

## EXPERIMENTAL STUDY

# Nerve growth factor and retinoic acid interactions in the control of small cell lung cancer proliferation

Chiara Fiorentini, Marco Facchetti, Alessandra Finardi, Sandra Sigala, Marcelo Páez-Pereda<sup>1</sup>, Emanuele Sher<sup>2</sup>, PierFranco Spano and Cristina Missale

Division of Pharmacology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Via Valsabbina 19, 25124 Brescia, Italy,

<sup>1</sup>Max-Planck Institute of Psychiatry, Department of Endocrinology, Kraepelinstrasse 10, 80804 Munich, Germany and <sup>2</sup>Lilly Research Centre, Erl Wood Manor, Sunninghill Road, Windlesham GU20 6PH, Surrey, UK

(Correspondence should be addressed to C Missale; Email: cmissale@med.unibs.it)

## Abstract

**Objective:** Nerve growth factor (NGF) has antiproliferative and differentiating effects in neuroendocrine tumors. In cell lines derived from small cell lung cancer (SCLC), NGF treatment stimulates NGF receptor expression, activates NGF secretion, inhibits proliferation and abrogates invasion. Since these effects are lost upon NGF withdrawal, it is relevant to identify other differentiation factors that may co-operate with the NGF system to control SCLC growth and differentiation.

**Design:** Retinoic acid (RA), which has been shown to inhibit cell transformation and proliferation, modulates the expression of NGF receptors and the sensitivity to NGF in different cell models. In the present study, we have investigated whether NGF and RA may interact to control the proliferation of SCLC cell lines.

**Methods:** SCLC cells were exposed to 50 ng/ml NGF or 1  $\mu$ M *all-trans* RA for different times. Cell proliferation was measured by the [<sup>3</sup>H]thymidine incorporation test and NGF receptor expression was evaluated by immunofluorescence.

**Results:** We found that RA increased the expression of both *trkA* and *p75* NGF receptors in NCI-N-592 and GLC8 cell lines and prevented the loss of both NGF production and NGF receptor expression occurring when NGF treatment was discontinued. As a result, RA, which did not inhibit the proliferation of untreated cells, abolished NGF withdrawal-related increase in cell proliferation both *in vitro* and *in vivo*, thus making permanent the antiproliferative effects of NGF.

**Conclusions:** These data suggest that combined treatments with NGF and RA or mimicking drugs may represent a strategy to be further investigated for the treatment of SCLC.

European Journal of Endocrinology 147 371–379

## Introduction

Small cell lung cancer (SCLC) is a very aggressive neuroendocrine tumor (1–5) characterized by early metastasis potential and rapid development of treatment resistance. Despite the rapid progress in understanding the molecular abnormalities underlying the pathogenesis of SCLC, there have been no major advances in the therapy for this tumor (1).

The proliferation of SCLC is controlled by different neurohormones and growth factors including insulin-like growth factor-I, bradykinin, neurotensin and serotonin (1, 6, 7). Moreover, like other tumors of neuroendocrine origin (8–11), the SCLC cell lines NCI-N-592 and GLC8 are sensitive to the antiproliferative action of nerve growth factor (NGF) (12). We have shown that only 50% of NCI-N-592 and GLC8 cells express *trkA* and *p75* receptors for NGF and that

NGF treatment increases their expression in the majority of cultured cells (12). As a result, NGF inhibits the proliferation rate and abrogates the *in vitro* invasive potential and *in vivo* tumorigenicity of these cell lines (12). Besides stimulating the expression of its receptors, NGF also stimulates its own production in these cell lines, thus establishing a potential autocrine mechanism constraining cell growth (12). The effects of NGF on SCLC cell lines, however, are lost when NGF treatment is discontinued, suggesting that the amount of secreted NGF may be too low to sustain an autocrine loop. Thus, it is relevant to identify other factors which, by interacting with the NGF system, may contribute to control SCLC growth and differentiation.

Some observations point to retinoic acid (RA) as a plausible candidate. Retinoids are active in the prevention and treatment of a variety of malignancies. In particular, they modulate the growth and differentiation of

different cancer cells including promyelocytic leukemia, breast cancer, and skin, head and neck cancers (13, 14) and are active as chemopreventive agents in animal models of mammary, skin, liver and prostate cancer (13, 14). Furthermore, it has been shown that RA modulates the sensitivity to NGF in different models with a mechanism involving the regulation of NGF receptor expression. For example, in immature sympathetic neurons, RA inhibits the expression of trkA (15, 16), while in neuroblastoma cells RA modulates NGF responsiveness by increasing trkA expression (17, 18). Exposure of pheochromocytoma PC12 cells to RA results in both stimulation of p75 NGF receptor expression and increased sensitivity to the differentiating effects of NGF (19). Moreover, in thyroid carcinoma cells, RA and NGF synergistically co-operate to inhibit cell proliferation (11).

On this basis, in the present study we investigated whether NGF and RA may interact to control the proliferation of SCLC cell lines. The results showed that RA induces the expression of NGF receptors in untreated NCI-N-592 and GLC8 cell lines and prevents the loss of both NGF production and NGF receptor expression occurring when NGF treatment is discontinued. As a result, subsequent RA administration to cells previously treated with NGF counteracts the increase of cell proliferation occurring during NGF withdrawal both *in vitro* and *in vivo*, thus making permanent the antiproliferative effects of NGF. These data suggest that combined treatments with NGF and RA or mimicking drugs may represent a strategy to be further investigated for the treatment of SCLC.

## Materials and methods

### Cell culture and treatment

The human SCLC cell lines NCI-N-592 and GLC8 were used. Cells were grown 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 under 5% CO<sub>2</sub>/95% O<sub>2</sub>. Cells were treated either with 1 µM *all-trans* RA for 8 or 15 consecutive days or with 50 ng/ml NGF (2.5 s mouse NGF; Alomone Labs, Jerusalem, Israel) for 15 consecutive days. At the end of NGF treatment, cells were divided into two groups and grown either in the standard medium lacking NGF or in the presence of 1 µM RA for an additional 40 days. RA and NGF were added to the cultures every 3 days at the time of each change of medium.

### Immunofluorescence

Cells were collected and, after two rinses with phosphate-buffered saline (PBS), fixed in PBS containing 3% paraformaldehyde and 2% sucrose for 10 min at room temperature. Cells were then rinsed with PBS, permeabilized with 0.5% saponine for 10 min at room

temperature and incubated for 1 h at room temperature with the following antibodies diluted in PBS containing 0.1% saponine: anti-trkA (1 µg/ml; Santa Cruz Biotechnology, Heidelberg, Germany), anti-p75 (5 µg/ml; MC192 mouse monoclonal, Boehringer Mannheim, Mannheim, Germany) and anti-NGF (20 ng/ml; 27/21 mouse monoclonal; Boehringer Mannheim). After three rinses with PBS, cells were incubated for 1 h at room temperature and in the dark with the appropriate secondary antibodies conjugated to either fluorescein or rhodamine. After three rinses, cells were resuspended in 1 ml PBS and analyzed using a multichannel cytometer (FACSTAR; Becton Dickinson, Milan, Italy). Total counts of 10<sup>4</sup> cells were accumulated for each group. Omission of the primary antibodies was performed as a control of non-specific staining.

### [<sup>3</sup>H]Thymidine incorporation

Untreated or treated cells were counted and plated in multiwell plates (16 mm; NUNC) at a density of 5 × 10<sup>5</sup> cells/well in the presence or the absence of the treatments. Twenty-four hours later, [<sup>3</sup>H]thymidine (0.5 µCi/ml; 84.8 Ci/mmol; Dupont-NEN, Milan, Italy) was added and cells were incubated for an additional 24 h at 37°C. Cells were rinsed three times with PBS, incubated for 10 min with ice-cold trichloroacetic acid and then 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.

### In vitro invasion assay

Polycarbonate filters (8 µm pore size) were coated with 40 µg Matrigel (Becton Dickinson, Heidelberg, Germany) and incubated at 37°C for 30 min. Cells were suspended in DMEM medium containing 0.1% bovine serum albumin (BSA), added to the inner side of the transwell (10<sup>5</sup> cells/well) and incubated for 24 h at 37°C. Cells on the upper side of the filter were removed with a cotton swab and cells attached to the bottom side of the membrane were fixed in methanol, stained with hematoxylin and counted.

### Measurement of NGF cell content

A two-site enzyme-linked immunosorbent assay (ELISA) system (Boehringer Mannheim with mAb 27/21) was used to quantitate NGF in cell extracts according to the manufacturer's instructions. Briefly, 96-well plates were coated with either anti-NGF antibody (0.1 µg/ml) or mouse IgG (1 µg/ml; blanks) for 2 h at 37°C. Cells were collected, rinsed with PBS, homogenized in 100 mM Tris-HCl containing 400 mM NaCl, 2% BSA, 0.1% Triton-X 100, 1 mM phenylmethylsulfonylfluoride, 7 µg/ml aprotinin, 4 mM EDTA (pH 7.0) and centrifuged at 100 000 g for

10 min at 4 °C. The supernatant was added with the same volume of 20 mM CaCl<sub>2</sub> containing 0.2% Triton-X 100 and aliquots of this preparation were incubated overnight at 4 °C in the coated microwells previously described. Wells were rinsed, incubated for 4 h at 37 °C with the anti-NGF antibody conjugated with β-galactosidase and then rinsed again. Chlorophenol red-β-D-galactopyranoside was then added and wells were incubated at 37 °C for 30–60 min and analyzed for absorbance at 574 nm.

### RNA isolation and PCR

Total RNA was isolated using the SV Total RNA Isolation System (Promega Italia, Milan, Italy). Four micrograms of each sample were transcribed into cDNA by standard methods using murine Moloney Leukemia Virus Reverse Transcriptase (Promega Italia) and oligo(dT)<sub>18</sub> (Promega Italia) as a primer. Aliquots of cDNA were used as a template in PCR reactions containing the oligonucleotides 5'-TCATCCACCCACCCAGTCTTC-3' encoding human NGF residues SSTMPVF and 5'-GGCAGCCTGTTTGTCTGTGT-3' encoding human NGF residues TDDKQAA. Reactions were performed for 28 cycles (95 °C 45 s; 53 °C 45 s; 72 °C 1 min) within the linear range of amplification. Omission of the reverse transcription reaction was performed as a control of the PCR specificity. Amplification with 5'-TAAAGACCTCTATGCCAACACAGT-3' and 5'-CACGATGGAGGGCCGACTCATC-3' primers respectively encoding human β-actin residues KDLYANTV and DESGPSIV (95 °C 30 s; 60 °C 30 s; 72 °C 1 min; 25 cycles) was performed as a control of the amount of cDNA used in each sample. The PCR products were resolved on 1% agarose gels stained with ethidium bromide and blotted on nylon membrane. Blots were hybridized with the NGF PstI/PstI cDNA fragment (kindly provided by Dr R dal Toso, R&C Scientifica, Vicenza, Italy) labeled with α<sup>32</sup>P-dCTP (Dupont-NEN) to a specific activity of at least 5 × 10<sup>8</sup> c.p.m./μg using a multiprime labeling system. Hybridization was performed in 50% formamide, 5 × SSC at 42 °C overnight and, after washing, blots were exposed to Kodak-X-Omat films.

### SDS-PAGE and immunoblotting

Untreated and treated cells were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.5% Triton-X 100 and 0.1% SDS and a complete set of protease inhibitors (Complete Mini Protease Inhibitors; Roche Molecular Biochemicals, Milan, Italy), incubated on ice for 20 min and centrifuged at 18 000 *g* for 2 min at 4 °C. Aliquots of cell extracts (50 μg protein/lane) were resolved on 12% SDS-PAGE, transferred onto PolyScreen PVDF membranes (Dupont-NEN) membranes and blotted for 1 h at room temperature in Tris-buffered saline (TBS) con-

taining 0.1% Tween 20 and 5% non-fat powdered milk. Membranes were incubated overnight at 4 °C with the monoclonal anti-p21<sup>Cip1/Waf1</sup> antibody (2 μg/ml; Becton Dickinson, Milano, Italy) and then with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1500 dilution; DAKO, Milan, Italy). The amount of proteins in each lane was checked by immunoreaction with a monoclonal anti-β-tubulin antibody (1:1500 dilution in TBS containing 5% non-fat powdered milk and 0.1% Triton-X 100; Neo-Markers, Fremont, CA, USA) for 2 h at room temperature and then with an HRP-conjugated secondary antibody (1:1500 dilution; DAKO). For detection, an ECL chemiluminescence system (Amersham International, Milan, Italy) was used.

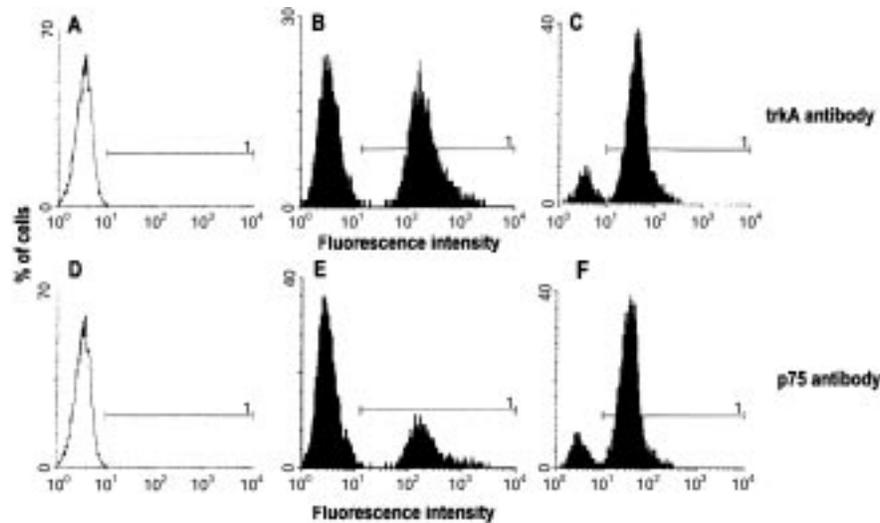
### In vivo studies

Twenty Nu/Nu female mice (20 g body weight (b.w.); Charles River Breeding Laboratories, Milano, Italy) were injected s.c. in the dorsal region with 10<sup>7</sup> NCI-N-592 cells. After the appearance of tumors of 30 ± 2 mm<sup>3</sup>, mice were divided into two groups and treated with either s.c. saline (*n* = 5) or s.c. NGF (1 μg/g b.w.; *n* = 15) once a day for 21 consecutive days. NGF-treated mice were further divided into two groups and treated with either saline (*n* = 5) or RA (1.5 μg/g b.w.; *n* = 10) by oral gavage for an additional 15 days. Tumor size was evaluated at the end of NGF treatment and during subsequent RA or saline administration. Tumor volumes were determined according to the formula  $V = A \times B^2 \times 0.4$ , where A and B are the larger and the smaller axes of the tumor.

## Results

### RA induces the expression of NGF receptors in SCLC cell lines

To evaluate whether RA may modulate NGF receptor expression in SCLC cell lines, NCI-N-592 and GLC8 cultures were treated with *all-trans* RA (1 μM) for 15 days and then tested for trkA and p75 levels by indirect immunofluorescence followed by flow cytometry (FACS). A representative FACS scan obtained with the NCI-N-592 cell line is reported in Fig. 1. Only 49 ± 3% (means ± S.E.M.; *n* = 3) of untreated cells expressed trkA (Fig. 1B) and 38 ± 4% (*n* = 3) expressed p75 (Fig. 1E). Exposure to 1 μM RA for 15 consecutive days resulted in a strong induction of NGF receptor expression. Of the RA-treated cells 82 ± 4% (*n* = 3) were in fact positive for trkA staining (Fig. 1C) and 86 ± 6% (*n* = 3) were positive for p75 expression (Fig. 1F). Figure 1A and D show the non-specific labeling obtained with omission of the primary antibodies. Similar results were obtained with the GLC8 cell line where RA treatment increased the percentages of cells positive



**Figure 1** RA induces the expression of NGF receptors in NCI-N-592 cells. Cells were grown in the absence or the presence of 1  $\mu$ M RA for 15 days, fixed, permeabilized, immunoreacted with anti-trkA and anti-p75 antibodies and analyzed by FACS as described in Materials and methods. Total counts of  $10^4$  cells were accumulated for each group. A representative FACS analysis is shown. (A) omission of the anti-trkA antibody, (B) trkA expression in untreated cells, (C) trkA expression in RA-treated cells, (D) omission of the anti-p75 antibody, (E) p75 expression in untreated cells and (F) p75 expression in RA-treated cells. Note the increase in the signal for both trkA and p75 in RA-treated cells.

for trkA and p75 from  $51 \pm 5$  to  $78 \pm 4$  and from  $47 \pm 3$  to  $73 \pm 6$  respectively.

We then investigated whether a 15-day RA treatment may also induce the production of endogenous NGF in these cell lines. Virtually no specific NGF labeling ( $5 \pm 1\%$ ) was detectable by FACS in untreated cells. A 15-day RA treatment did not induce NGF production as shown by the observation that only  $9 \pm 2\%$  of RA-treated cells were positive for NGF labeling. These data thus suggest that RA does not activate in SCLC cell lines the silent autocrine loop mediated by NGF.

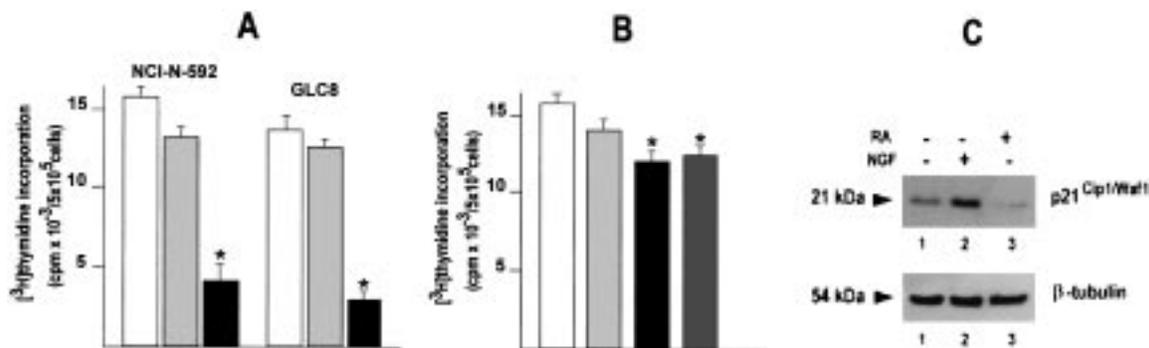
### **RA inhibits proliferation of SCLC cell lines only when given in combination with NGF**

We have previously shown that NGF has two major functional effects on SCLC cells: inhibition of cell proliferation and inhibition of invasion (12). Here we investigated the effects of RA on these two parameters. The effects of a 15-day administration of 1  $\mu$ M RA on the *in vitro* proliferation of NCI-N-592 and GLC8 cells were evaluated by measuring [ $^3$ H]thymidine incorporation. As a positive control, a group of cells was exposed to 50 ng/ml NGF for 15 days. As shown in Fig. 2A, the proliferation rate of both cell lines, which was strongly inhibited by NGF, was not significantly affected by RA treatment. To evaluate whether synergistic interactions may occur between NGF and RA in the induction of growth arrest, NCI-N-592 cells were simultaneously treated with NGF and RA for 8 days and tested for [ $^3$ H]thymidine incorporation. The length of treatment was selected on the basis of previous time-course

experiments showing that an 8-day NGF treatment only slightly inhibited the proliferation rate of this cell line (12). As reported in Fig. 2B, an 8-day NGF treatment, but not RA treatment, inhibited cell proliferation by about 30%. When NGF and RA were given together, no further inhibition of NCI-N-592 proliferation was observed, suggesting that RA does not potentiate the antiproliferative effect of NGF. In line with these observations are the data obtained by measuring the expression of p21<sup>Cip1/WAF1</sup>, a regulatory protein involved in cell cycle arrest (20). As reported in Fig. 2C (upper panel), p21<sup>Cip1/WAF1</sup> was poorly expressed in the proliferating NCI-N-592 cell line. Exposure of these cells to NGF for 15 days resulted in a remarkable p21<sup>Cip1/WAF1</sup> induction. By contrast, RA treatment did not increase p21<sup>Cip1/WAF1</sup> levels in NCI-N-592 cells.

The data reported in Fig. 3 show that RA, although ineffective in the proliferation test, efficiently inhibited the capacity of NCI-N-592 and GLC8 cells to invade Matrigel-coated membranes, suggesting that it impairs their *in vitro* invasive potential.

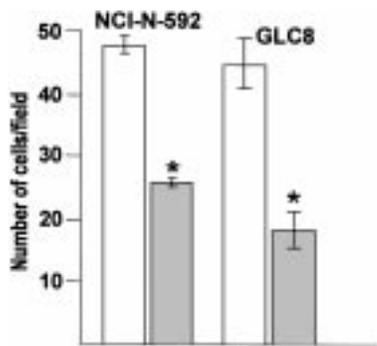
As reported in Fig. 4, although inactive in untreated cells, RA efficiently counteracted the increase in SCLC cell proliferation occurring during NGF withdrawal. Inhibition of NCI-N-592 cell proliferation induced by chronic NGF treatment (50 ng/ml, 15 days) was reversible when NGF administration was discontinued (Fig. 4A). In particular, the cell proliferation rate lasted at the inhibited values for 8 days after NGF withdrawal then gradually increased, reaching, on day 36, the same values as those detected before NGF treatment. When NGF-treated NCI-N-592 cells were



**Figure 2** RA does not inhibit the proliferation of SCLC cells *in vitro*. (A) Cells were grown in the absence or the presence of either 1 μM RA or 50 ng/ml NGF for 15 days. The cell proliferation rate was evaluated by the [<sup>3</sup>H]thymidine incorporation test. Bars represent the means ± S.E.M. of three independent experiments. Untreated cells (open bars), RA-treated cells (lightly shaded bars) and NGF-treated cells (solid bars). \**P* < 0.001 vs untreated cells (Student's *t*-test). (B) Cells were grown in the absence or the presence of 1 μM RA and 50 ng/ml NGF either given alone or in combination for 8 days. The cell proliferation rate was evaluated by the [<sup>3</sup>H]thymidine incorporation test. Bars represent the means ± S.E.M. of three independent experiments. Untreated cells (open bars), RA-treated cells (lightly shaded bars), NGF-treated cells (solid bars) and NGF + RA-treated cells (darker shaded bars). \**P* < 0.001 vs untreated cells. (C) Western blot analysis of p21<sup>Cip1/WAF1</sup> expression in untreated and treated NCI-N-592 cells. Cells were treated either with 1 μM RA or 50 ng/ml NGF for 15 days. Lane 1: untreated cells, lane 2: NGF treatment and lane 3: RA treatment. These experiments revealed that RA does not inhibit SCLC cell proliferation and does not potentiate the antiproliferative effect of NGF, when the two compounds are given together. Note that NGF, but not RA, induces the expression of p21<sup>Cip1/WAF1</sup>, a molecular marker of growth arrest.

cultured for up to 36 days in a medium lacking NGF and containing 1 μM RA, [<sup>3</sup>H]thymidine incorporation failed to increase to the values found before NGF treatment (Fig. 4A). RA, administered in parallel to one group of untreated NCI-N-592 cells, did not significantly affect their proliferation rate at any time tested (not shown). Similar results were obtained with the GLC8 cell line (Fig. 4A, inset). Neither morphological changes nor cell death were detected during NGF and RA treatment. The possibility that NGF and RA may interact to control SCLC cell proliferation also *in vivo* was then investigated. We have previously reported that chronic NGF administration to Nu/Nu mice trans-

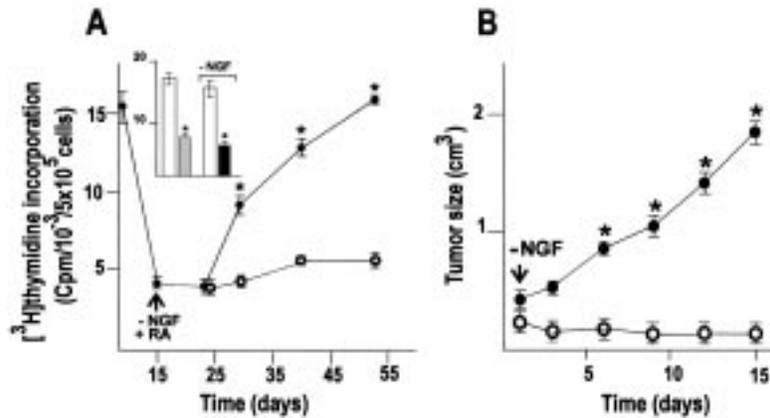
planted with SCLC cells strongly inhibited the growth of the established tumors (12). In the present study, Nu/Nu mice transplanted with NCI-N-592 cells and treated with NGF (1 μg/g b.w. once a day for 21 consecutive days) were divided into two groups and treated with either saline or RA (1.5 μg/g b.w.) for 15 days. As reported in Fig. 4B, the tumor size exponentially increased in the group of NGF-treated mice switched to saline administration. By contrast, tumors did not grow in mice treated with RA. The tumor size at the end of RA treatment was in fact the same as at the end of NGF treatment (Fig. 4B). RA administration to untreated mice did not significantly inhibit tumor growth (not shown).



**Figure 3** Effect of RA on SCLC cell invasive potential. Untreated (open bars) and RA-treated (shaded bars) cells (1 μM, 15 days) were analyzed for *in vitro* invasion as described in Materials and methods. Bars represent the means ± S.E.M. of three independent experiments. \**P* < 0.001 vs untreated cells (ANOVA followed by the Fisher PLSD). Note the remarkable reduction in the number of cells that invaded the Matrigel-coated membranes in the RA-treated group.

**Endogenous NGF contributes to the antiproliferative effect of RA in SCLC cell lines**

As we have shown previously, the expression of trkA and p75 in NCI-N-592 and GLC8 cells was increased by chronic exposure to NGF (12). Evaluation of NGF receptor expression during NGF withdrawal showed that both trkA and p75 were progressively lost when NGF administration was discontinued. The percentage of NCI-N-592 cells expressing trkA and p75, 81 ± 3 and 80 ± 2 in NGF-treated cells, decreased during NGF withdrawal reaching, within 36 days, the values of 45 ± 2.5% and 28 ± 1% respectively. Exposure to 1 μM RA during NGF withdrawal abolished these decreases. The extent of cells expressing trkA and p75 in cultures treated with RA during NGF withdrawal was in fact superimposable on that found during NGF treatment (88 ± 3% in the case of trkA and 76 ± 4% in the case of p75). Moreover, in contrast with untreated cells



**Figure 4** Effects of sequential treatments with NGF and RA on the proliferation rate of SCLC cells *in vitro* and *in vivo*. (A) Cells were treated with 50 ng/ml NGF for 15 days. On day 15, cells were divided into two groups and grown either in the standard medium lacking NGF (●) or in the standard medium containing 1 μM RA (○) for an additional 36 days. Cell proliferation was measured at the times indicated by the [<sup>3</sup>H]thymidine incorporation test. Points are the means ± S.E.M. of three independent experiments. \**P* < 0.001 versus RA-treated cells (Student's *t*-test). Statistical analysis was applied to the points corresponding to individual times. (Inset) Effect of RA on the proliferation rate of NGF-treated GLC8 cells. Bars represent the means ± S.E.M. of three independent experiments. Untreated cells (open bars), NGF-treated cells (shaded bar) and RA-treated cells (solid bar). \**P* < 0.001 vs untreated cells (Student's *t*-test). (B) Effects of subsequent treatments with NGF and RA on the growth of established tumors in athymic mice. Twenty Nu/Nu mice were injected with 10<sup>7</sup> NCI-N-592 cells in the dorsal region. After tumor formation, mice were treated with either saline (*n* = 5) or NGF (1 μg/g b.w.; *n* = 15) for 21 days and examined for tumor growth. On day 21, NGF-treated mice were divided into two groups and treated with either saline (●) or 1.5 μg/g b.w. RA (○) for 15 days and examined for tumor growth at the times indicated. Points are the means ± S.E.M. of five (saline) or ten (RA) animals. \**P* < 0.001 vs RA-treated mice (Student's *t*-test). Statistical analysis was applied to points corresponding to individual times. These experiments revealed that RA administration to cells previously exposed to NGF prevents the increase of cell growth due to NGF withdrawal.

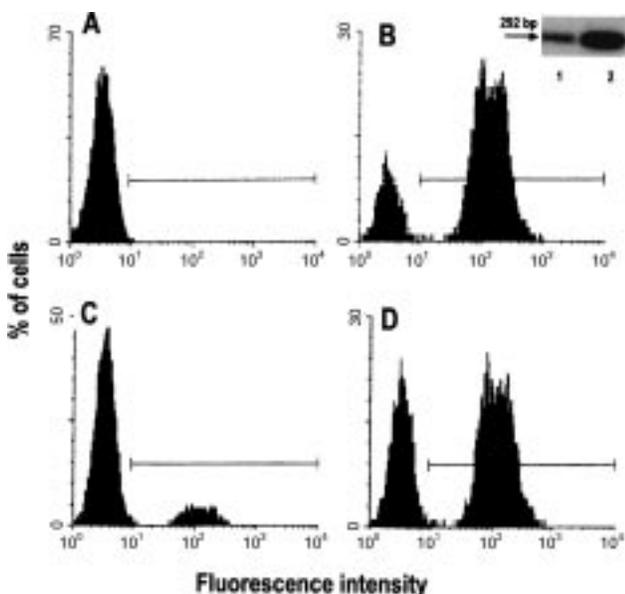
where RA did not affect NGF expression, RA administration counteracted the loss of NGF production occurring when NGF treatment was discontinued. As reported in Fig. 5, 79 ± 5% of NGF-treated cells were positive for NGF expression (Fig. 5B). That NGF levels measured by FACS in NGF-treated cells reflect the production of endogenous NGF has been shown by measuring the expression of NGF mRNA by RT-PCR followed by Southern blot (Fig. 5B, inset). The percentage of NGF-positive cells was decreased to 12 ± 1 during NGF withdrawal (Fig. 5C). Exposure of NGF-treated cells to RA during NGF withdrawal maintained the percentage of NGF-positive cells at the value of 68 ± 2 (Fig. 5D). This observation was confirmed by measuring intracellular NGF levels by a two-site ELISA. NGF content, which was undetectable in untreated cells, was 510 ± 46 pg/10<sup>6</sup> cells in NGF-treated cells and was undetectable after 36 days of NGF withdrawal and 473 ± 50 pg/10<sup>6</sup> cells after a 36-day treatment with RA of cells previously exposed to NGF.

As shown in Fig. 6, the effect of RA on NGF production in SCLC cells was completely prevented by a specific anti-NGF monoclonal antibody, suggesting that it was mediated by secreted NGF. Similarly, endogenous NGF also contributed, at least in part, to the antiproliferative effects of RA on NGF-treated SCLC cells. The inhibitory effect of RA on SCLC cell proliferation was in fact partially inhibited when RA was

administered in the presence of the anti-NGF monoclonal antibody (Fig. 6).

## Discussion

Lung cancer continues to be the leading cause of cancer death. Although our understanding of the pathogenesis of these tumors has made rapid progress, there have been no similar therapeutical advances and the overall 5-year survival is still under 13% (1). It is therefore crucial to identify and develop novel strategies for the prevention and treatment of this disease. Chemoprevention with retinoids provides an extremely attractive approach (21). RA has been shown in fact to inhibit cell transformation and proliferation in different models (21–24). In particular, it has been shown that retinoids block tumor promotion by inhibiting proliferation, inducing differentiation and/or apoptosis (13, 14, 25) and influencing cell–cell and cell–substrate interactions (23, 26–29). Clinically, retinoids have been shown to decrease the incidence of second primary tumors in patients with head and neck cancer and to reduce the development of skin cancer in patients with xeroderma pigmentosum (13, 14). Furthermore, retinoids achieve remission in acute promyelocytic leukemia by inducing growth inhibition, granulocyte differentiation, activation of the caspase

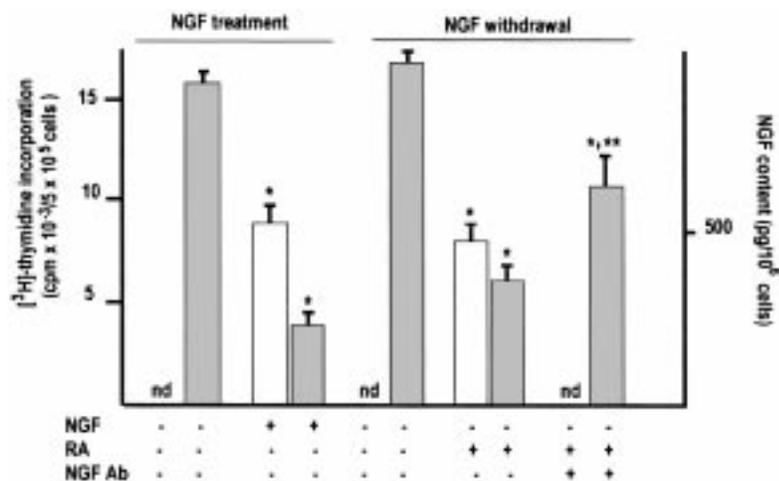


**Figure 5** Effects of sequential treatments with NGF and RA on NGF production in NCI-N-592 cells. Cells were treated with 50 ng/ml NGF for 15 days. On day 15, cells were divided into two groups and grown either in the standard medium lacking NGF or in the standard medium containing 1 μM RA for an additional 36 days. Cells were fixed, permeabilized, immunoreacted with anti-NGF antibodies and analyzed by FACS as described in Materials and methods. Total counts of 10<sup>4</sup> cells were accumulated for each group. A representative FACS analysis is shown. (A) Non-specific labeling, (B) NGF treatment, (C) NGF withdrawal and (D) RA treatment during NGF withdrawal; (B inset) NGF mRNA expression in untreated (lane 1) and NGF-treated (lane 2) NCI-N-592 cells measured by RT-PCR and Southern blot as described in Materials and methods. These experiments showed that RA administration to NGF-treated SCLC cells prevents the loss of endogenous NGF production occurring during withdrawal of NGF treatment.

system and apoptosis (13, 14, 25, 30, 31). Retinoids have also been reported to play a central role in the differentiation of some lung cell populations during development (32, 33), to suppress lung carcinogenesis in the experimental animal (34, 35), to increase the neuroendocrine differentiation (36) of the SCLC cell line NCI-H82 and to block its transition to a treatment-resistant phenotype (37). Most lung cancer cell lines, including SCLC, are, however, refractory to the antiproliferative action of RA (38, 39).

Increasing evidence now suggests that NGF is another differentiation factor for various neuroendocrine tumors (8–10) including SCLC (12). In particular, we have reported that NGF inhibits the proliferation and abrogates the tumorigenic and invasive potential of two SCLC cell lines. These effects were accompanied by biochemical alterations such as induction of NGF receptor expression and production and secretion of endogenous NGF (12). The concentrations of secreted NGF, however, appeared to be too low to sustain an autocrine loop. As a result, the antiproliferative and antitumorigenic effects of NGF on NCI-N-592 and GLC8 cells were reversible when NGF treatment was discontinued.

In this paper, we report that NGF and RA efficiently interact to control the proliferation rate of NCI-N-592 and GLC8 cell lines. The evaluation of the functional effects of NGF and RA on SCLC cells revealed that, while NGF exerted a strong antiproliferative effect, RA did not affect cell proliferation when given alone for up to 15 days. Similarly, the simultaneous administration of RA did not enhance the antiproliferative effect of short-term NGF treatment, suggesting that there are no synergistic interactions. Along the same lines, in NCI-N-592 cells NGF, but not RA, strongly induced the expression of p21<sup>Cip1/WAF1</sup>, a cell cycle-related protein involved in growth arrest (20).



**Figure 6** The effects of RA on cell proliferation and NGF production are mediated by endogenous NGF. Cells were treated with 50 ng/ml NGF for 15 days. On day 15, cells were divided into two groups and grown either in the standard medium lacking NGF or in the standard medium containing 1 μM RA in the absence or the presence of a monoclonal anti-NGF antibody (Ab; 50 ng/ml) for an additional 36 days. Proliferation was evaluated by [<sup>3</sup>H]thymidine incorporation and intracellular NGF content by a two-site ELISA. [<sup>3</sup>H]Thymidine incorporation (shaded bars) and NGF cell content (open bars); nd, not detectable refers to NGF cell content. \*P < 0.001 vs untreated; \*\*P < 0.001 vs RA treatment (Student's *t*-test). These data suggested that the ability of RA to maintain the production of NGF in SCLC cells during withdrawal of NGF treatment is mediated by the endogenous NGF secreted into the culture medium. Note also that the antiproliferative effects of RA in NGF-treated cells is partially dependent on secreted NGF.

Transcription of p21<sup>Cip1/WAF1</sup> is induced in fact in different cell lines undergoing differentiation (40–42) and disruption of the p53-p21<sup>Cip1/WAF1</sup> pathway is an important step in tumorigenesis (20). Although inactive as an inhibitor of SCLC cell proliferation, RA abrogated the invasive potential of these cell lines however. In line with these observations, it has been reported that RA inhibits the invasive potential of human meningiomas and melanoma cell lines without modifying their proliferation rate (23, 27, 43). Besides inhibiting invasion, RA increased the expression of trkA and p75 NGF receptors in NCI-N-592 and GLC8 cells and prevented the decrease of NGF receptors occurring during NGF withdrawal. Moreover, RA also prevented the loss of endogenous NGF production occurring when NGF treatment was discontinued, thus sustaining the autocrine loop mediated by endogenous NGF. The effect of RA on NGF production was completely antagonized by an anti-NGF antibody, suggesting that it was mediated by secreted NGF. In line with this, we report that in untreated cells, which lack endogenous NGF, RA did not activate NGF expression. Thus, it can be suggested that, by maintaining the expression of NGF receptors, RA indirectly activates an NGF-mediated signal essential to induce the expression of the NGF gene. On this subject, analysis of the NGF promoter revealed the presence of at least two AP-1 consensus sequences which mediate NGF mRNA induction by a variety of stimuli (44). Since AP-1 is one of the downstream signals activated by NGF receptors, NGF may indeed activate and maintain its own production.

The effects of RA on NGF receptor expression and NGF production have a functional implication in the regulation of SCLC cell proliferation both *in vitro* and *in vivo*. While inactive in untreated cells, RA counteracted in fact the increase of the *in vitro* cell proliferation occurring during NGF withdrawal. Similarly, the increase of tumor growth occurring in nude mice when NGF treatment was discontinued was completely blocked by subsequent RA administration. In line with our observations, RA has been reported to inhibit the proliferation of thyroid papillary tumor cells only when given in combination with NGF (11) and to potentiate the antitumorigenic effect of other cytokines (45, 46).

Two mechanisms apparently contribute to the anti-proliferative effects of RA in NGF-treated cells, one that is NGF dependent, since it was inhibited by the inactivation of secreted NGF, and one that is apparently independent of secreted NGF. On the one hand, RA, by inducing NGF receptor expression, maintains activated the NGF-inducible, NGF-mediated autocrine loop that constrains cell proliferation. On the other hand, other putative mechanisms of interaction may be invoked to explain the mechanism of the antiproliferative action of RA that is apparently independent of secreted NGF. For instance, one possible point of cross-talk might

involve the NGF-responsive transcription factor NGFI-B which can form functional heterodimers with the retinoid X receptor for RA and regulate gene transcription (47, 48). Furthermore COUP-TE, a transcription factor involved in the antiproliferative effect of RA (49), has been shown to up-regulate the NGF-responsive transcription factor NGFI-A (50), providing another putative mechanism of interaction.

In conclusion, these data suggest the existence of a cross-talk between NGF and RA in SCLC cells and may provide the framework to further investigate novel strategies for the management of this tumor.

## Acknowledgements

We thank Francesco Clementi (University of Milan) for helpful discussions. This study was supported by the Italian Association of Cancer Research (AIRC).

## References

- 1 Carney DN. Biology of small cell lung cancer. *Lancet* 1992 **339** 843–846.
- 2 Gazdar AF, Helman L, Israel MA, Russell EK, Linnoila RI, Mulshine JL *et al.* Expression of neuroendocrine cell markers L-dopa decarboxylase, chromogranin A, and dense core granules in human tumors of endocrine and non endocrine origin. *Cancer Research* 1988 **48** 4078–4082.
- 3 Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A *et al.* Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature* 1985 **316** 823–826.
- 4 Sher E, Pandiella A & Clementi F. Voltage-operated calcium channels in small cell lung carcinoma cell lines: pharmacological, functional and immunological properties. *Cancer Research* 1990 **53** 3892–3896.
- 5 Codignola A, Tarroni P, Clementi F, Pollo A, Lovallo M, Carbone E *et al.* Calcium channel subtypes controlling serotonin release from human small cell lung carcinoma cell lines. *Journal of Biological Chemistry* 1993 **268** 26240–26247.
- 6 Cattaneo MG, Codignola A, Vicentini LM, Clementi F & Sher E. Nicotine stimulates a serotonergic autocrine loop in human small cell lung carcinoma. *Cancer Research* 1993 **53** 5566–5568.
- 7 Codignola A, Tarroni P, Cattaneo MG, Vicentini LM, Clementi F & Sher E. Serotonin release and cell proliferation are under the control of alpha-bungarotoxin-sensitive nicotinic receptors in small cell lung carcinoma cell lines. *FEBS Letters* 1994 **342** 286–290.
- 8 Missale C, Boroni F, Sigala S, Zanellato A, Dal Toso R, Balsari A *et al.* Nerve growth factor directs the differentiation of the bipotential cell line GH<sub>3</sub> into the mammothroph phenotype. *Endocrinology* 1994 **135** 290–298.
- 9 Polack M, Scharfmann R, Seilheimer B, Eisenbarth G, Dressler D, Verma IM *et al.* Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line. *PNAS* 1993 **90** 5781–5785.
- 10 Missale C, Boroni F, Losa M, Giovanelli M, Zanellato A, Dal Toso R *et al.* Nerve growth factor suppresses the transforming phenotype of human prolactinomas. *PNAS* 1993 **90** 7961–7965.
- 11 Paez-Pereda M, Missale C, Grubler Y, Arzt E, Schaaf L & Stalla GK. Nerve growth factor and retinoic acid inhibit proliferation and invasion in thyroid tumor cells. *Molecular and Cellular Endocrinology* 2000 **167** 99–106.
- 12 Missale C, Codignola A, Sigala S, Finardi A, Paez-Pereda M, Sher E *et al.* Nerve growth factor abrogates the tumorigenicity of human small cell lung cancer cell lines. *PNAS* 1998 **95** 5366–5371.

- 13 Kurie JM. The biological basis for the use of retinoids in cancer prevention and treatment. *Current Opinions in Oncology* 1999 **11** 497–502.
- 14 Niles RM. Recent advances in the use of vitamin A (retinoids) in the prevention and treatment of cancer. *Nutrition* 2000 **16** 1100–1101.
- 15 Rodriguez-Tebar A & Rohrer H. Retinoic acid induces NGF-dependent survival response and high-affinity NGF receptors in immature chick sympathetic neurons. *Development* 1991 **112** 813–820.
- 16 Holst A, Rodriguez-Tebar A, Michaille JJ, Dhoulailly D, Backstrom A, Ebendal T *et al.* Retinoic acid-mediated increase in trkA expression is sufficient to elicit NGF-dependent survival of sympathetic neurons. *Molecular and Cellular Neuroscience* 1995 **6** 185–198.
- 17 Haskell BE, Stach RW, Werrbach-Perez K & Perez-Polo JR. Effect of retinoic acid on nerve growth factor receptors. *Cell and Tissue Research* 1987 **247** 67–73.
- 18 Kogner P, Barbany G, Persson H, Soderhall S, Ahstrom L & Bjork O. Expression of nerve growth factor receptor mRNAs and clinical response to retinoic acid in neuroblastoma. *Progress in Clinical and Biological Research* 1994 **385** 147–153.
- 19 Scheibe RJ & Wagner JA. Retinoic acid regulates both expression of the nerve growth factor receptor and sensitivity to nerve growth factor. *Journal of Biological Chemistry* 1992 **267** 17611–17616.
- 20 Johnson DG & Walker CL. Cyclins and cell cycle checkpoints. *Annual Reviews of Pharmacology and Toxicology* 1999 **39** 295–312.
- 21 Lotan R. Retinoids in cancer chemoprevention. *FASEB Journal* 1996 **10** 1031–1039.
- 22 Bertram JS. Inhibition of neoplastic transformation *in vitro* by retinoids. *Cancer Surveillance* 1983 **3** 243–262.
- 23 Pérez Pereda M, Hopfner U, Pagotto U, Renner U, Uhl E, Artz E *et al.* Retinoic acid stimulates meningioma cell adhesion to the extracellular matrix and inhibits invasion. *British Journal of Cancer* 1999 **81** 381–386.
- 24 Simon D, Koehrl J, Reiners C, Boerner AR, Schmutzler C, Mainz K *et al.* Redifferentiation therapy with retinoids: therapeutic option for advanced follicular and papillary thyroid carcinoma. *World Journal of Surgery* 1998 **22** 569–574.
- 25 Idres N, Benoit G, Flexor MA, Lanotte M & Chabot GG. Granulocytic differentiation of human NB4 promyelocytic leukemia cells induced by all-trans retinoic acid metabolites. *Cancer Research* 2001 **61** 700–705.
- 26 McGarvey TW, Silberman S & Persky B. The effect of butyric acid and retinoic acid on invasion and experimental metastasis of murine melanoma cells. *Clinical and Experimental Metastasis* 1990 **8** 433–448.
- 27 Jacob K, Wach F, Holzapfel U, Hein R, Lengyel E, Buettner R *et al.* *In vitro* modulation of human melanoma cell invasion and proliferation by all-trans-retinoic acid. *Melanoma Research* 1998 **8** 211–219.
- 28 Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Hori M & Tada M. Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. *Journal of Biological Chemistry* 1998 **273** 12725–12731.
- 29 Hossein MZ & Bertram JS. Retinoids suppress proliferation, induce cell spreading, and up-regulate connexin-43 only in post-confluent 10T1/2 cells: implications for the role of gap junctional communication. *Cell Growth and Differentiation* 1994 **5** 1253–1261.
- 30 Gianni M, Ponzanelli I, Mologni L, Reichert U, Rambaldi A, Terao M *et al.* Retinoid-dependent growth inhibition, differentiation and apoptosis in acute promyelocytic leukemia cells. Expression and activation of caspases. *Cell Death and Differentiation* 2000 **7** 447–460.
- 31 Fujimura S, Suzumiya J, Anzai K, Ohkubo K, Hata T, Yamada Y *et al.* Retinoic acids induce growth inhibition and apoptosis in adult T-cell leukemia (ATL) cell lines. *Leukemia Research* 1998 **22** 611–618.
- 32 Jetten AM, Nervi C & Vollberg TM. Control of squamous differentiation in tracheobronchial and epidermal epithelial cells: role of retinoids. *National Cancer Institute Monographs* 1992 **13** 93–100.
- 33 Chytil F. Retinoids in lung development. *FASEB Journal* 1996 **10** 986–992.
- 34 Kelloff GJ, Boone CW, Steele VK, Perloff M, Crowell J & Doody LA. Development of chemopreventive agents for lung and upper aerodigestive tract cancers. *Journal of Cellular Biochemistry* 1993 **17** 2–17.
- 35 Moon RC, Mehta RG & Rao KJ. Retinoids and cancer in experimental animals. In *The Retinoids*, pp 573–595. Eds MB Sporn, AB Roberts & DS Goodman. New York: Raven Press, 1994.
- 36 Doyle LA, Giangiulo D, Hussain A, Park HJ, Yen RW & Borges M. Differentiation of human variant small cell lung cancer cell lines to a classic morphology by retinoic acid. *Cancer Research* 1989 **49** 6745–6751.
- 37 Kalemkerian GP, Jasti RK, Celano O, Nelkin BD & Mabry M. All-trans-retinoic acid alters *myc* gene expression and inhibits *in vitro* progression in small cell lung cancer. *Cell Growth and Differentiation* 1994 **5** 55–60.
- 38 Gerads J, Chen JY, Russel EK, Yankaskas JR, Nieves L & Minna JD. Human lung cancer cell lines exhibit resistance to retinoic acid treatment. *Cell Growth and Differentiation* 1993 **4** 799–809.
- 39 Kim YH, Dohi DE, Han GR, Zou CP, Oridate N, Walsh GL *et al.* Retinoid refractoriness occurs during lung carcinogenesis despite functional retinoid receptors. *Cancer Research* 1995 **55** 5603–5610.
- 40 Jiang H, Lin J, Su ZZ, Collart FR, Huberman E & Fisher PB. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21WAF1/Cip1 expression in the absence of p53. *Oncogene* 1994 **9** 3397–3406.
- 41 Steinman RA, Hoffman B, Iro A, Guillouf C, Lieberman DA & El-Houseini ME. Induction of p21(WAF1/CIP1) during differentiation. *Oncogene* 1994 **9** 3389–3396.
- 42 Poluha W, Poluha DK, Chang B, Crosbie NE, Schonhoff CM, Kilpatrick DL *et al.* The cyclin-dependent kinase inhibitor p21WAF1 is required for survival of differentiating neuroblastoma cells. *Molecular and Cellular Biology* 1996 **16** 1335–1341.
- 43 Helige C, Smolle J, Zellnig G, Hartmann E, Fink-Puches R, Kerl H *et al.* Inhibition of K1735-M2 melanoma cell invasion *in vitro* by retinoic acid. *Clinical and Experimental Metastasis* 1993 **11** 409–418.
- 44 Cartwright M, Martin S, D'Mello S & Heinrich G. The human nerve growth factor gene: structure of promoter region and expression in L929 fibroblasts. *Molecular Brain Research* 1992 **15** 67–75.
- 45 Lancillotti F, Giandomenico V, Affabris E, Fiorucci G, Romeo G & Rossi GB. Interferon alpha-2b and retinoic acid combined treatment affects proliferation and gene expression of human cervical carcinoma cells. *Cancer Research* 1995 **55** 3158–3164.
- 46 Lingen MW, Polverini PJ & Bouck NP. Retinoic acid and interferon alpha act synergistically as antiangiogenic and antitumor agents against human head and neck squamous cell carcinoma. *Cancer Research* 1998 **58** 5551–5558.
- 47 Forman BM, Umesono K, Chen J & Evans RM. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* 1995 **81** 541–550.
- 48 Perlman T & Jansson L. A novel pathway for vitamin A signalling mediated by RXR heterodimerization with NGFI-B and Nurr1. *Genes and Development* 1995 **9** 769–782.
- 49 Lin B, Chen GQ, Xiao D, Kolluri SK, Cao X, Su H *et al.* Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition and apoptosis by retinoic acid in cancer cells. *Cancer Research* 2000 **55** 3158–3164.
- 50 Pipaon C., Tsai SY & Tsai MJ. COUP-TF upregulates NGFI-A gene expression through SP1 binding site. *Molecular and Cellular Biology* 1999 **19** 2734–2745.

---

Received 20 November 2001

Accepted 16 May 2002