. Keratinocytes were incubated with a mouse MoAb directed against p75 *(A)* or a rabbit polyclonal Ab to p140 *(B)*. Controls were obtained by replacing the primary antibody with irrelevant mouse *(A)* or rabbit *(B)* sera.

(264 bp) that specifically hybridizes with the corresponding probe after Southern blot analysis (Fig 6, *lane 1*). The negative result in Fig 6, *lane 2* rules out the possibility that NGF expression in keratinocyte cultures originates from contaminating murine 3T3 fibro-

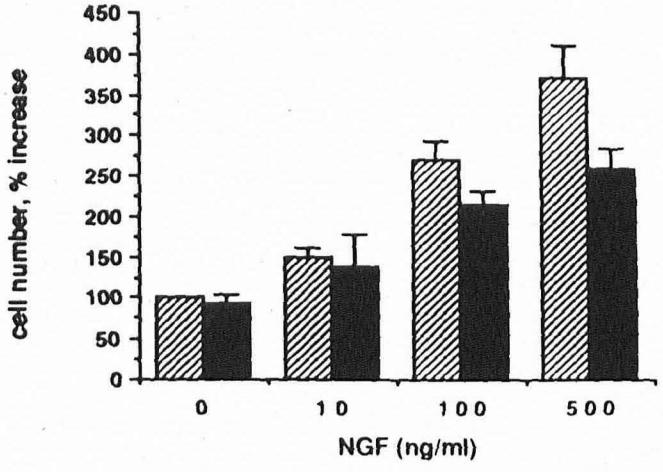

Figure 4. Effect of NGF on the proliferation of cultured human keratinocytes. Keratinocytes were cultivated in DMEM/F12 serum-free medium containing 0.1% BSA, EGF, and various concentrations of NGFβ *(hatched bars)* w/wo the addition of anti-NGF neutralizing MoAb *(solid bars)*. Keratinocyte growth with EGF alone corresponds to 100 on the y axis. Data are expressed as means ± SEM of triplicate cultures from five independent experiments. Student t test was used for comparison of the means. NGFβ (500, 100 ng/ml) versus control (EGF alone), $p < 0.001$. NGFβ (10 ng/ml) versus control, $p < 0.01$. NGFβ (500 ng/ml) versus NGFβ + anti-NGF MoAb, $p < 0.05$.

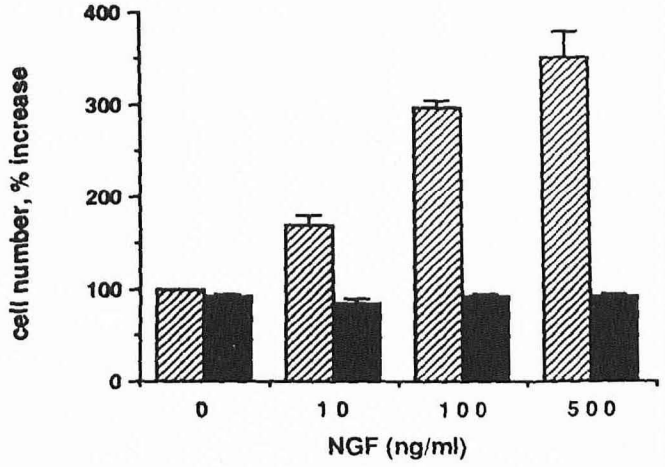

Figure 5. Effect of K252a on NGF-induced keratinocyte proliferation. Keratinocytes were cultivated in DMEM/F12 serum-free medium containing 0.1% BSA, EGF, and various concentrations of NGFβ *(hatched bars)*, w/wo the addition of K252a *(solid bars)*. Keratinocyte growth with EGF alone corresponds to 100 on the y axis. Data are expressed as means ± SEM of triplicate cultures from five independent experiments. Student t test was used for comparison of the means. NGFβ (500, 100, 10 ng/ml) versus control (EGF alone), $p < 0.001$. NGFβ (500, 100 ng/ml) versus NGF + K252a, $p < 0.001$. NGFβ (10 ng/ml) versus NGF + K252a, $p < 0.002$.

blasts. A sample of keratinocyte mRNA that was not reverse transcribed but was otherwise processed identically to the sample in lane 1 fails to show any specific band, thus confirming that the NGF band comes from keratinocyte mRNA and not from contaminating genomic DNA (Fig 6, *lane 3*). Figure 6, *lane 4* shows a negative control performed in the same experimental conditions without any template. Our data confirm that human keratinocytes express NGFβ at the mRNA level.

ELISA To determine NGF levels in keratinocyte conditioned medium, 1×10^4 and 1×10^5 cells per well were seeded. In both

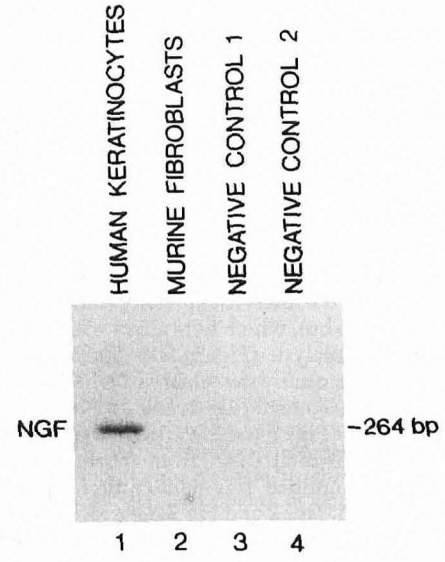

Figure 6. Autoradiogram of the NGFβ mRNA expression by RT-PCR an Southern blot analysis. A strong band of the predicted size (264 bp) is see after 50 cycles *(lane 1)*. The different control lanes are explained in the tex

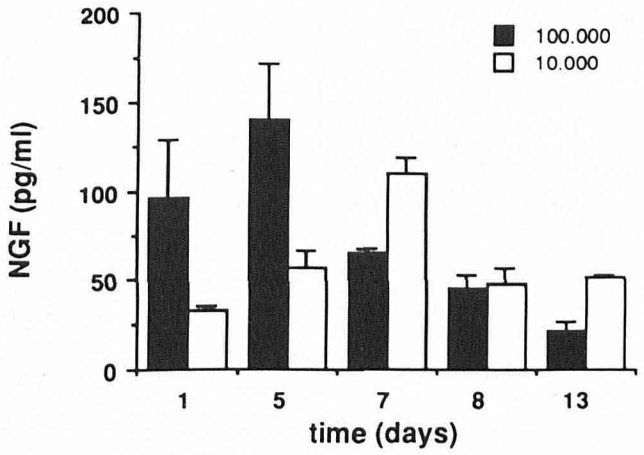

Figure 7. NGFβ levels secreted by normal human keratinocytes, measured by ELISA. 10,000 *(open bars)* or 100,000 *(solid bars)* cells were cultivated in DMEM/F12 on 3T3 cells and culture supernatants collected at different times after seeding. NGF levels are given in pg/ml and results are expressed as means ± SEM of triplicate from three independent experiments.

types of cultures, NGF was detected in the conditioned media after the first day of incubation. NGF levels increased from day 1 to day 5 after seeding proportionally to the different cell number. At day 7, when cells reach the confluence, NGF levels dramatically decreased through day 13 (Fig 7), thus indicating that confluent keratinocytes secrete lower levels of NGF as compared to growing cells. To further confirm this observation, at day 7, a smaller number of seeded keratinocytes, which at this time are still growing, produced greater NGF concentrations than did a higher cell number already at confluence. From day 8 through day 13, all cells have reached the confluence despite the seeding number and secrete equal amounts of NGF. NGF was undetectable in culture supernatants from murine fibroblasts (3T3).

DISCUSSION

This study demonstrates that human keratinocytes in culture express both the low- and the high-affinity receptors mRNA species for NGF and also display a proliferative response to this factor. Thus, non-neuronal cells such as human keratinocytes can, like neurons, co-express both NGF receptor classes. However, unlike neuronal cells, the exposure of human keratinocytes to NGF results in a proliferative rather than a differentiative response. This poses important questions concerning relationships between structural-functional properties of the receptors and the cascade of intracellular events involved in mediating the different physiologic response to NGF.

Recent work has demonstrated that expression of the trk proto-oncogene is, at least in PC12 cells or primary neurons, critical for NGF signal transduction [29,39,40]. Much uncertainty exists, however, concerning the functional role of the p75 receptor component. Although some have suggested that p140 trk alone may constitute the high-affinity NGF receptor [41], and p75 the low-affinity NGF binding sites [42], others have proposed that both receptor components are individually low-affinity receptors, the co-expression and consequent association being necessary for the generation of high-affinity binding sites [29]. Data suggesting that the binding of NGF can convert the low-affinity to the high-affinity receptors [43] and/or that the NGF receptor components may perhaps not interact directly at the level of the receptors themselves [44] could, in part, account for these discrepancies. Despite reports of the expression of similar receptors alone or in combination on cultured Schwann cells or glial cells, respectively [45], and of NGF effects on other cells such as melanocytes [22] and lymphocytes [7], the exact nature and functional requirements of the receptors underlying the NGF effects in non-neural cells have yet to be fully defined.

Results in this paper support the concept that keratinocytes also have the potential of co-expressing p140 trk and p75 NGF-R components. As tyrosine kinase activation is a common feature in the proliferative responses to many growth factors [46], the occurrence of the trk proto-oncogene in human keratinocytes suggests that this receptor component may be important for the proliferative action of NGF on these cells. Trk has been associated with induction of DNA synthesis in variant NIH 3T3 cells when stimulated with NGF [47]. Because the p75 protein lacks tyrosine kinase activity, this receptor component may not be required for the proliferative action of NGF in human keratinocytes. Indeed, the results presented in this paper seem to confirm that NGF effect on keratinocyte proliferation can be exerted through the p140 trk high-affinity NGF-R, whereas blocking p75 protein is irrelevant to NGF mitogenic activity. Whether p75 exerts any effects on the cell surface NGF binding characteristics or at the post-receptor level remains, however, an open question. The concentrations of NGF effective in exerting a proliferative action on keratinocytes are relatively higher than those exerting differentiative effects on neuronal or PC12 cells expressing both NGF-R classes. Possible explanations may include differences per se in the sensitivity of cells to the differentiative versus proliferative action of NGF, differences in the amount or splicing processes of one or the other NGF-R species, and/or cellular shedding of soluble p75 forms keeping NGF in solution [48]. In addition, EGF is routinely added to our culture medium, and its presence may conceivably explain the high doses of NGF needed to stimulate keratinocyte proliferation, perhaps competing with the NGF mitogenic effect. Indeed, EGF is a potent mitogen for keratinocyte and, like NGF, acts via a receptor that possesses an intracellular tyrosine kinase domain [46]. Preliminary data indicate that, without EGF, NGF, at doses as low as 10 ng/ml, is able to induce keratinocyte proliferation (data not shown).

The experiments described here show furthermore that cultured normal human keratinocytes synthesize and secrete NGF. These data confirm the work of others with both human [21] and murine keratinocytes [49], although the present report is the first to quantitate by a sensitive ELISA method the amounts of NGF released. The *in vitro* bioassays for NGF employed in earlier studies do not allow, unfortunately, a precise comparison with our data. The present study also demonstrates that keratinocytes secrete greater amounts of NGF when subconfluent as compared to confluent and more differentiated cells, suggesting that NGF secretion is growth-regulated, in agreement with the study of Di Marco and co-workers [21]. This finding and the proliferative activity of NGF seems to suggest an autocrine role of NGF in human epidermis, and a possible involvement of this molecule in certain physiopathologic cutaneous conditions. For instance, NGF has been shown to accelerate wound healing in mice [50]. This beneficial effect could reflect not only keratinocyte hyperproliferation but also a trophic activity of NGF on sensory neurons, intensely re-innervating the skin during wound healing.

Conceivably, NGF could also be involved in disorders of keratinocyte proliferation such as psoriasis, the latter being characterized by keratinocyte hyperproliferation, inflammatory cell infiltration, and alteration of certain cytokines [51]. In addition to its growth-promoting activity on keratinocytes, NGF is also reported to activate mast cells and T lymphocytes [3,7], which invade the psoriatic lesion [52]. Interleukin-6, which is expressed in high levels in psoriatic skin and which stimulates proliferation of human keratinocytes [53], can enhance NGF secretion [54]. Interestingly, agents used in the treatment of psoriasis, e.g., UVB [49], steroids [21], and retinoic acid [55], modulate NGF expression.

Immunohistochemical studies have shown a consistent correlation between NGF-R expression on keratinocytes and the presence of intraepidermal sensory nerves [56]. Moreover, sensory fibers co-expressing NGF-R and neuropeptide immunoreactivity are seen in close proximity to the basal epidermal cells [23]. As NGF can regulate the expression of neuropeptides in sensory neurons [57,58], perhaps there exists a functional interrelationship between peptidergic sensory neurons, neuropeptides, and NGF at the skin level.

Further work will be required to better understand the role of NGF in normal as well as pathologic conditions in the skin. Human keratinocytes in culture should provide a useful model to study these NGF effects *in vitro*.

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