Evidence for Nerve Growth Factor-mediated Paracrine Effects in Human Epidermis

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Abstract. Nerve growth factor (NGF) is critical to the development and maintenance of the peripheral nervous system, but its possible roles in other organ systems are less well characterized. We have recently shown that human epidermal melanocytes, pigment cells derived from the neural crest, express the NGF receptor (p75 NGF-R) in vitro (Peacocke, M., M. Yaar, C. P. Mansur, M. V. Chao, and B. A. Gilchrest. 1988. *Proc. Natl. Acad. Sci. USA.* 85:5282–5286). Using cultured human skin-derived cells we now demonstrate that the melanocyte p75 NGF-R is functional, in that NGF stimulation modulates melanocyte gene expression; that exposure to an NGF gradient is chemotactic for melanocytes and enhances their dendricity; and that keratinocytes, the dominant epidermal cell type, express NGF messenger RNA and hence are a possible local source of NGF for epidermal melanocytes in the skin. These combined data suggest a paracrine role for NGF in human epidermis.

MAMMALIAN nerve growth factor (NGF)[1] is a 26-kD protein that was first identified in mouse submaxillary gland approximately 40 years ago as an activity that stimulated growth and neurite development of embryonal sensory and sympathetic neural cells (Cohen et al., 1954). Twenty years later the NGF protein was sequenced (Varon and Shooter, 1967), and more recently the NGF gene has been cloned (Ulrich et al., 1983). NGF consists of three types of subunits α, β, and γ which interact to form a ~130-kD complex. It has a well-established role in the development and survival of the central and peripheral nervous systems (Levi-Montalcini, 1987; Edgar, 1985; Thoenen and Edgar, 1985). In the PC12 rat pheochromocytoma cell line (Greene and Tischler, 1976), a widely used model system for studying NGF effects on neural cells, NGF induces neurite outgrowth and gene expression, proving that p75 nerve growth factor-receptor (NGF-R) expressed by these cells is functional (Greenberg et al., 1985; Dickson et al., 1986; Edgar and Thoenen, 1978). Neural crest–derived Schwann cells are capable of synthesizing both NGF and p75 NGF-R, at levels determined in part by their maturity and by injury (Rush, 1984; Heumann et al., 1987). There is increasing evidence that NGF may modulate inflammatory responses in association with tissue injury and that it stimulates wound healing (Pearce and Thompson, 1986; Otten et al., 1989; Matsuda et al., 1988). Specifically, NGF causes proliferation and histamine release by mast cells (Pearce and Thompson, 1986), promotes differentiation of specific granulocytes, enhances T-lymphocyte–dependent antibody synthesis and induces growth and differentiation of human B-lymphocytes (Otten et al., 1989; Matsuda et al., 1988).

All known effects of NGF are mediated by the 26-kD beta subunit through its receptor. There are two types of NGF receptors, one a low molecular weight protein of ~75-kD p75 NGF-R (Chao et al., 1986; Johnson et al., 1986), and the other a higher molecular weight protein of ~140 kD. Both are necessary for the high affinity binding of NGF which is necessary for the cellular response. The higher molecular weight receptor was recently found to be the proto-oncogene, trk, which is a member of the tyrosine kinase receptor family (Klein et al., 1991; Kaplan et al., 1991; Kaplan et al., 1991).

We have recently demonstrated that human epidermal melanocytes can be induced to express the p75 NGF-R in vitro in response to a variety of pharmacologic and physiologic stimuli (Peacocke et al., 1988). A functional role for p75 NGF-R in melanocytes was not established. However, the possibility that NGF may modulate melanocyte behavior is further suggested by the finding that skin epithelium, the principal tissue compartment in which melanocytes reside postnatally, expresses high levels of NGF at least during embryogenesis (Davies et al., 1987). Melanocytes migrate to the epidermis early in embryogenesis (Holbrook K. A., A. M. Vogel, C. A. Foster, and R. Underwood. 1987. *Clin. Res.* 35:691*A*), and subsequently produce melanin pigment in organelles called melanosomes and transfer the pigment to surrounding keratinocytes via extensive dendrites. Melanin pigmentation is the principal determinant of skin color, and increased melanogenesis (tanning) following sun ex-

1. *Abbreviations used in this paper*: NGF, nerve growth factor; p75 NGF-R, NGF receptor.

posure is the body's major source of protection against further ultraviolet injury. After ultraviolet irradiation of human skin, melanin production, melanosome transfer, melanocyte number, and dendricity are all increased (Gilchrest et al., 1979), but the mediators of these phenomena are unknown. We now report that the p75 NGF-R in melanocytes is functional, in that NGF added to melanocyte culture affects the expression of the proto-oncogenes c-fos and c-myc as well as the expression of p75 NGF-R and beta-actin genes. We further show that NGF is chemotactic for melanocytes and induces dendrite formation in vitro as well as expression of p75 NGF-R on the cell surface, as measured by indirect immunofluorescence. Finally, we show that keratinocytes, the most abundant cell in the epidermis, known to elaborate a wide variety of cytokines (Kupper, 1989), express NGF mRNA and thus may provide a paracrine source of this protein to modulate melanocyte function in normal skin.

Materials and Methods

Tissue and Cell Culture

Primary keratinocyte (Gilchrest et al., 1979) and melanocyte (Gilchrest et al., 1984) cultures were prepared from newborn foreskins as described. At confluence dishes were washed twice with 0.02% EDTA, incubated in 0.25% trypsin at 37°C, and disaggregated to form a single cell suspension. Keratinocytes or melanocytes were inoculated on dishes coated with human fibronectin 10 $\mu g/cm^2$ (Gilchrest et al., 1980). Cultures were maintained at 37°C in 8% CO_2 and provided three times weekly with serum-free Medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with 10 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), 10 ng/ml EGF (Bethesda Research Laboratories, Gaithersburg, MD), 10^{-9} M triiodothyronine (Sigma Chemical Co.), 10 μg/ml transferrin (Sigma Chemical Co.), 1.4×10^{-6} M hydrocortisone (Calbiochem-Behring Corp., La Jolla, CA), 2 mg/ml BSA (Sigma Chemical Co.), and 150 μg/ml crude bovine hypothalamic extract (Maciag et al., 1981).

The human epidermal squamous cell carcinoma line SCC-12.B.2 (Rheinwald et al., 1983), kindly provided by Dr. James Rheinwald, was maintained on an irradiated 3T3 feeder layer in a 3:1 mixture of DME (Gibco Laboratories) and Ham's F12 (Gibco Laboratories) supplemented with 5% FBS and antibiotics. For experimental use, cultures were handled identically to the normal keratinocytes.

Melanocyte Migration Studies

A drop of medium 3-7 mm in diameter containing 100 ng of the 26-kD NGF purified from mouse submaxillary gland (CR 2.5S-NGF; Collaborative Research, Bedford, MA) was placed in the middle of fibronectin-coated tissue culture dishes (Gilchrest et al., 1985). In control dishes, the NGF was replaced with either 100 ng transferrin or NGF mixed with neutralizing rabbit antiserum to NGF (Collaborative Research) in sufficient volume to neutralize the added NGF (5 μl/ng NGF as reported by the provider). First passage melanocytes were then plated in the same dishes in a second 3-mm-diam drop of medium ~2 cm away from the site of growth factor application and left to attach for 1 h, a time period previously shown to be sufficient for attachment of nearly 100% of the cells (Gilchrest et al., 1985). The dishes were then gently flooded with 2 ml of growth medium and returned to the incubator. To assure that NGF indeed bound to the fibronectin coated surface, paired dishes containing no cells were processed as above; and 24 h and 5 d after growth medium addition, dishes were fixed with 4% (vol/vol) formaldehyde for 30 min and processed for immunofluorescence as described (Stanley et al., 1980). The first antibody used was either rabbit antiserum to the 2.5S-NGF or normal rabbit serum as control (Collaborative Research). The second antibody used was fluorescein-tagged goat anti-rabbit IgG (Cooper Biomedicals, Malvern, PA). Cultures containing melanocytes were examined daily for 5 d using an inverted phase microscope (160 ×; Zeiss Oberkochen, Germany) without reference to culture identity for gross differences in cells morphology and location in the dish. On day 5 cultures were stained with the BioRad (Richmond CA.) silver stain kit and then counter stained with 1% Rhodanile blue for 20 min. Briefly, for silver staining, cultures were fixed with 40% methanol in PBS for 30 min and after several washes with PBS were incubated with oxidizer for 5 min, silver reagent for 20 min, and several changes of developer for 10 min. The development was stopped by incubation with 5% acetic acid in PBS.

DNA Probes

The probes for c-fos and c-myc were genomic DNA fragments received from the American Type Culture Collection (Rockville, MD) (ATCC #41042, #41010) (Alitalo et al., 1983; Miller et al., 1984). For c-fos a 3.0-kb Nco–Xho fragment was used, and for c-myc a 1.4-kb Cla–EcoRI was used. The probe for glyceraldehyde-3-phosphate dehydrogenase was a 1.2 kb human cDNA received from the American Type Culture Collection (ATCC #57090) (Tso et al., 1985). A 600-bp HindIII–XbaI fragment was used. The probe for p75 NGF-R was an EcoRI 0.8-kb fragment of human p75 NGF-R cDNA received from M. V. Chao (Johnson et al., 1986). The probe for beta-actin was a 2.1-kb BamHI fragment of human beta-actin cDNA received from P. Gunning (Gunning et al., 1983). The probe for human tyrosinase was received from the American Type Culture Collection (ATCC #59510) (Kown et al., 1987); a 1.8-kb EcoRI fragment of the tyrosinase cDNA was used. All fragments were oligo labeled with ^{32}P for a specific activity of 2×10^8 cpm/μg of DNA.

Northern Blot Analysis

Melanocytes were stimulated with phorbol 12-tetradcanoate 13-acetate (TPA) 50 ng/ml, known to induce p75 NGF-R at the cell surface within 24 h (Peacocke et al., 1988), and NGF 50 ng/ml was added to cultures 72 h later. Keratinocytes were grown under standard conditions on fibronectin coated plates in serum-free medium to near confluence. Cultures were then supplemented with 5 μg/ml cycloheximide, known to enhance steady state levels of many mRNA species (Paulsson et al., 1987). Total RNA was extracted from cultured melanocytes by lysis of cells with 4 M guanidine thiocyanate immediately before and .5, 1, 2, 4, 8, and 24 h after addition of NGF, and from keratinocyte cultures 14 h after cycloheximide supplementation. Total RNA of SCC-12.B.2 was extracted from near confluent cells under standard conditions.

RNA was isolated through a 5.7 M cesium chloride gradient (Chirgwin et al., 1979; Glisin et al., 1974). The RNA was resuspended in sterile distilled water, and the concentration of RNA was determined by absorbance at 260 nm. The purity of the sample was determined by A_{260}/A_{280} and was always 2.0 or greater. RNA was reprecipitated with 3 M sodium acetate and ethanol and stored at −70°C. Either 10 or 30 μg RNA (constant for each northern blot), was size fractionated through a 1% agarose gel containing formaldehyde (2.2 M). The RNA was then transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL) and immobilized by shortwave UV illumination. The blot was then prehybridized overnight at 42°C in a solution containing 50% formamide, 10% dextran sulfate, 0.6 M NaCl, 50 mM NaH_2PO_4–H_2O, 5 mM EDTA, 0.02% polyvinylpyrolidone, 0.02% Ficoll, 0.02% BSA, and denatured salmon sperm DNA at 10 μg/ml. Blots were hybridized overnight at 42°C to a ^{32}P-labeled oligo denatured DNA probe with specific activity of 2×10^8 cpm/μg of DNA. The blots were then washed three times for 20 min at room temperature in 0.3 M NaCl/0.03 M sodium citrate/0.1% SDS and then twice for 30 min at 45°C in 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS and twice for 30 min at 55°C in the same solution. Wet blots were sealed in a plastic bag and autoradiography was performed at −70°C with XAR film (Eastman Kodak Co., Rochester, NY) and intensifying screens. Analysis of autoradiographic band density was performed with a densitometer using the one dimensional GSXL software program (LKB, Piscataway, NJ) with manual identification of peaks and baselines.

PCR Amplification Method

Oligonucleotide primers were synthesized on a Bio-search DNA synthesizer. The 5′ primer spanned the first 20 bases of the mature human beta-NGF mRNA and the 3′ primer spanned the last 20 bases of the mature human beta-NGF mRNA (Ulrich et al., 1983).

RNA was reverse transcribed into cDNA as described (Rappolee et al., 1988). A 20 μl reverse transcription reaction mixture containing 10 μg of total cellular RNA, 1×PCR buffer (10 mM Tris, HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin) 1 mM dNTPs, 25 U of RNAsin (Promega Biotech, Madison, WI), 0.5 μg of oligo (dT) and 200 U of BRL (Gaithersburg, MD) Molony murine leukemia virus reverse transcriptase was incubated at 37°C for 60 min, heated to 95°C for 5 min, and then quickly chilled on ice. PCR was performed at a final concentration of 1× PCR buffer, 1 mM dNTPs, 0.1 μg each of the 5′ and 3′ primers, 1 μg

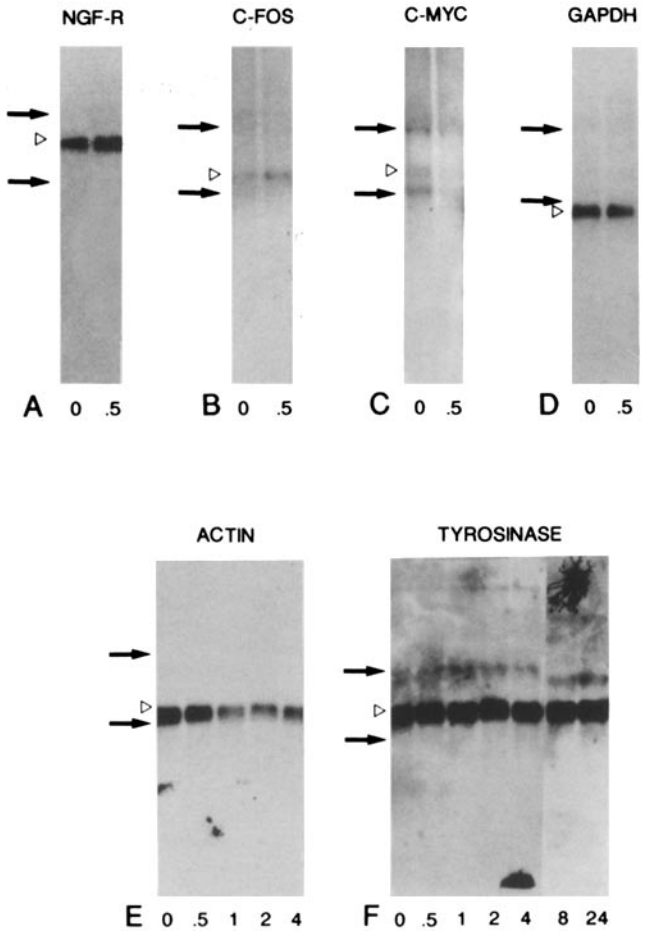

Figure 1. Modulation of melanocyte gene expression by NGF. Representative northern blots display hybridization of the indicated cDNA probes to total cellular RNA harvested from melanocytes at the indicated times (before and .5, 1, 2, 4, 8, and 24 h) after NGF addition to the cultures. (*A*) p75 NGF-R 3.8-kb mRNA is nearly twice its basal level 30 min after NGF addition. (*B*) C-fos 2.2-kb mRNA is more than four times the basal level, previously shown to be induced by TPA treatment of the melanocytes (Peacocke et al., 1988), 30 min after NGF addition. (*C*) C-myc 2.2–2.4-kb mRNA is decreased by >60% 30 min after NGF addition. (*D*) GAPDH 1.4-kb mRNA used as a housekeeping probe shows slightly less mRNA in the second lane (30 min after NGF addition) of the same northern shown in *A–C*. (*D*) Beta-actin 2.1-kb mRNA is decreased by >50% 30 min after NGF addition and by >80% 2 h after NGF addition. (*E*) Tyrosinase 2.3-kb mRNA level is constant for at least 24 h after addition of NGF. For all panels, solid arrows indicate location of the 28S and 18S ribosomal RNA, and open arrow heads indicate the location of the hybridized cDNA. Quantification of expression was performed by densitometry analysis.

of cDNA or as a control against possible contaminating genomic DNA molecules, 1 μg of nonreverse transcribed RNA from the same sample, and 2.5 U of Thermus aquaticus DNA polymerase (Taq polymerase) (Perkin-Elmer/Cetus, Norwalk, CT) in a total volume of 100 μl. For asymmetric PCR amplification to generate single stranded DNA, one primer (either the 5′ or 3′ primer) was used at a concentration of 50 pM and the other primer at a concentration of 1 pM. The mixture was overlaid with mineral oil and then amplified through 40 cycles with the Perkin-Elmer/Cetus thermal cycler. The amplification profile involved denaturation at 95°C for 45 s, primer annealing at 45°C for 45 s, and extension at 72°C for 45 s. Samples of amplified RNA and cDNA were separated over a 3% agarose gel and stained with 0.005% ethidium bromide (Sigma Chemical Co.).

Single-stranded DNA was sequenced using the chain-termination method of Sanger (Sanger et al., 1987).

Results

Nerve Growth Factor Effect on Melanocyte Gene Expression

To determine if p75 NGF-R in melanocytes is functional, we examined the ability of exogenously added NGF to affect the expression of the proto-oncogenes c-fos and c-myc, as well as the beta-actin gene, all of which are known to be stimulated by NGF in PC12 cells (Greenberg et al., 1985). Genes for p75 NGF-R and tyrosinase, a well studied melanocytic enzyme required for melanin synthesis, were also studied. Cultured melanocytes grown under standard conditions were stimulated with 50 ng/ml TPA to induce the receptor, and 50 ng/ml NGF was added to cultures 72 h later. Total RNA was extracted from cultures immediately before and .5, 1, 2, 4, 8, and 24 hours after addition of NGF and used for northern blots. p75 NGF-R mRNA level increased 30 min after NGF stimulation (Fig. 1 *A*), with further increase 4 h after stimulation (Fig. 2). In a total of three experiments, p75 NGF-R mRNA was 1.8 ± 0.2 (mean ± SEM) its basal level ($p < 0.02$, paired t test). 30 min after NGF stimulation there was

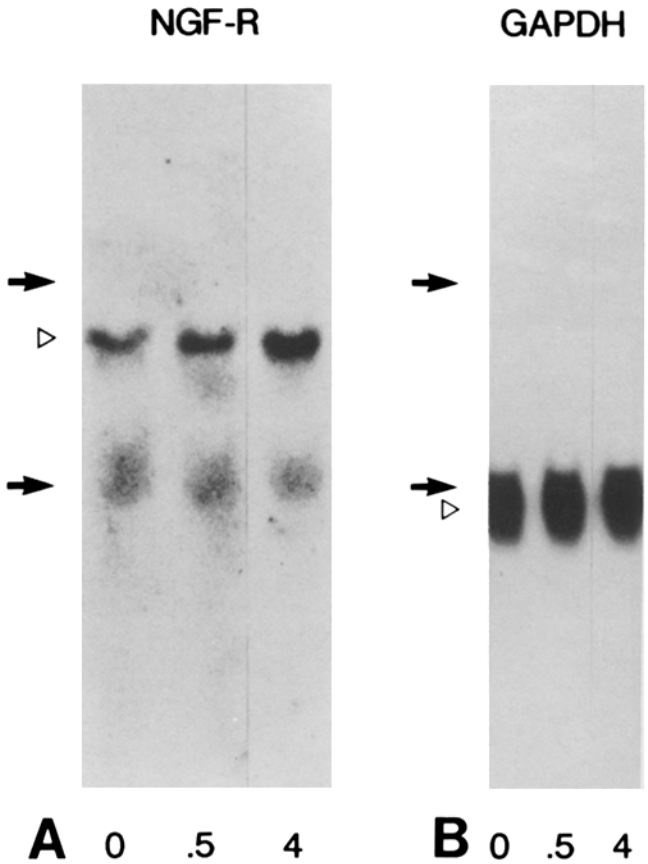

Figure 2. NGF induction of p75 NGF-receptor message in cultured human melanocytes. The same northern blot of total cellular RNA harvested before and .5 and 4 h after NGF addition has been hybridized with cDNA probes for p75 NGF-R and the constitutively expressed GAPDH. (*A*) p75 NGF-R 3.8-kb mRNA level is elevated to nearly twice its TPA-induced basal level (Peacocke et al., 1988) 30 min after NGF addition and more than 2.5 times its basal level 4 h after NGF addition. (*B*) GAPDH 1.4-kb mRNA shows equal amounts of mRNA in each lane.

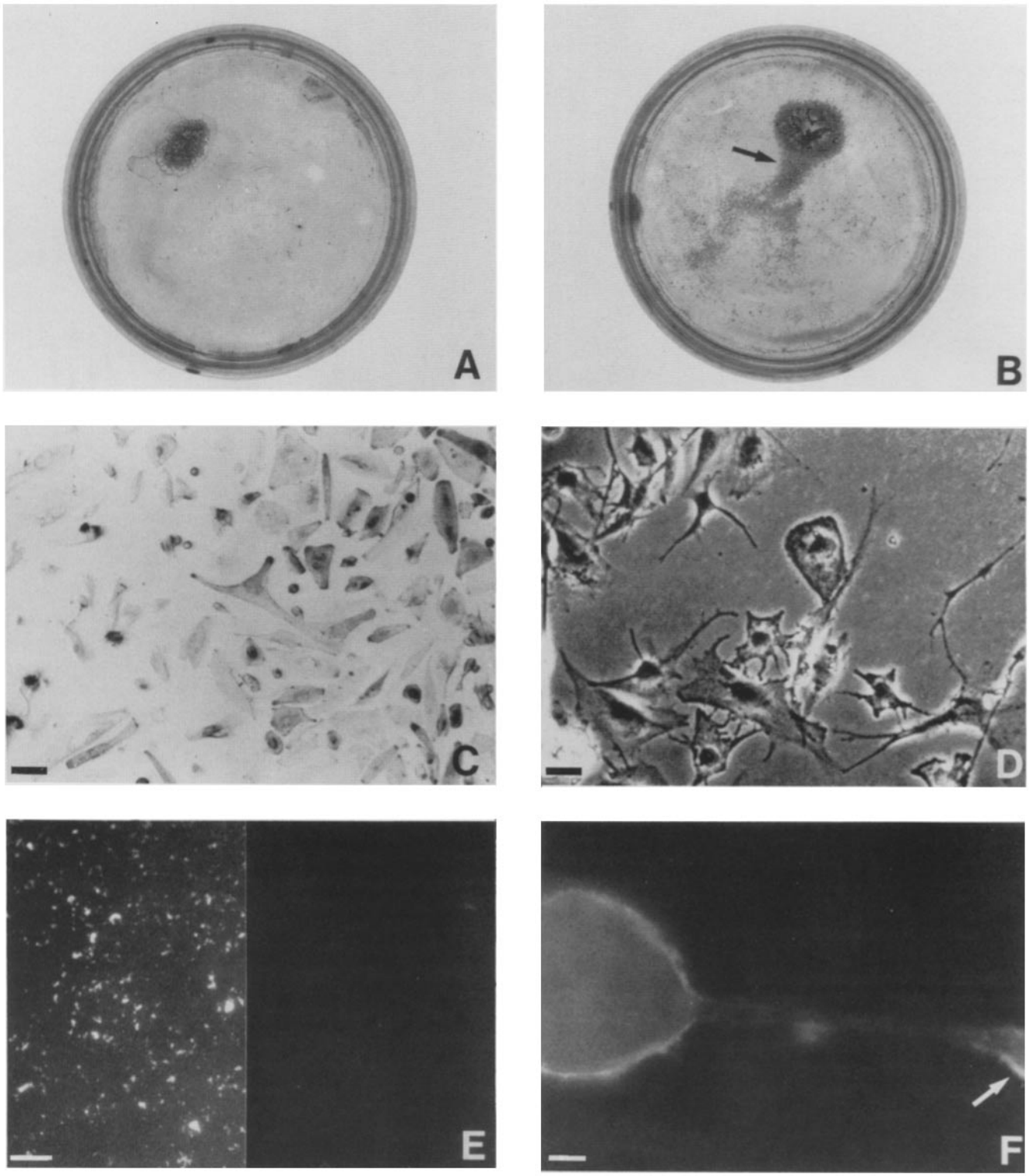

Figure 3. Stimulation of melanocyte migration and dendricity in an NGF gradient. Dishes were prepared as described, incubated for 5 d then were stained with silver stain followed by Rhodanile blue stain. (*A*) In a dish containing transferrin, proliferation and radially symmetric migration of melanocytes from their attachment site is apparent. (*B*) In a dish containing NGF, the melanocyte colony is teardrop in shape with preferential migration (*arrow*) toward the NGF source. There is also local proliferation of melanocytes at the center of the dish where NGF was applied. Dark staining at the edge of the dish represents concentrated background staining with Rhodanile blue. (*C*) Cells at the edge of the melanocyte colony facing away from the NGF attachment site in an NGF-containing dish are polygonal to bipolar with few dendrites. Cells in the center of the colony displayed a similar morphology. (*D*) Melanocytes at the migration front closest to the NGF source in the same dish (shown at 40% higher magnification) demonstrate extensive dendrite formation. (*E*) Center of fibronectin-coated tissue culture dish 5 d after NGF was applied and covered with growth medium. Indirect immunofluorescence using rabbit anti-NGF IgG displays abundant amount of NGF particles sticking to the dish surface (*left*). Control dish incubated with normal rabbit serum (*right*) is negative, proving that the bright particles are indeed deposits of surface bound NGF. (*F*) Immunofluorescence of

also a transient induction of c-fos transcripts to 2.7 ± 1.1 times the basal level ($p < 0.007$, paired t test) (Fig. 1 *B*) accompanied by supression of c-myc transcripts (Fig. 1 *C*). The beta-actin mRNA species, strongly expressed by melanocytes under basal conditions, was decreased by >50% in abundance as early as 60 min after NGF stimulation, with further down regulation to less than 20% of the basal level at 2–4 h after stimulation (Fig. 1 *E*). In a total of three experiments, beta-actin mRNA level was decreased by 72 ± 8.9% at these time points ($p < 0.03$, paired *t* test). Tyrosinase mRNA levels remained unchanged through 24 h (Fig. 1 *F*). To exclude the possibility that altered melanocyte gene expression was due to an active contaminant in the NGF preparation, melanocytes were grown in hormone supplemented medium with 20% FBS and lacking TPA, conditions under which they do not express p75 NGF-R on their surface (Peacocke et al., 1988). No modulation of gene expression was observed .5, 1, and 4 h after addition of 50 ng/ml NGF to these cultures (data not shown), establishing that functional p75 NGF-R was required for melanocyte gene modulation following NGF stimulation.

Nerve Growth Factor Effect on Melanocyte Migration and Dendricity

Because NGF affected gene expression in melanocytes we wanted to determine whether it might serve as a chemotropic signal for them, as reported for other neural crest derived cells (Gundersen and Barrett, 1979). Because NGF firmly and readily adheres to surfaces (Pearce et al., 1973; Levi-Montalcini and Angeletti, 1968), we chose to test this hypothesis by fixing NGF to fibronectin-coated culture dishes in which melanocytes were subsequently grown. Three experiments using different cell donors were performed. Melanocytes were plated in a 3-mm-diam area of tissue culture dishes 2 cm from a second area of the same size to which NGF, NGF plus neutralizing anti-NGF antibodies, or transferrin as a second control had previously been fixed. The dishes (two to five per condition) were then gently flooded with growth medium and returned to the incubator. Paired dishes lacking cells were processed identically as controls, and immunofluorescent staining was performed after 1 and 5 d in growth medium on dishes containing NGF to confirm continued adherence of the NGF to the dish surface (Fig. 3 *E*). Fluorescent deposits were comparable at both times and were virtually restricted to the area on which NGF had previously been fixed; there were scattered fluorescent deposits elsewhere on the dish surface. Thus, NGF attachment to the fibronectin coated surface was confirmed. After 5 d, in dishes containing growth medium and transferrin or in dishes containing growth medium and both NGF and anti-NGF antibodies, proliferation and radially symmetric migration of melanocytes from the attachment site was apparent (Fig. 3 *A*). In contrast, in dishes containing growth medium and NGF, as early as 2 days and definitely by 5 days the melanocyte colony was teardrop in shape with preferential migration toward the NGF source (Fig. 3 *B*). Survival and local proliferation of initially small clusters of melanocytes, presumably dislodged from the attachment site at the time the dish was flushed with medium, were frequently observed in NGF-containing dishes near the site of NGF application. Similar survival and proliferation of isolated melanocytes in the center of control dishes were not observed despite daily inspection. Instead, the occasional scattered attached cells noted 24 h after plating invariably disappeared after an additional 24–48 h, suggesting that the presence of NGF permitted survival of melanocytes that were too sparsely seeded to survive under the basal culture conditions. Furthermore, while all cells in transferrin-containing dishes and most cells in NGF-containing dishes had the expected polygonal to bipolar morphology characteristic of the culture system used (Gilchrest et al., 1985) (Fig. 3 *C*), and failed to bind the anti-p75 NGF-R mAb ME 20.4, melanocytes at the NGF migration front were strikingly more dendritic (Fig. 3 *D*) and demonstrated positive immunofluorescent staining for the p75 NGF-R (Fig. 3 *F*). The appearance of p75 NGF-R on the surface of melanocytes in the presumptive NGF gradient is consistent with induction of the receptor by its ligand, as has been reported for other growth factors and their receptors (Sinkovics, 1988; Earp et al., 1988; Lingham et al., 1988), and with the induction of p75 NGF-R message by NGF stimulation in the present study (Fig. 1 and 2).

Nerve Growth Factor Expression by Keratinocytes

If NGF influences melanocyte behavior within the epidermis, there must be a source of NGF in the skin. Since ~90% of epidermal cells are keratinocytes, we sought to demonstrate NGF production in cultured postnatal keratinocytes. Keratinocytes were grown under standard conditions on fibronectin coated plates in serum-free medium to near confluence. Cultures were then supplemented with 5 μg/ml cycloheximide, known to enhance steady-state levels of many mRNA species (Paulsson et al., 1987) and total RNA was extracted from keratinocytes 14 h after cycloheximide supplementation. Using sequence specific beta-NGF primers and the DNA thermal cycling technique (Rappolee et al., 1988), samples of reversed transcribed keratinocyte RNA showed a single band of the predicted size that when used as a cDNA probe in northern blot analysis hybridized to the known 1.3- and 1.5-kb NGF mRNA transcripts (Fig. 4). Further, the PCR product was confirmed by sequence analysis to be 100% homologous to mature beta-NGF mRNA (Fig. 5). Samples of keratinocyte RNA that were not reversed transcribed but were otherwise processed identically to the first samples failed to show the specific band, thus confirming that the NGF band indeed came from keratinocyte mRNA and not from contaminating genomic DNA molecules (Fig. 4). To rule out the possibility that NGF expression in the keratinocyte cultures originated from rare admixed contaminating cells such as fibroblasts or melanocytes, a well differentiated squamous carcinoma cell line SCC-12.B.2 (Rheinwald

a dendritic melanocyte at the NGF migration front using anti-p75 NGF-R antibody. The cell displays a bright fluorescent rim around the cell body and the tip of the dendrite (*arrow*). *C* and *D* were obtained before fixation and staining of the dish shown in *B*. Bars: (*C*) 5 μm; (*D*) 3.5 μm; (*E* and *F*) 1 μm.

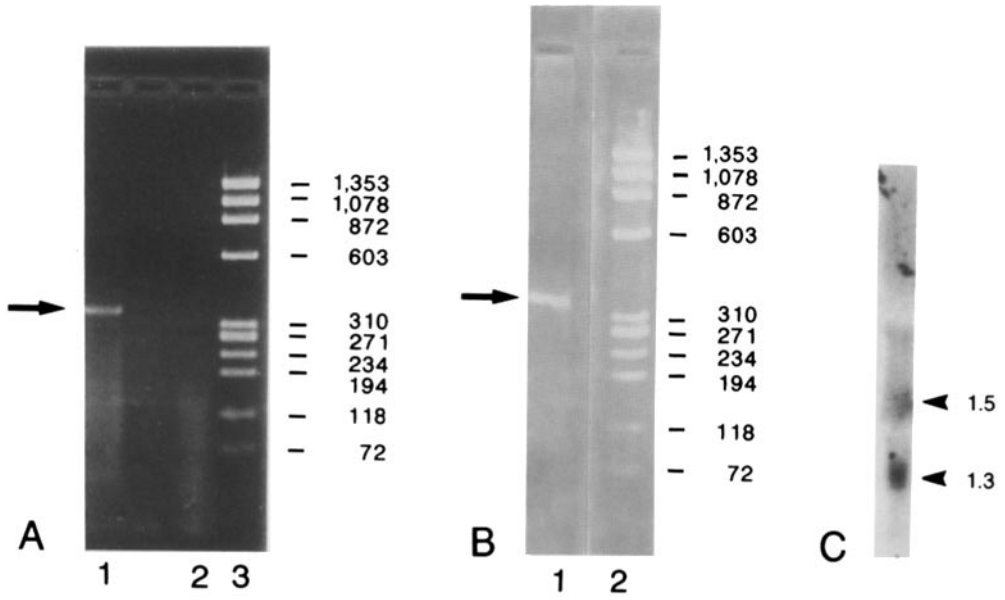

Figure 4. NGF gene expression by human keratinocytes. (*A*) Ethidium bromide–stained PCR product of reverse transcribed keratinocyte mRNA. A strong band of the predicted 354-bp size is seen after 40 cycles (lane *1*). No band is seen in nonreverse transcribed keratinocyte mRNA, excluding the possibility that the NGF band was generated from contaminating genomic DNA molecules (lane *2*). Lane *3* is $\phi\chi$174 RF DNA digested with HoeIII, used as standard. (*B*) Reverse-transcribed mRNA from the established keratinocyte line SCC-12.B.2. A strong band of the predicted 354-bp size is seen after 40 cycles (lane *1*). (*C*) ^{32}P-labeled PCR product used as a cDNA probe of total keratinocyte RNA in northern blot analysis. RNA (20 μg) from keratinocyte samples hybridize with the probe, demonstrating the two known NGF mRNA transcripts of 1.3 and 1.5 kb.

et al., 1983) was grown under standard conditions to near confluence and then total cellular RNA was extracted, reversed transcribed, and processed identically to the keratinocytes using the thermal cycling technique. The cell line also demonstrated a single band at the predicted size, ruling out the possibility that NGF expression in keratinocyte cultures originated from contaminating cells.

Discussion

Our experiments demonstrate that cultured keratinocytes derived from human skin express NGF message and that melanocytes respond to exogenous NGF stimulation by directed migration, altered morphology, and modulation of gene expression. These results confirm that the p75 NGF-R in melanocytes is functional, and that the human melanocyte may provide an alternative model to the rat pheochromocytoma PC12 line for studies of NGF effects in the nervous system.

Two of the early melanocyte responses to NGF are induction of c-fos and p75 NGF-R and down regulation of c-myc and beta-actin. Similar to other growth factors including EGF and insulin (Greenberg et al., 1985), NGF acts at least in part by stimulating tyrosine kinase (Kaplan et al., 1991). Interestingly, NGF stimulates c-fos, c-myc, and beta-actin gene expression in PC12 cells; while in melanocytes it stimulates only c-fos and inhibits c-myc and beta-actin gene expression.

In PC12 cells, neuronal differentiation following NGF stimulation is a transcription-dependent process (Greenberg et al., 1985), suggesting that the early induction of c-fos mRNA and repression of beta-actin mRNA we observed may be necessary initial events in melanocytes for dendrite outgrowth and possibly for other aspects of differentiation. NGF has been shown to direct developing or regenerating axons along a concentration gradient both in vivo (Levi-Montalcini, 1976) and in vitro (Gunderson and Barrett, 1979). However, recent in vivo work suggests that during murine development NGF is not responsible for attracting sensory nerve fibers to their target fields, but is rather involved in their survival (Davies et al., 1987; Rohrer et al., 1988). Our data suggest NGF may play both roles. During human embryogenesis, melanocytes migrate from the neural crest to the epidermis, where they become dendritic; and similar migration from the base of hair follicles to interfollicular epidermis occurs, for example, postnatally during wound healing or in depigmented skin in response to phototherapy (Quevedo et al., 1987). The melanocyte migration and dendricity we observed in an NGF gradient may thus mimic these events and may also explain the preferential extension of melanocyte dendrites in the presence of ker-

PRIMER 1

5' ***TCATCATCCCATCCCATCTT*** CCACAG
GGGCGAATTCTCGGTGTGGACAGTG
TCAGCGTGTGGGTTGGGGATAAGAC
CACCGCCACAGACATCAAGGGCAAG
GAGGTGATGGTGTTGGGAGAGGTGA
ACATTAACAACAGTGTATTCAAACA
GTACTTTTTTGAGACCAAGTGCCGG
GACCCAAATCCCGTTGACAGCGGGT
GCCGGGGCATTGACTCAAAGCACTG
GAACTCATATTGTACCA ***CGACTCACA***
CCTTTGTCAAG 3'

PRIMER 2

Figure 5. Verification of the keratinocyte gene product as NGF. Nucleotide sequence of the amplified keratinocyte cDNA generated by PCR with primers located at the beginning and end of human beta-NGF coding region. Nucleotide sequence is 100% homologous to the published nucleotide sequence of the corresponding mature mRNA region of human beta-NGF.

atinocytes both in vivo (Quevedo et al., 1987) and in vitro (Yaar, M., M. Peacocke, J. Bhawan, P. R. Gordon, and B. A. Gilchrest. 1988. *Clin. Res.* 36:705*A*). Hence, we speculate that NGF produced by keratinocytes may mediate in part keratinocyte–melanocyte interactions. Although the NGF mRNA level we have demonstrated in cultured keratinocytes is rather low and, as shown for other cell types, may be expressed more readily in vitro than in vivo (Shelton and Reichardt, 1986), NGF expression in vivo may be induced by specific physiologic stimuli. We have previously shown induction of p75 NGF-R on cultured melanocytes by a variety of sublethal injuries including growth factor depletion and UV irradiation (Peacocke et al., 1988). The latter may have particular biologic relevance because UV is absorbed in the skin and has both short-term and long-term effects on epidermal cells, including increased melanocyte dendricity and melanin production as part of the tanning response against further damage. Recently it was reported that UV irradiation induces NGF mRNA in the murine PAM 212 keratinocyte cell line (Tron et al., 1990). Although translation of NGF protein was not rigorously demonstrated, this study also identified NGF-like activity in PAM 212 conditioned medium. Thus, in vivo, as a cytokine released by keratinocytes, NGF may also modulate melanocyte gene expression to enhance dendrite formation and in consequence increase melanin transfer, among other adaptive behaviors.

Further studies will be required to delineate these and other suspected roles for NGF in mediating cell–cell interactions outside the nervous system.

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