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Nerve growth factor abrogates the tumorigenicity of human small cell lung cancer cell lines

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ABSTRACT **Nerve growth factor (NGF) has antiproliferative and differentiating effects on adenomas of neuroendocrine origin. Cell lines derived from small-cell lung carcinoma (SCLC), a very aggressive neuroendocrine tumor, express NGF receptors. The role of NGF in the control of proliferation and progression of this carcinoma, however, has never been investigated. Chronic exposure of NCI-N-592 and GLC8 SCLC cell lines to NGF remarkably inhibited their proliferation rate both *in vitro* and *in vivo*, prevented their anchorage-independent clonal growth in soft agar, impaired their invasive capacity *in vitro*, and abolished their tumorigenic potential in nude mice. The proliferative response of SCLC cell lines to nicotine was also remarkably impaired by *in vitro* NGF treatment. Furthermore, NGF treatment activates in SCLC cell lines the expression and secretion of NGF. NGF thus reverts SCLC cell lines to a noninvasive, nontumorigenic phenotype that does not respond to nicotine and produces NGF.**

Small-cell lung cancer (SCLC) is a very aggressive human tumor representing about 25% of all lung cancers (1). Although our understanding of the biology of SCLC is expanding rapidly, there have been no recent major advances in the treatment of this tumor, and overall survival has not changed significantly since the late 1970s (1). SCLC has some phenotypical properties of a neuroendocrine tumor such as expression of L-dopa decarboxylase (1, 2), bombesin/gastrin-releasing peptide (1, 3), neuron-specific enolase (1), and voltage-operated calcium channels of neuronal type (4–6). Proliferation of SCLC is controlled by autocrine loops sustained by the secretion of different neurohormones and growth factors such as bombesin, insulin-like growth factor I, bradykinin, neurotensin, cholecystokinin, and vasopressin (1). A mitogenic loop mediated by serotonin (5-HT) and facilitated by neuronal-type nicotinic receptors through stimulation of 5-HT release (7, 8) has been recently shown to be operative in SCLC cell lines (9, 10).

Despite the observation that SCLC cell lines express (11) the tyrosine kinase trkA receptor for NGF (12–14), the role of this neurotrophin in the control of proliferation, invasiveness, and tumorigenic potential of this tumor has never been investigated. This issue seems of critical relevance as it is now emerging that NGF is an antiproliferative and differentiation factor for various tumors of neuroendocrine origin (15–21). In particular, NGF has been consistently reported to induce differentiation of the pituitary tumor cell line GH_3 (15) and the insulinoma cell line RINm5F (16, 17) and to suppress cell growth and tumorigenicity of human prolactin-secreting adenomas (18, 19). In addition, we found that an autocrine loop mediated by NGF is operative in both normal pituitary lactotroph cells (20) and slowly proliferating, nontumorigenic prolactinomas but not in tumorigenic prolactinoma cells (18, 21). The impact of this mechanism is such that the breakdown of the NGF-mediated autocrine loop in nontumorigenic prolactinomas brings about their neoplastic progression to a tumorigenic phenotype (21).

These data prompted us to investigate whether NGF can modify the phenotype of an aggressive carcinoma of neuroendocrine origin such as the SCLC. The results show that chronic NGF treatment remarkably inhibits the proliferation rate of two SCLC cell lines, prevents their anchorage-independent clonal growth in soft agar, impairs their invasive potential *in vitro*, and abrogates their tumorigenicity *in vivo*. In addition, administration of NGF to nude mice xenografted with SCLC cells suppresses the growth of the established tumors. These effects are accompanied by relevant biochemical changes. Chronic exposure of SCLC cell lines to NGF activates the production and secretion of NGF and brings about the loss of the mitogenic response to nicotine. These findings suggest that NGF may induce a switch of gene expression in SCLC cell lines, reverting them to a more differentiated, nontumorigenic phenotype and may represent the basis to develop new strategies for the therapy of this aggressive tumor.

MATERIALS AND METHODS

Cell Cultures and Treatments. The human SCLC cell lines NCI-N-592 and GLC8 were kindly provided by Prof. G. Gaudino (Dept. of Biomedical Sciences and Oncology, University of Torino, Italy). Cells were grown in suspension in RPMI 1640 medium, supplemented with 10% fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO_2/95% O_2. Cells were treated with 50 ng/ml NGF (human recombinant NGF kindly provided by Genentech) for 15 consecutive days. NGF was added to the culture medium every 3 days, at the time of each medium change. In NGF withdrawal experiments, cells treated with NGF for 15 days were transferred to fresh medium lacking NGF, and the culture was continued for an additional 25 days.

[^{3}H]Thymidine Incorporation. Cells were plated in multiwell plates (16 mm, NUNC) at the density of 10^6 cells per well. [^{3}H]Thymidine (0.5 μCi/ml/84.8 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN) was added, and cells were incubated for 24 h at 37°C. In another set of experiments, untreated and NGF-treated cells were exposed either to nicotine (0.1 pM–100 nM) or to 100 nM 5-HT for 48 h. After a 24-h incubation at 37°C with nicotine or 5-HT, [^{3}H]thymidine was added to samples,

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Abbreviations: NGF, nerve growth factor; SCLC, small-cell lung carcinoma; 5-HT, 5-hydroxytryptamine.

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and the incubation was continued for an additional 24 h. Cells were rinsed three times with 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4 (PBS), and incubated for 10 min with ice-cold trichloroacetic acid. Cells were then incubated for 20 min at room temperature with 1 M NaOH and then with 1 M HCl. The two media were collected together and analyzed for radioactivity.

Growth in Soft Agar. Cells were suspended in 0.3% agar with complete RPMI medium, plated at the density of 5×10^4 cells in 85-mm dishes previously coated with 0.5% agar, and maintained at 37°C for 40 days. On day 20, colonies were counted and divided into small (<10 cells) and large (>50 cells) colonies. Photomicrographs were made on a Leica microscope at ×10 magnification.

***In Vitro* Invasion Assay.** *In vitro* invasion was determined in 24 transwell chambers as previously described (22, 23) with minor modifications. Polycarbonate filters (8-μm pore size) were coated with 40 μg of Matrigel (Collaborative Research) and incubated at 37°C for 30 min. Cells, suspended in DMEM containing 0.1% BSA, were then added to the inner side of the transwell (10^5 cells/well). After a 24-h incubation at 37°C, cells on the upper face of the filter were removed with a cotton swab. Cells attached to the bottom side of the membrane were fixed in methanol, stained with hematoxilin, and counted.

Immunofluorescence. Cells were fixed in PBS, 3% paraformaldehyde, 2% sucrose for 10 min, permeabilized in PBS, 0.5% saponine for 10 min at room temperature, and labeled for 1 h at room temperature with either a rabbit antiserum directed to trkA (1 μg/ml; Santa Cruz Biotechnology), or a mouse monoclonal antibody against the human $p75^{NGFR}$ (5 μg/ml; Boehringer Mannheim) or a mouse monoclonal antibody against NGF (20 ng/ml, clone 27/21; Boehringer Mannheim) dissolved in PBS/saponine. Cells were then incubated for 1 h at room temperature and in the dark in PBS/saponine containing the appropriate secondary antibodies conjugated to either fluorescein or rhodamine and analyzed using a multichannel cytometer (FACSTAR, Becton Dickinson). Total counts of 10^4 cells were accumulated for each group. Isotype control with irrelevant antibodies and omission of the primary antibodies were performed to exclude the possibility of cross-reactivity. For the study of cell cycle, cells were fixed with 70% acetone at 4°C for 20 min and double-labeled with propidium iodide and the anti-trkA antibody and analyzed by FACS.

Poly(A)$^+$ RNA Isolation, PCR, and Hybridization. Poly(A)$^+$ RNA was isolated according to Badley *et al.* (24), and 1 μg of each sample was transcribed into cDNA by standard methods using murine Moloney leukemia virus reverse transcriptase (GIBCO/BRL) and oligo$(dT)_{18}$ as a primer. Aliquots of each cDNA were used as template in PCR. The oligonucleotides 5′-TCATCCACCCACCCAGTCTTC-3′, encoding human NGF residues SSTMPVF, and 5′-GGCAGCCTGTTT-GTCGTCTGT-3′, encoding human NGF residues TDD-KQAA, were used to amplify NGF. Reactions were performed for 30 cycles (94°C, 45 sec; 53°C, 45 sec; 72°C, 1 min). The α3, α7, β2, and β4 subunits of nicotinic receptors were amplified by using primers and PCR conditions described by Codignola *et al.* (25). Amplification with 5′-TAAAGACCTCTATGC-CAACACAGT-3′ and 5′-CACGATGGAGGGCCGGACT-CATC-3′ primers encoding human β-actin residues KDLY-ANTV and DESGPSIV, respectively, was used as an internal control. The PCR products were analyzed by Southern blot with either a specific ^{32}P-labeled *Pst*I restriction fragment of the NGF cDNA or with oligonucleotide probes specific for the different subunits of nicotinic receptor (25).

NGF Secretion. A two-site ELISA (kit from Boehringer Mannheim with mAb 27/21) was used to quantitate NGF in cell extracts and culture media. Blanks consisted of samples added to microwells coated with mouse IgG instead of the NGF antibody.

***In Vivo* Studies.** Fifteen Nu/Nu female mice (20 g body weight, Charles River Breeding Laboratories) were divided into two groups and injected s.c. in the dorsal region either with 10^7 NCI-N-592 cells ($n = 7$) or with 10^7 NCI-N-592 cells previously treated with 50 ng/ml NGF for 15 days ($n = 8$) and examined for tumor growth. In another set of experiments, 20 Nu/Nu female mice were injected s.c. in the dorsal region with 10^7 NCI-N-592 cells. After the appearance of tumors of 30 ± 2 mm^3, mice were divided into two groups, treated with either s.c. saline or s.c. NGF (1 μg/g body weight) once a day for 21 consecutive days, and examined for tumor growth. Tumor volumes were determined using the formula $V = A \times B^2 \times 0.4$, where A and B are the larger and smaller axis of the tumor, respectively.

RESULTS

Expression of NGF Receptors in SCLC Cell Lines. Immunofluorescence studies were performed to investigate the presence of NGF receptors in NCI-N-592 and GLC8 cell lines. The cytofluorimetric analysis of NCI-N-592 cells showed that only 43% ± 4% and 54% ± 3% of the cells in culture were labeled by the antibodies against $p75^{NGFR}$ and trkA, respectively. Exposure of the cultures to 50 ng/ml NGF for 7 days resulted in the induction of the expression of both NGF receptors in the majority of cultured cells ($p75^{NGFR}$, 74% ± 2.6%; trkA, 82% ± 4%). Similar results were obtained with the GLC8 cell line, where 47.5% ± 3% and 68% ± 4% of untreated cells were positive for $p75^{NGFR}$ and trkA expression, respectively. A 7-day NGF treatment increased to 85 ± 2 and 78 ± 3 the percentages of cells expressing $p75^{NGFR}$ and trkA, respectively. A 15-day exposure to 50 ng/ml NGF did not produce any further modification of NGF receptor expression.

***In Vitro* Proliferation, Colony Formation, and Invasion.** As shown in Fig. 1, chronic exposure of NCI-N-592 cells to NGF resulted in a remarkable inhibition of cell proliferation. This effect was evident after an 8-day treatment (37% inhibition) and reached its maximum (70% inhibition) on the 12th day. After this time, the cell proliferation rate did not decrease

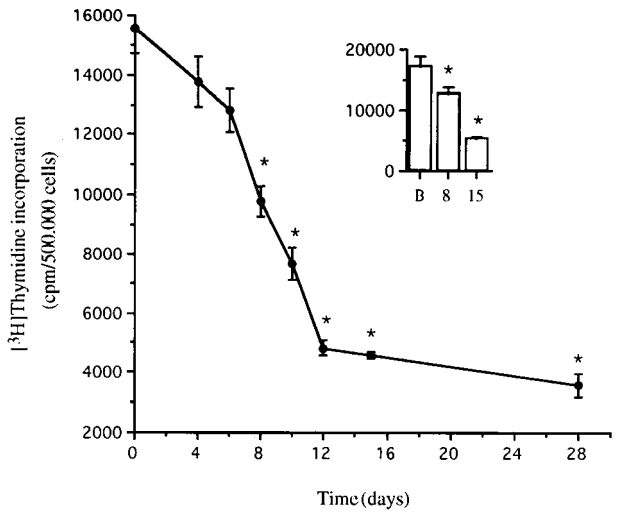

FIG. 1. Effects of NGF on SCLC cell proliferation rate. Cells were exposed to 50 ng/ml NGF for different times. [^{3}H]Thymidine incorporation was evaluated as described in *Materials and Methods*. The data are expressed as incorporated cpm/5×10^5 cells. The complete time course of [^{3}H]thymidine incorporation in NCI-N-592 cells is reported. (*Inset*) [^{3}H]Thymidine incorporation in GLC8 cells. Points are the means ± SEM of three experiments, each performed in triplicate. *, $P < 0.001$ versus untreated cells by Student's t test. Statistical analysis was applied to the points corresponding to individual times.

further, even if cells were chronically exposed to NGF for an additional 16 days. Similarly, addition of NGF to GLC8 cells inhibited their proliferation rate with a time course very similar to that observed with NCI-N-592 cells. [^{3}H]Thymidine incorporation was 30 and 70% inhibited after an 8-day and a 15-day NGF treatment, respectively (Fig. 1, *Inset*). Cell cycle analysis showed that NGF treatment promoted the accumulation of the majority of cells in the G_0/G_1 phase of the cell cycle with a small percentage of cells distributed in the other phases of cell cycle. Analysis of NGF receptor expression showed that both cells blocked in G_0/G_1 and cycling cells expressed trkA (not shown).

The ability of SCLC cells to form colonies in soft agar was also remarkably reduced by NGF treatment. As shown in Fig. 2, although untreated NCI-N-592 cells had a high potential of growing as colonies in soft agar (Fig. 2*A*), the cells that were previously exposed to NGF for 15 days lost this property (Fig. 2*B*). The anchorage-independent clonal growth of GLC8 cells before and after a 15-day NGF treatment is reported in Fig. 2 *C* and *D*, respectively. Analysis of the size distribution of colonies revealed that untreated NCI-N-592 and GLC8 cells formed 468 ± 11 and 336 ± 15 (mean ± SEM of four plates) big colonies/plate, whereas cells pretreated with NGF formed only a few small colonies/plate.

Both untreated and NGF-treated SCLC cell lines were further analyzed for their capacity to invade Matrigel-coated membranes. As shown in Table 1, NGF-treated cells exhibited a remarkable reduction of their *in vitro* invasive capacity compared with untreated cells.

***In Vivo* Tumorigenicity.** The data reported in Table 2 demonstrate that tumor formation occurs in all mice injected with NCI-N-592 cells. By contrast, mice injected with NGF-treated NCI-N-592 cells showed virtually no sign of tumor formation. Only one mouse over eight developed a small tumor nodule that did not significantly grow within 1 month. Similar results were obtained with GLC8 cells injected in a small group of nude mice (not shown).

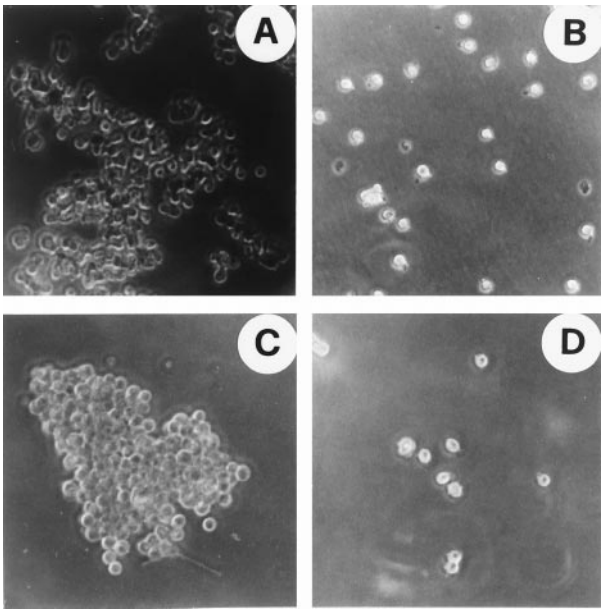

FIG. 2. Growth of SCLC cell lines in soft agar. SCLC cells were grown in the absence or in the presence of 50 ng/ml NGF for 15 days. Untreated NCI-N-592 (*A*), NGF-treated NCI-N-592 (*B*), untreated GLC8 (*C*), and NGF-treated GLC8 (*D*) cells were plated in soft agar with complete RPMI medium and maintained at 37°C for 40 days. On day 20, colonies were counted and divided into big (>50 cells) and small (<10 cells) colonies. Photomicrographs were made with a Leica microscope at ×10 magnification.

Table 1. *In vitro* invasiveness of SCLC cells

	Cells/field	
	NCI-N-592	GLC8
Untreated cells	53.6 ± 1.8	19.6 ± 0.9
NGF-treated cells	28 ± 0.9*	1 ± 0.4*

Untreated and NGF-treated cells were plated on Matrigel-coated filters. After a 24 h incubation cells attached to the lower face of the filter were fixed, stained and counted.
*$P < 0.001$ versus untreated cells by ANOVA followed by the Fisher PLSD.

The effects of NGF administration on the growth of established tumors in nude mice xenografted with NCI-N-592 cells are reported in Fig. 3. Mice with tumors of 30 ± 2 mm^3 (Fig. 3*A*) were treated with either saline or NGF (1 μg/g body weight, s.c.) once a day for 21 consecutive days. The tumor growth curves are reported in Fig. 3*D*. Tumors grew of the same extent in saline- and NGF-treated mice during the first 10 days of administration. However, at later times tumors did not grow further in NGF-treated mice, although they grew exponentially in saline-treated animals. At the end of the treatment, the tumor size was 14-fold smaller in NGF-treated animals (Fig. 3*C*) than in saline-treated controls (Fig. 3*B*). None of the 10 mice injected with NGF died during treatment, and no important side effects were noted.

NGF Production in SCLC Cell Lines. Extremely low copies of NGF mRNA were present in SCLC cells. Exposure of NCI-N-592 and GLC8 cultures to 50 ng/ml NGF for 15 days resulted in a 4- and 6-fold increase of NGF mRNA levels, respectively (not shown). In line with this observation, the cytofluorimetric analysis of cells stained with the monoclonal antibody against NGF showed that only 14% ± 2% of NCI-N-592 cells and 12% ± 1% of GLC8 cells were immunopositive for NGF. However, a strong NGF staining was detectable in the majority (82% ± 3% of NCI-N-592 and 85% ± 3% of GLC8 cells) of NGF-treated cells. Quantification of NGF production by a two-site ELISA revealed the presence of 2 ± 0.3 ng/10^6 cells in cell extracts from NGF-treated NCI-N-592 cells and 3.7 ± 0.5 ng/10^6 cells in cell extracts from NGF-treated GLC8 cells. The concentrations of NGF in the media conditioned for 24 h by cells pretreated with NGF were 2.8 ± 0.5 ng/ml for NCI-N-592 cells and 5 ± 1 ng/ml for GLC8 cells.

SCLC Cell Proliferation in Response to Nicotine. NGF treatment dramatically blunted the mitogenic response of SCLC cell lines to nicotine. In line with previous observations (7, 8), (−)nicotine induced a dose-dependent stimulation of [^{3}H]thymidine incorporation in NCI-N-592 cells with a maximal effect of 164% ± 4.2% over basal at the concentration of 10 nM and an EC_{50} value of 8 ± 0.6 pM (Fig. 4). NGF treatment remarkably reduced the mitogenic efficiency of (−)nicotine. In NGF-treated NCI-N-592 cells, the maximal mitogenic effect of (−)nicotine (EC_{50} = 20 ± 0.5 pM) was reduced to a 45% ± 3% increase over basal (Fig. 4). The same results were obtained with the GLC8 cell line (Fig. 4, *Inset*).

The expression of the α3, α7, β2, and β4 subunits of the nicotinic receptor in SCLC cell lines was studied by PCR amplification and Southern blot. The results clearly suggest

Table 2. *In vivo* tumorigenic capacity of NGF-treated SCLC cells

	Mice with tumors
Untreated SCLC cells	7/7
NGF-treated SCLC cells	0/8

Female athymic Nu/Nu mice were xenografted in the dorsal region with either 10^7 NCI-N-592 cells or 10^7 NCI-N-592 cells previously exposed to 50 ng/ml NGF for 15 days. Tumor volume in mice receiving untreated cells was calculated by determining perpendicular diameters with callipers. Experiment was terminated 2 months after cell injection.

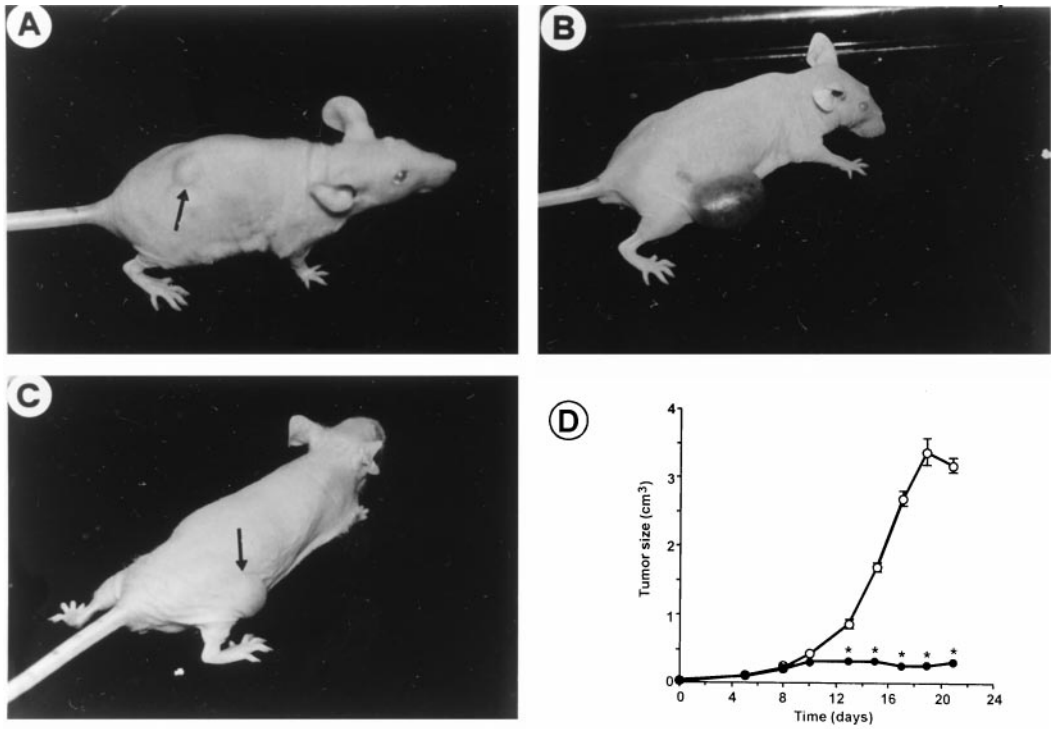

FIG. 3. Effect of *in vivo* NGF treatment on SCLC tumor growth in athymic mice. 20 Nu/Nu mice were xenografted with 10^7 NCI-N-592 cells in the dorsal region. After the formation of tumors of 30 ± 2 mm^3, animals were divided into two groups, treated with either saline or NGF (1 μg/g body weight; s.c.) once a day for 21 consecutive days, and examined for tumor growth. (*A*) Tumor size before treatments. (*B*) Tumor size in saline-treated controls. (*C*) Tumor size in NGF-treated mice. (*D*) Tumor growth curve. Points are the means ± SEM of 10 animals. *, $P < 0.001$ versus saline-treated mice by Student's *t* test. Statistical analysis was applied to points corresponding to individual times. ○, saline; ●, NGF.

that NGF treatment did not significantly modify the expression of any nicotinic receptor subunit (data not shown).

The effects of exogenous 5-HT on SCLC cell proliferation were also investigated. NGF treatment did not modify the ability of 100 nM 5-HT to stimulate SCLC cell proliferation rate. In NCI-N-592 cells, 5-HT stimulation of [^{3}H]thymidine incorporation was 61% ± 10% before and 64% ± 18% after NGF treatment and in GLC8 cells was 58% ± 9% and 53% ± 6% before and after exposure to NGF, respectively.

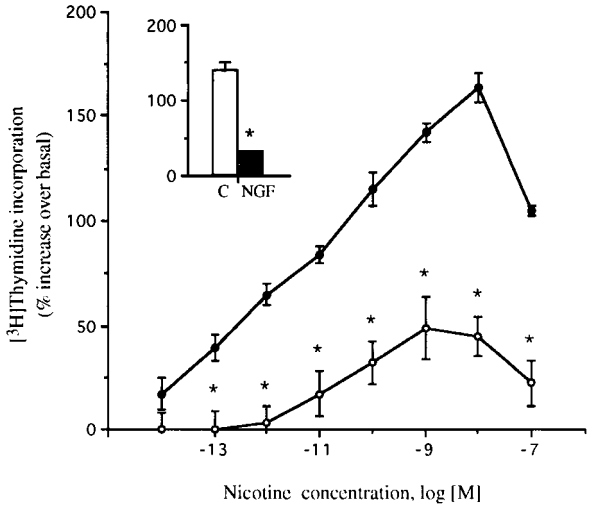

FIG. 4. Effects of (−)nicotine on [^{3}H]thymidine incorporation in NGF-treated SCLC cells. Untreated (●) and NGF-treated (○) NCI-N-592 cells were exposed to different concentrations of (−)nicotine for 48 h and analyzed for [^{3}H]thymidine incorporation. The data are expressed as percentage increases over basal. Points are the means ± SEM of four experiments, each performed in triplicate. *, $P < 0.001$ versus the corresponding control value by Student's *t* test. Statistical analysis was applied to the points corresponding to individual (−)nicotine concentrations. (*Inset*) Effects of 10 nM (−)nicotine on [^{3}H]thymidine incorporation in untreated and NGF-treated GLC8 cells. Each bar is the mean ± SEM of three independent experiments. *, $P < 0.001$ versus untreated cells by Student's *t* test.

The Effects of NGF on SCLC Cell Lines Are Reversible. The inhibitory effect of NGF on SCLC cell proliferation rate was unmodified 8 days after cells were transferred to the medium lacking NGF. After this time, however, the cell proliferation rate gradually increased reaching, on the 20th day, the same values detected in untreated cells. Similarly, the expression of both NGF receptors and NGF progressively decreased to the levels measured before NGF treatment within 15 days after NGF withdrawal. As *in vitro*, also *in vivo* the effects of NGF were reversible. After NGF treatment withdrawal, the tumors started to grow again, reaching the size of 3.5 ± 0.1 cm^3 within 20 days.

DISCUSSION

NGF, which was originally identified as a peptide essential for development and survival of different neuronal populations (26–29), is now recognized to have the characteristics of a pleiotropic cytokine (30). As the majority of cytokines, this neurotrophic factor may be involved in tumor growth and progression as suggested by the finding that different tumor cell lines express NGF receptors (31–36). The role of NGF in the control of tumor cell proliferation, however, has never been investigated thoroughly. Anecdotal data suggested that NGF may inhibit the growth of some neuroblastoma (36–39), one melanoma (40, 41), and one colon carcinoma (41) cell lines *in vitro*. On the other hand, some tumor cell lines do not respond, and others seem to proliferate in response to NGF (42–45).

NGF consistently inhibits the growth and promotes differentiation of cell lines derived from neuroendocrine tumors

(15–18), and we had previously shown that the loss of NGF production is associated with neoplastic progression of pituitary adenomas (21). The data reported here indicate that NGF abrogates the tumorigenicity and the invasive potential of two cell lines derived from human SCLC, a very aggressive carcinoma of neuroendocrine origin (1).

Chronic, but not short term NGF administration remarkably inhibited SCLC cell proliferation rate. This inhibition was due to the accumulation of the majority of cells in the G_0/G_1 phase of the cell cycle. A small percentage (20–25%) of NGF-treated cells, however, was still cycling, and this may account for the observation that inhibition of [^{3}H]thymidine incorporation promoted by NGF never was above 75%. Analysis of NGF receptor expression, showing that both cycling and noncycling cells express trkA, argues against the possibility that SCLC cell lines contain a population of cells refractory to NGF action and rather suggests that the doubling of each cell was slowed down by NGF treatment. These results are in line with previous studies showing that a 3-day exposure to NGF did not affect [^{3}H]thymidine incorporation in two SCLC cell lines (HTB 119 and HTB 120) (11) and that a longer NGF treatment had a clear antiproliferative effect on the NCI-H69 SCLC cell line (46). A long-term pretreatment of SCLC cells with NGF also inhibited their anchorage-independent clonal growth in soft agar. The few colonies produced grew as very small colonies whose size did not further increase even if kept in culture for up to 40 days. Thus, NGF-pretreated SCLC cells could sustain only a limited number of divisions in agar even if supplemented with serum. In addition, NGF treatment remarkably impaired the *in vitro* invasive capacity of SCLC cell lines and completely restrained their tumorigenicity in nude mice. The observation that no initial tumor formation was detectable after injection of NGF-treated cells indicates that the establishment of early tumor growth was impaired. These findings thus strongly indicate that NGF not only has antiproliferative effects on SCLC cells but reverts them to a less severe, nontumorigenic phenotype. On the other hand, one report suggested that a 10-day NGF exposure only marginally stimulates the growth of HTB 119 and HTB 120 SCLC cell lines in soft agar without changing their proliferation rate *in vitro* (11). The different profiles of NGF receptor expression in the cell lines used in that study may explain the apparent discrepancy with our present results. NCI-N-592 and GLC8 cells express both $p75^{NGFR}$ and trkA receptors, whereas those used by Oelmann *et al.* (11) apparently express only trkA. One possibility is, therefore, that although trkA could mediate the stimulatory effects of NGF, coexpression of both $p75^{NGFR}$ and trkA could be a crucial condition for the inhibitory effect of NGF on tumor growth to occur. NGF binding to $p75^{NGFR}$ induces ceramide production in T9 glioma cells, an effect related to inhibition of cell proliferation and differentiation (47), and promotes activation of the nuclear transcription factor NFκB in Schwann cells (48). Furthermore, $p75^{NGFR}$ and trkA collaborate to activate a specific $p75^{NGFR}$-associated protein kinase (49), suggesting that cross-talk pathways occur between $p75^{NGFR}$ and trkA-dependent signaling. Our preliminary observation that the ability of NGF to inhibit SCLC proliferation was partially reduced by a monoclonal antibody against $p75^{NGFR}$ (not shown), suggesting that $p75^{NGFR}$ does indeed contribute to the effects of NGF, gives support to this view. The type of response of a tumor cell to NGF thus may be strictly dependent on the pattern of expression of NGF receptor subtypes.

In line with the data obtained *in vitro*, chronic NGF administration to athymic mice xenografted with SCLC cells strongly suppressed the growth of the established tumors so that, at the end of the treatments, the tumor size was 14-fold smaller in NGF-treated than in saline-treated mice.

Profound biochemical alterations have also been observed in SCLC cells exposed to NGF. The expression of NGF receptors, which is variable in NCI-N-592 and GLC8 SCLC cell lines, was sensitive to induction by NGF. In addition, NGF-treated cells expressed NGF mRNA and produced and secreted NGF. Quantification of secreted NGF revealed that its concentrations in the culture media are biologically significant to activate trkA but not $p75^{NGFR}$. Thus, as $p75^{NGFR}$ seems to contribute to the biological effects of NGF in SCLC, the amount of secreted NGF is probably too low to sustain an autocrine loop. As a result, the effects of NGF on these cell lines are reversible upon NGF withdrawal. The possibility, however, exists that neuroendocrine cells in the lung express an NGF-mediated autocrine loop and that the loss of this mechanism could contribute to SCLC development and progression.

The mitogenic response to nicotine is one of the most peculiar properties of SCLC cells. Activation of nicotinic acetylcholine receptors on SCLC cells stimulates a series of Ca^{2+}-dependent events, including the release of the potent autocrine growth factor 5-HT, ultimately resulting in a remarkable stimulation of cell proliferation (7, 8). The impact of these events is such that the role of tobacco smoking on lung cancer development and progression has been considered also in the light of a direct pathogenetic interaction of nicotine with neuroendocrine cells of the lung. Here, we report a striking impairment of the mitogenic response to nicotine in SCLC cell lines that had been chronically exposed to NGF. The mechanisms underlying this effect are still a matter of investigation. NGF treatment does not modify the expression of nicotinic receptor subunits. An effect on the expression of 5-HT receptors, which mediate the mitogenic effects of 5-HT secreted in response to nicotine (7, 8), can also be excluded as exogenous 5-HT stimulated SCLC cell proliferation rate by the same extent before and after NGF treatment. Thus, transducing steps downstream nicotinic receptor activation could be selectively modulated.

In conclusion, we report here that chronic exposure of two SCLC cell lines to NGF reverts them to a slowly proliferating, noninvasive, nontumorigenic phenotype characterized by indifference to nicotine. An attractive mode of anticancer therapy is represented by gene therapy. The constitutive expression of either antisense RNA inhibiting the production of oncogenes (50) and growth factors (51, 52) or of genes encoding for tumor suppressors (53, 54) was shown to be effective in reducing tumorigenicity and malignancy. This study, showing that NGF abrogates tumorigenicity of two SCLC cell lines and strongly inhibits the growth of established tumors in nude mice, could provide a potential therapeutic framework for NGF gene targeting in the therapy of those human SCLC that are sensitive to the antitumorigenic activity of this growth factor.

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