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Growth-regulated Synthesis and Secretion of Biologically Active Nerve Growth Factor by Human Keratinocytes*

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Eddi Di Marco, Pier Carlo Marchisio‡, Sergio Bondanza, Adriano Tito Franzi§, Ranieri Cancedda, and Michele De Luca¶

From the Laboratorio Differenziamento Cellulare, Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy, the ‡Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Torino, 10126 Torino, Italy, and the §Istituto di Anatomia Umana Normale, Università di Genova, 16132 Genova, Italy

Nerve growth factor (NGF) transcripts were identified in normal human keratinocytes in primary and secondary culture. The expression of the NGF mRNA was strongly down-regulated by corticosteroids and was maximal when keratinocytes were in the exponential phase of growth. Immunofluorescence studies on growing keratinocytes colonies and on elutriated keratinocytes obtained from growing colonies and mature stratified epithelium showed specific staining of the Golgi apparatus only in basal keratinocytes in the exponential phase of growth. The keratinocyte-derived NGF was secreted in a biologically active form as assessed by neurite induction in sensory neurons obtained from chick embryo dorsal root ganglia. Based on these data we suggest that the basal keratinocyte is the cell synthesizing and secreting NGF in the human adult epidermis. The paracrine secretion of NGF by keratinocytes might have a major role in regulating innervation, lymphocyte function, and melanocyte growth and differentiation in epidermal morphogenesis as well as during wound healing.

Nerve growth factor (NGF)[1] is a polypeptide hormone, member of a family of neurotrophic factors (for a recent review see Ref. 1), responsible for the maintenance, development, and differentiation of several cell types of neural crest origin (2, 3) and of central cholinergic neurons (4). NGF, which acts as a retrograde messenger between target tissues and innervating neurons (3), has also chemotactic properties, since neurites from peripheral sympathetic ganglia orient their growth toward a source of NGF (neurotropism) (5, 6).

During wound healing of human skin, a widespread sprouting of sensory nerve fibers has been observed. This hyperinnervation has an important role in the re-epithelialization of the wound, since neurotransmitters like the vasoactive intestinal polypeptide have been shown to stimulate adenylate cyclase activity and proliferation of cultured human keratinocytes *in vitro* (7). Synthesis of NGF has already been demonstrated in the developing skin of the mouse embryo (8, 9). Both the presumptive dermis and epidermis of the developing mouse whisker pad contains NGF mRNA (8), but the cellular type responsible for the NGF synthesis has not been identified, and little is known about the adult animal.

In this study we have investigated whether human epidermal cells are able to synthesize and secrete biologically active NGF. As a model system, we used human epidermal keratinocytes cultured in conditions that allow a full epidermal differentiation (10, 11). Since cultured keratinocytes (i) are normal cells, *i.e.* non-transformed (11), (ii) are able to reconstitute *in vitro* epithelial sheets closely resembling the normal human epidermis (12), and (iii) maintain virtually the same differentiation features and gene expression patterns of its *in vivo* counterpart (11, 13–15), so as to be used in routine grafting for large skin and mucosal defects (14, 16–18), this system has many advantageous features for investigating epidermal physiology.

We report that normal human keratinocytes synthesize and secrete biologically active NGF. NGF synthesis is limited to the basal layer of the epidermis, depends upon the keratinocyte growth phase, and is regulated by corticosteroids. Since NGF synthesis is virtually limited to proliferating keratinocytes, we suggest that the paracrine secretion of NGF might have a major role in regulating innervation, lymphocyte function, and melanocyte growth and differentiation in epidermal morphogenesis, as well as during wound healing.

EXPERIMENTAL PROCEDURES

Cell Culture—Human epidermal keratinocytes were obtained from skin biopsies of healthy volunteers and cultivated on a feeder layer of lethally irradiated 3T3-J2 cells (a gift from Prof. Howard Green, Harvard Medical School, Boston, MA) as described (10, 12, 19). In brief, skin biopsies were minced and trypsinized (0.05% trypsin, 0.01% EDTA) at 37 °C for 3 h. Cells were collected every 30 min, plated (2.5×10^4/cm^2) on lethally irradiated 3T3-J2 cells (2.4×10^4/cm^2), and cultured in 5% CO_2 and humidified atmosphere in keratinocyte growth medium, which consisted of Dulbecco's modified Eagle's (DMEM) and Ham's F-12 media (3:1 mixture) containing fetal calf serum (10%), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (20 pM), epidermal growth factor (10 ng/ml) (a gift from Dr. Carlos George Nascimento, Chiron Corp., Emeryville, CA), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml). Primary cultures were passaged in secondary cultures as described (19). 3T3-J2 cells were cultured in DMEM containing fetal calf serum (10%), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml). Conditioned medium was prepared from growing keratinocyte colonies or from irradiated feeder layer by exposing the cultures for 3 days to DMEM containing glutamine (4 mM) and bovine serum albumin (0.1%). The conditioned medium was then centrifuged, aliquoted, and stored at −80 °C. Nor-

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¶ To whom correspondence should be addressed: Laboratorio di Differenziamento Cellulare, IST, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV No. 10, 16132, Genova, Italy. Tel.: 010-352958/355504; Fax: 010-352999.

[1] The abbreviations used are: NGF, nerve growth factor; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid.

mal human melanocytes were isolated from cultured stratified squamous epithelium and cultured as previously described (19).

Counterflow Centrifugation-Elutriation and 3,4-Dihydroxyphenylalanine Reaction—Counterflow centrifugation-elutriation was performed as previously described (20). Briefly, growing keratinocyte colonies or reconstituted epidermis were trypsinized and cells (30×10^6) were suspended in the elutriation buffer (Hanks' balanced salt solution containing 3% newborn calf serum, 0.01% sodium bicarbonate, 0.1% EDTA, 0.5% penicillin-streptomycin (10,000 IU/ml)). The elutriation was done at room temperature in a Beckman centrifuge J2-21 equipped with an elutriation rotor (J2-6B) and a Sanderson elutriation chamber. A Masterflex peristaltic pump was utilized for the counterflow. Cells were loaded at a rotor speed of 1000 ± 10 rpm and counterflow rate of 5 ml/min in order to allow cells to separate inside the chamber. Subsequently, while the rotor speed remained constant, the counterflow rate was increased stepwise to 6.5, 10, 15, 25, and 50 ml/min. For each step, 150 ml of elutriate was collected. The cells elutriated from each fraction were centrifuged, resuspended in Hanks' balanced salt solution, and stained with Giemsa. α-Naphthyl esterase activity and tyrosinase activity were determined as described (19, 20).

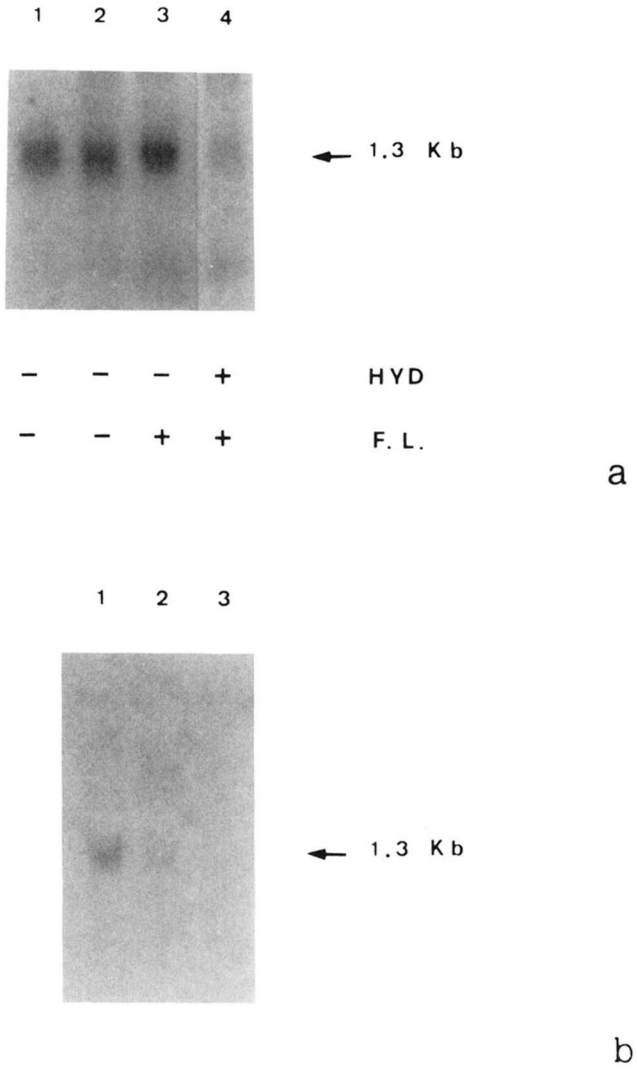

FIG. 1. **Northern blot analysis.** *Panel a*, 5 μg of poly(A^+) RNA from mouse submandibular glands (*lane 1*) and from growing keratinocyte colonies (*lanes 2–4*) were hybridized with the βNGF cDNA probe. *HYD*, hydrocortisone; *F.L.*, feeder layer of lethally irradiated 3T3-J2 cells. *Panel b*, 30 μg of total RNA from growing keratinocyte colonies cultured on feeder layer, in the absence of hydrocortisone (*lane 1*), from lethally irradiated 3T3-J2 cells (*lane 2*), and from isolated melanocytes (*lane 3*), were hybridized with the βNGF cDNA probe. Equal amounts of total or poly(A^+) RNA were loaded in each lane as assessed by ethidium bromide staining and β actin hybridization (not shown).

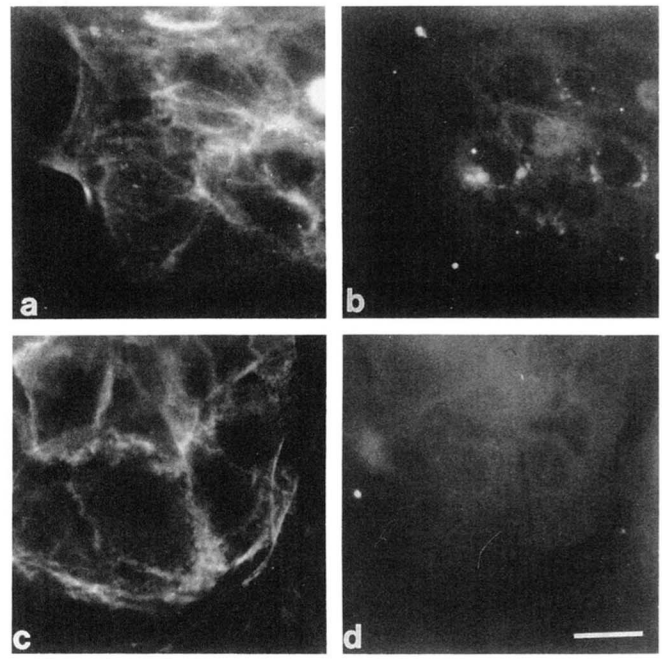

FIG. 2. **Immunofluorescence staining of growing keratinocyte colonies.** Growing keratinocyte colonies cultured in the presence (*panels c* and *d*) or in the absence (*panels a* and *b*) of hydrocortisone were subjected to immunofluorescence staining using the anti-NGF monoclonal antibody MAS-432 (*panels b* and *d*) and simultaneously with fluorescein-tagged phalloidin for F-actin (*panels a* and *c*). *Bar*, 5 μm.

RNA Blotting—The βNGF human cDNA probe (clone N8D9) was a gift from Dr. Pietro Calissano (University of Rome, Italy). Total cellular RNA was isolated by lysing the cells with 4.2 M guanidine thiocyanate followed by cesium chloride gradient centrifugation as described (21). Poly(A^+) RNA was prepared by an oligo(dT)-cellulose column (Pharmacia) chromatography. Two mg of total cellular RNA, dissolved in 1 ml of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), were denatured in boiling water for 2 min followed by addition of an equal volume of 1 M sodium chloride. The sample was then applied to the column and the poly(A^-) fraction was eluted with 50 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 M sodium chloride, 0.1% SDS. The poly(A^+) fraction was eluted using 2 ml of H_2O kept at 37 °C and precipitated with 300 mM sodium acetate, pH 5, and 2.5 volumes of absolute ethanol. Thirty μg of total RNA or 1–5 μg of poly(A^+) were size-fractionated through a 1.5% agarose gel and transferred to a nylon membrane (GeneScreen Plus, Du Pont-New England Nuclear) in 1.5 M sodium chloride, 0.15 M sodium citrate. After immobilization by shortwave UV exposure, blots were prehybridized at 42 °C for 3 h in 50% deionized formamide, 0.75 M sodium chloride, 25 mM sodium phosphate, 5 mM EDTA, 0.2 mg/ml salmon DNA, 0.5% SDS. Hybridization buffer was identical to the above buffer with the addition of the ^{32}P-labeled βNGF probe (2×10^6 cpm/ml) and 10% dextran sulfate. A final wash was done at 65 °C for 30 min in 15 mM sodium chloride, 1 mM sodium phosphate, 1 mM EDTA, 0.1% SDS. All filters were autoradiographed on x-ray films (Hyperfilm-MP, Amersham) with intensifying screens at −70 °C.

Immunofluorescence—Anti-mouse NGF mAb (MAS 432, clone 27/21) was purchased from Sera-Lab. Anti-mouse NGF mAb (αD11, Ref. 22), able to inhibit human NGF biological activity,[2] was a gift from Drs. Cattaneo and Calissano (University of Rome, Italy). Keratinocytes (1×104 cells/cm^2) were plated onto 24-well Costar plates, containing 1.4-cm^2 round glass coverslips previously coated with feeder layer, and cultured in the presence or in the absence of hydrocortisone. When keratinocyte colonies were evident in phase contrast microscopy, coverslip-attached cells were fixed in 3% formaldehyde (from paraformaldehyde) in phosphate-buffered saline (PBS), pH 7.6, containing 2% sucrose for 5 min at room temperature and permeabilized for 3–5 min at 0 °C in HEPES-Triton X-100 buffer (20 mM HEPES, pH 7.4, 300 mM sucrose, 50 mM sodium chloride, 3

[2] A. Cattaneo, personal communication.

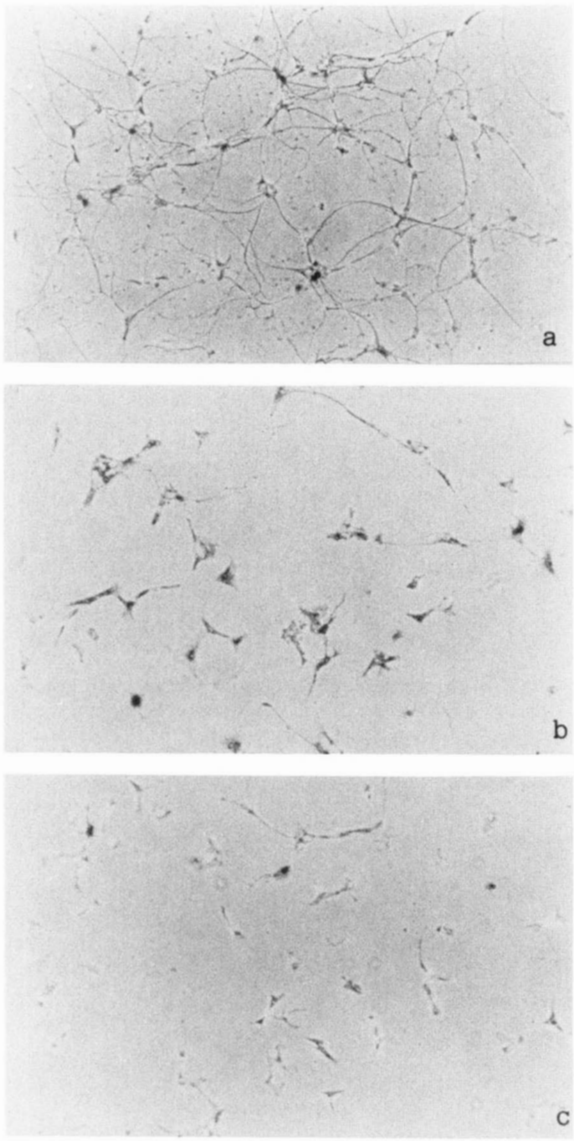

FIG. 3. ***In vitro* bioassay of the NGF.** Sensory neurons from dorsal root ganglia were isolated from 9-day-old chick embryos. Cultures were exposed for 18 h to medium conditioned by growing keratinocyte colonies (1:5 dilution) in the absence (*panel a*) and in the presence (*panel b*) of the anti-NGF mAb αD11. *Panel c* shows cells exposed to medium conditioned by irradiated 3T3-J2 cells (1:5 dilution).

mM magnesium chloride, and 0.5% Triton X-100). Indirect immunofluorescence was performed as previously reported (23). Briefly, the primary antibody (10–30 μg/ml) was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min. After rinsing in PBS, 0.2% bovine serum albumin, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (DAKOPATTS, Copenhagen, Denmark) for 30 min at 37 °C in the presence of 2 μg/ml fluorescein-labeled phalloidin (F-PHD; Sigma). Coverslip were mounted in 50% glycerol/PBS, and observations were carried out in a Zeiss Axiophot photomicroscope. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1000 ISO and developed in T-Max developer for 10 min at 20 °C.

In Vitro Bioassay of the NGF—2.5 S mouse NGF was a gift from Dr. R. A. Murphy (University of Alberta, Edmonton, Canada). Sensory neurons were prepared and cultured from dorsal root ganglia obtained from 9-day-old chick embryos. Ganglia were trypsinized (0.05% trypsin, 0.02% EDTA) at 37 °C for 30 min, and isolated neurons were suspended in Ham's F-12 medium containing fetal calf serum (5%), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml). Contaminating fibroblasts were eliminated by incubating the cell suspension on plastic for 30 min at 37 °C. Nonattached sensory

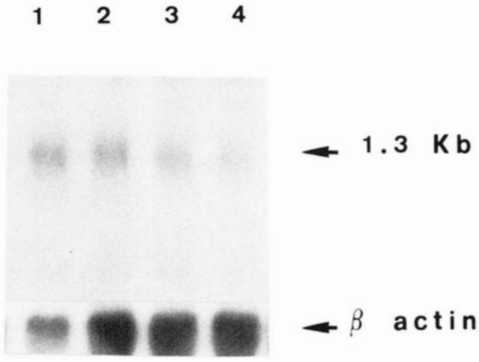

FIG. 4. **Northern blot analysis.** Two μg of poly(A⁺) RNA obtained from mouse sub-mandibular glands (*lane 1*), growing keratinocyte colonies (*lane 2*), and the reconstituted epidermis 1 day (*lane 3*) and 5 days (*lane 4*) after confluence were hybridized with the human βNGF cDNA.

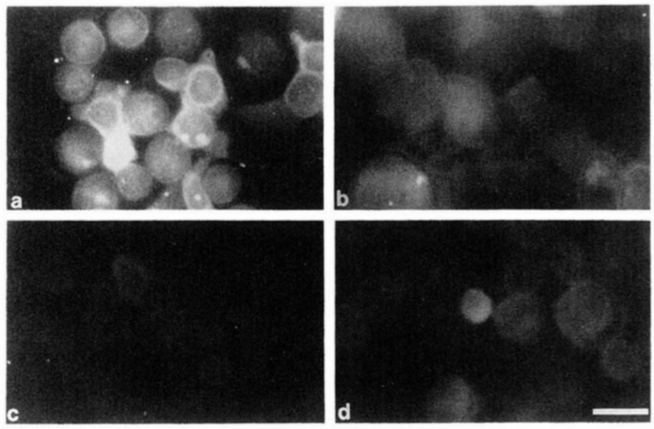

FIG. 5. **Immunofluorescence staining of elutriated cells.** Keratinocytes obtained from growing colonies (*panels a* and *b*) and reconstituted epidermis (*panels c* and *d*) were subjected to counterflow centrifugation-elutriation. An homogeneous population of basal cells (*panels a* and *c*) and terminally differentiated cells (*panels b* and *d*) were centrifuged and subjected to immunofluorescence staining using the anti-NGF mAb MAS-432. *Bar*, 5 μm.

neurons were then plated in 24-well plates (50,000 cells/well) precoated with poly-L-lysine (10 μg/well) and were exposed to NGF, 1:5 dilution of medium conditioned by keratinocyte colonies or irradiated 3T3-J2 cells in the presence or in the absence of anti-NGF mAbs (MAS 432 or α:D11), and unrelated mAbs. After 18 h at 37 °C, neurons were fixed in 3% formaldehyde, 2% sucrose, PBS for 5 min, stained with 0.5% crystal violet, 20% methanol for 15 min, washed, dried, and observed under a phase contrast microscope.

RESULTS

Specific Synthesis of NGF by Human Keratinocytes—Polyadenylated RNA was isolated from colonies in the exponential phase of growth and analyzed by Northern hybridization for the presence of the NGF mRNA. As shown in Fig. 1*a*, a 1.3-kilobase transcript, consistent in size with the NGF mRNA detected in mouse sub-mandibular glands (*lane 1*) (24), was expressed in normal human keratinocytes (*lane 3*). It is known that fibroblasts of embryonic origin are able to synthesize NGF (8). To make sure that the expression of the NGF mRNA was not due to the presence of the irradiated feeder layer, polyadenylated RNA was obtained from keratinocytes grown in the absence of 3T3 (19) and analyzed as above. As shown in Fig. 1 (*panel a, lane 2*), the NGF mRNA was expressed in keratinocytes grown in the absence of feeder layer. To further confirm this, RNA obtained from keratinocyte colonies, irradiated 3T3-J2 cells and isolated melanocytes, was hybridized

as above. As shown in Fig. 1 (*panel b*), there was a strong expression of NGF mRNA in the keratinocyte culture (*lane 1*), a minimal expression in the feeder layer (*lane 2*), and no expression in the pure melanocyte culture (*lane 3*). Equal amounts of RNA were loaded in each lane as assessed by ethidium bromide staining and β actin hybridization (not shown).

Steroids hormones can regulate NGF synthesis differentially in mouse sub-mandibular glands *in vivo* and in continuous cell lines *in vitro* (25). In cultured human keratinocytes the expression of the NGF mRNA was strongly inhibited by the addition of hydrocortisone (Fig. 1*a*, *lane 4*).

The presence and localization of the NGF was then analyzed by immunofluorescence. Growing keratinocyte colonies were fixed and immunostained as described under "Experimental Procedures." Fig 2*b* shows the presence of immunoreactive NGF in the Golgi apparatus of basal keratinocytes grown in the absence of hydrocortisone. In agreement with the Northern hybridization data, immunoreactive NGF was undetectable in keratinocytes grown in the presence of hydrocortisone (Fig. 2*d*). Very poor staining was occasionally seen in the feeder layer, and no immunoreactive NGF was detectable in the cytoplasm of melanocytes present in the culture (not shown).

The Keratinocyte-derived NGF Is Secreted in a Biologically Active Form—To investigate whether NGF was secreted by keratinocytes in a biologically active form, sensory neurons from dorsal root ganglia were isolated from 9-day-old chick embryos and used for an *in vitro* bioassay. Medium conditioned by growing keratinocyte colonies (Fig. 3*a*) determined a neurite outgrowth similar to that induced by 10 ng/ml purified NGF (not shown). The keratinocyte-induced neurite outgrowth was completely abolished by the addition of the anti-NGF mAb αD11 (Fig. 3*b*). Identical amounts of medium conditioned by irradiated 3T3-J2 cells (Fig. 3*c*) or isolated melanocytes (not shown) were uneffective. Unrelated mAbs did not inhibit the neurite outgrowth (not shown).

NGF Synthesis Occurs Only in the Basal Layer of the Cultured Keratinocytes and Is Growth-regulated—The behavior of keratinocytes both in terms of growth and differentiation *in vitro* is different in growing colonies *versus* reconstituted, fully differentiated epithelium and parallels the behavior *in vivo* during wound healing (11). Polyadenylated RNA, isolated from growing keratinocyte colonies and from the reconstituted stratified epithelium, was analyzed by Northern hybridization for the presence of the NGF mRNA. As shown in Fig. 4, when compared with growing colonies (*lane 2*), a decrease of NGF mRNA expression was observed at 1 day after confluence (*lane 3*). Four days later the NGF mRNA was barely detectable (*lane 4*). Equal amounts of RNA were loaded in each lane as assessed by ethidium bromide staining (not shown) and β actin hybridization (Fig. 4, *arrowhead*).

The percentage of colony-forming cells decreases when keratinocyte colonies reach confluence (26).[3] The possibility of a nonspecific decrease of NGF expression due to the relative decrease in basal cells after stratification of the cultured epithelium was ruled out by the elutriation experiment. Homogeneous populations of basal and terminally differentiated cells from growing and confluent cultures were obtained by counterflow centrifugation-elutriation. The separation was monitored either by microscopical examination and by determination of the α-naphthyl esterase activity (Ref. 20, data not shown). Fractions were centrifuged and immunostained with the anti-NGF mAb (MAS-432). As shown in Fig. 5, the anti-NGF mAb stained only basal keratinocytes obtained from growing colonies (*panel a*), whereas a comparable number of basal cells from confluent cultures (*panel c*) and differentiated cells from both growing and confluent cultures (*panels b* and *d*) showed no evident NGF staining.

Taken together these data demonstrate that human keratinocytes present in the basal layer of epidermis are able to synthesize and secrete biologically active NGF in a growth-regulated fashion, suggesting a specific role of NGF during wound healing.

DISCUSSION

Normal human keratinocytes *in vitro* and *in vivo* possess a very intense secretory activity, *i.e.* they are able to synthesize and release several polypeptides that have important roles in regulating specific processes during wound healing (for a recent review see Ref. 27). In most cases synthesis and secretion of such hormones (parathyroid hormone-like protein, multiple bone resorption-stimulating factors, apolipoprotein E) (28–30) or growth factors (interleukin 1, transforming growth factor α, transforming growth factor β, basic fibroblast growth factor) (31–34), are finely modulated, further suggesting the regulatory role of the epidermis in several biological processes.

In this paper we identify the basal keratinocyte as the cell synthesizing and secreting nerve growth factor in the human adult epidermis and probably in the whole skin. Since NGF mRNA and protein expression are growth- and hormonally regulated, we postulate that this paracrine NGF secretion might have a key role in regulating wound healing at different levels, all of which await further investigation.

First, the sprouting of sensory nerve fibers, typical of healing wounds, disappears when wound healing is completed (7). Since it is well known that NGF supports the survival and differentiation of sensory neurons, induces neural processes, regulates the expression of neurotransmitters and the connection between the neuron and the target (35), we suggest that proliferating keratinocytes might regulate skin innervation during wound healing, both in terms of nerve fibers chemotaxis and survival. Preliminary evidences seem to sustain this hypothesis. In fact in the reconstructive surgery of the lip, grafting with autologous skin flaps (containing resting keratinocytes) does not allow a quick recovery of normal sensory neuron function, which, in contrast, is rapidly obtained by the autologous graft of *in vitro* reconstituted mucosa (containing growing epithelial cells).[4]

Second, we and others have reported that human keratinocytes are able to finely regulate melanocyte growth, differentiation, and physiological organization in the reconstituted epidermis (19, 34, 36). Since melanocytes, which are neural crest-derived cells, can be induced to express the NGF receptor (37), we are currently investigating whether some aspects of the keratinocyte-melanocyte interactions *in vitro* and *in vivo* could be regulated through a paracrine release of NGF. In particular, we have previously shown that the close keratinocyte-melanocyte contact observed in culture is essential for keratinocyte-induced melanocyte proliferation and differentiation (19). Since growing keratinocytes are highly migratory cells (38), a mechanism must exist by which melanocytes follow the growth of keratinocytes in the healing of wounds. NGF has a specific chemotactic activity for sympathetic axons and sensory neurites. Moreover NGF is able to specifically regulate the expression of a laminin-binding integrin (39) and to phosphorylate cytoskeleton-associated proteins in PC12 cells (40). This is of particular interest, since NGF receptors

[3] M. De Luca, unpublished data.

[4] M. De Luca, P. Mangiante and R. Cancedda, submitted for publication.

are constitutively expressed in many melanoma cells (both *in vivo* and *in vitro*) and in dysplastic nevi (41) and since very recent studies (42, 43) have shown that a class of NGF receptors is encoded by the *trk* protooncogene.

Third, interleukin 1, platelet-derived growth factor, tumor necrosis factor (44), and macrophages (45) have been shown to increase transcription and stability of NGF mRNA in cultured rat fibroblasts and during regeneration of rat sciatic nerve. In addition NGF has been shown to regulate growth and differentiation of human B lymphocytes positively (46). Thus, cytokines released by fibroblasts and macrophages in the injured skin might positively regulate NGF synthesis by basal keratinocytes and in turn influence the immune response during wound healing.

The new proposed role for NGF in the basal epidermis has been inferred from *in vitro* studies. Experiments are in progress to confirm such a role for NGF *in vivo* as well.

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