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# PREVENTION OF NEOCARZINOSTATIN-INDUCED CELL DEATH AND MORPHOLOGIC CHANGE IN SK-N-SH HUMAN NEUROBLASTOMA CELLS BY CONTINUOUS EXPOSURE TO NERVE GROWTH FACTOR

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**Abstract**—Neocarzinostatin is an antineoplastic agent that induces differentiated morphology in human (SK-N-SH) neuroblastoma cells in culture. We have compared this morphological differentiation with that induced by the endogenous differentiation inducer, nerve growth factor (NGF), and have explored the effects of exposure to NGF upon the morphological changes induced by neocarzinostatin in SK-N-SH cells. Both NGF and neocarzinostatin induced process outgrowth in these cells. The processes formed in the presence of NGF however, were shorter and thinner than those induced by neocarzinostatin. Furthermore, only neocarzinostatin induced enlargement of the somata of the cells, and caused cell death in a concentration-dependent fraction of the culture. These distinguishing features of treated cells allowed us to determine whether or not NGF exposure altered responsiveness of the cells to neocarzinostatin. NGF (100–1000 ng/mL) protected SK-N-SH cells from the morphological and cytocidal effects of neocarzinostatin (1-hr exposure, 0.017 to 0.033 μg/mL). Protection from neocarzinostatin required that NGF be continuously present for a period beginning 24 hr prior to neocarzinostatin exposure and continuing for the duration of the experiment, implying that the protection afforded by NGF has a latency necessitating pretreatment, and is reversible. These results suggest that neocarzinostatin is taken up by the cells and can exert its effects once NGF is removed, even after neocarzinostatin is washed out of the medium. The signal transduction cascade triggered by NGF receptor binding may prevent the action of neocarzinostatin or the expression of the cellular changes induced in SK-N-SH cells by neocarzinostatin.

Neuroblastoma is a childhood tumor of the neural crest. It is thought by some to represent a maturational arrest of neuroblasts in this region of the peripheral nervous system [1]. Several pharmacological agents have been identified which can induce mature neuron-like morphology in neuroblastoma cells in culture [2–4]. These drugs and their ability to produce "differentiation induction" have been proposed both as therapeutic agents for neuroblastoma and for the production of an *in vitro* model for normal neural crest differentiation. As part of a program to examine the relationship between pharmacological induction of "differentiation" in neuroblastoma cells and maturation of normal neural crest cells, we have determined that one proposed differentiation-inducing agent, neocarzinostatin, produces biochemical changes which in these cells differ significantly from those seen in normal neural crest cell development [5–7]. To characterize better the neocarzinostatin-neuroblastoma system with respect to known modulators of neural crest development, and to begin to examine the mechanism of "differentiation" induction by neocarzinostatin, we have studied the effects of nerve growth factor (NGF)‖, an endogenous "differentiation inducer," upon the changes induced by neocarzinostatin in murine and human neuroblastoma cells in culture.

Neocarzinostatin is an antineoplastic natural product that induces neuronal morphology in murine C1300–NB41A3 neuroblastoma cells in culture [4]. Three days after a 1-hr exposure at 37° to neocarzinostatin, these cells extend multiple processes, enlarge by a factor of 4, and exhibit multiple prominent nucleoli. Within 2 days of this morphological change, the cells begin to die, and the culture is acellular by 14 days after treatment. Our preliminary studies on neocarzinostatin were done on NB41A3 neuroblastoma cells because of the ready availability of a model for neuroblastoma growth *in vivo* using these cells. However, since other studies have suggested that NB41A3 cells are not morphologically responsive to NGF [8], and other neuroblastoma cell lines have been shown to have various defects of NGF-related second messenger pathways [9–11], this particular cell line is not an appropriate one in which to study the interaction between NGF and neocarzinostatin. Indeed, our preliminary studies have suggested that, unlike the case for apoptosis in normal neurons, the morphological changes and programmed death of C1300 cells treated with neocarzinostatin are unaffected by the presence of NGF [8].

We recently began studies on the effects of neo-

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‖ Abbreviations: NGF, nerve growth factor; and NCS neocarzinostatin.

carzinostatin upon NGF-responsive human neuroblastoma cell lines in culture. We found that cells of the SK-N-SH line exhibit one of two responses to neocarzinostatin. A subset of the cells in any given culture dies within 6 hr of a 1-hr exposure to the drug. The remaining cells appear unchanged until 3–4 days after treatment, when they elongate and extend two thick processes from the cell body. These process-bearing cells do not increase in number after this time. However, unlike the morphologically-altered C1300 cells, they can be maintained in tissue culture for at least 2 weeks in standard serum-containing medium [12]. Because of the known NGF-responsiveness of SK-N-SH cells [13, 14] and the relationship between NGF and programmed cellular events, including neurite extension and apoptosis [15], we have examined the effects of NGF upon the response of SK-N-SH cells to neocarzinostatin.

## MATERIALS AND METHODS

*Chemicals.* Neocarzinostatin was a gift from Dr. William T. Bradner (Bristol Myers-Squibb, Wallingford, CT). It was obtained as a sterile solution (0.5 mg/mL) in 0.015 M sodium acetate buffer, pH 5. NGF was obtained from Boehringer Mannheim (Indianapolis, IN) and stored at −20° in 100-μL aliquots at a concentration of 100 μg/mL in F-10 Nutrient solution (GIBCO, Grand Island, NY).

*Cell culture.* C1300 murine neuroblastoma cells of the subline NB41A3 and SK-N-SH human neuroblastoma cells (passage number 34) were obtained from the American Type Culture Collection (Rockville, MD). NB41A3 cells were maintained as adherent monolayers in 75-$cm^2$ tissue culture flasks (GIBCO) at 37°, and were fed twice weekly with Ham's medium containing 15% horse serum and 5% fetal bovine serum. SK-N-SH cells were maintained as adherent monolayers in 75-$cm^2$ tissue culture flasks (GIBCO) at 37° and 5% $CO_2$, and were fed twice weekly with Minimal Essential Medium containing 10% fetal bovine serum. All media components were obtained from GIBCO. All studies described herein were performed with SK-N-SH cells between passage numbers 35 and 42.

*Treatment of cell cultures with neocarzinostatin and/or NGF.* For treatment studies of cultured cells, cells were plated on 6-well tissue culture plates (GIBCO; approximately $10^5$ cells/well) in complete medium and allowed to incubate at 37° overnight. For studies involving treatment with neocarzinostatin, the drug was diluted to the appropriate concentration in 3 mL of fresh medium overlying the cells. Cultures were incubated at 37° and 5% $CO_2$ for 1 hr. Subsequently, the medium was removed from each well, and cells were washed twice with complete medium (1 mL/wash). Complete medium was then replaced, and the cells were incubated for the duration of the experiment. For studies involving treatment with NGF, the treatment time was divided into three periods: a period beginning 24 hr prior to neocarzinostatin treatment and ending just before the addition of neocarzinostatin; a 1-hr period cotemporal with neocarzinostatin treatment; and a period beginning with the washes after neocarzinostatin treatment and continuing for the duration of the experiment. In the experiments described herein, NGF was given during none, any one, any combination of two, or all three of these periods. All cells were given fresh medium twice weekly, and in cases where prolonged NGF exposure was indicated, the fresh medium contained the appropriate concentration of NGF.

All experiments included control wells for the addition of each agent alone for an identical time period as that indicated for the co-treatment wells, and a set of "positive internal control" wells where NGF exposure was continuous throughout all three periods described above.

*Cell culture growth rates.* Cell culture growth rates were determined as we have described previously [4, 12]. Briefly, cell counts were performed daily by counting the number of cells per high power field. Three fields were counted per well, and the average cell count was determined. The variation in count from field to field was less than 10% in most cases, and never greater than 30%. Average cell counts for each well on each day were normalized to the average cell count for that well on day 0.

*Light microscopy and photomicrography.* Cultures were examined daily by phase contrast inverted light microscopy using a Zeiss Axiomat 35 microscope. Photomicrography was performed using Tmax film (ASA 100; Eastman Kodak, Rochester, NY).

## RESULTS

*Effects of neocarzinostatin alone and in combination with NGF upon cell death in culture.* Neocarzinostatin exposure (1 hr, 37°) resulted in a reduction in SK-N-SH cell number within 24 hr after treatment. At its nadir, the cell count in 14 wells treated with 0.017 μg/mL neocarzinostatin was 23 ± 4% of the day 0 count. For five experiments in which cells were treated with 0.033 μg/mL neocarzinostatin, it was 15 ± 3% of the day 0 count. These data suggest that there was a concentration dependence of the fraction of the cell culture which died soon after neocarzinostatin treatment, although the concentration range used in these studies was insufficient to make that assertion. These cells did not undergo neuron-like morphological change prior to their death. Instead they went from being stellate and adherent to plastic to being round, refractile, and non-adherent.

SK-N-SH cells are known to express the high-affinity NGF receptor [10] and to respond to NGF with neurite extension under the culture conditions we have used (see below). Because of the role of NGF in preventing apoptotic cell death in NGF-responsive neurons subjected to apoptosis-inducing conditions [15], we next examined the effects of NGF exposure upon the induction of cell death by neocarzinostatin. SK-N-SH cells were exposed continuously to NGF (100 or 1000 ng/mL) from 24 hr before neocarzinostatin treatment (0.017 or 0.033 μg/mL, 1 hr, 37°) through the termination of the experiment. As shown in Fig. 1A, maintenance of NGF in the medium attenuated the decrease in cell number in the initial days after neocarzinostatin treatment, and resulted in increased culture recovery in the ensuing week. The magnitude of this effect

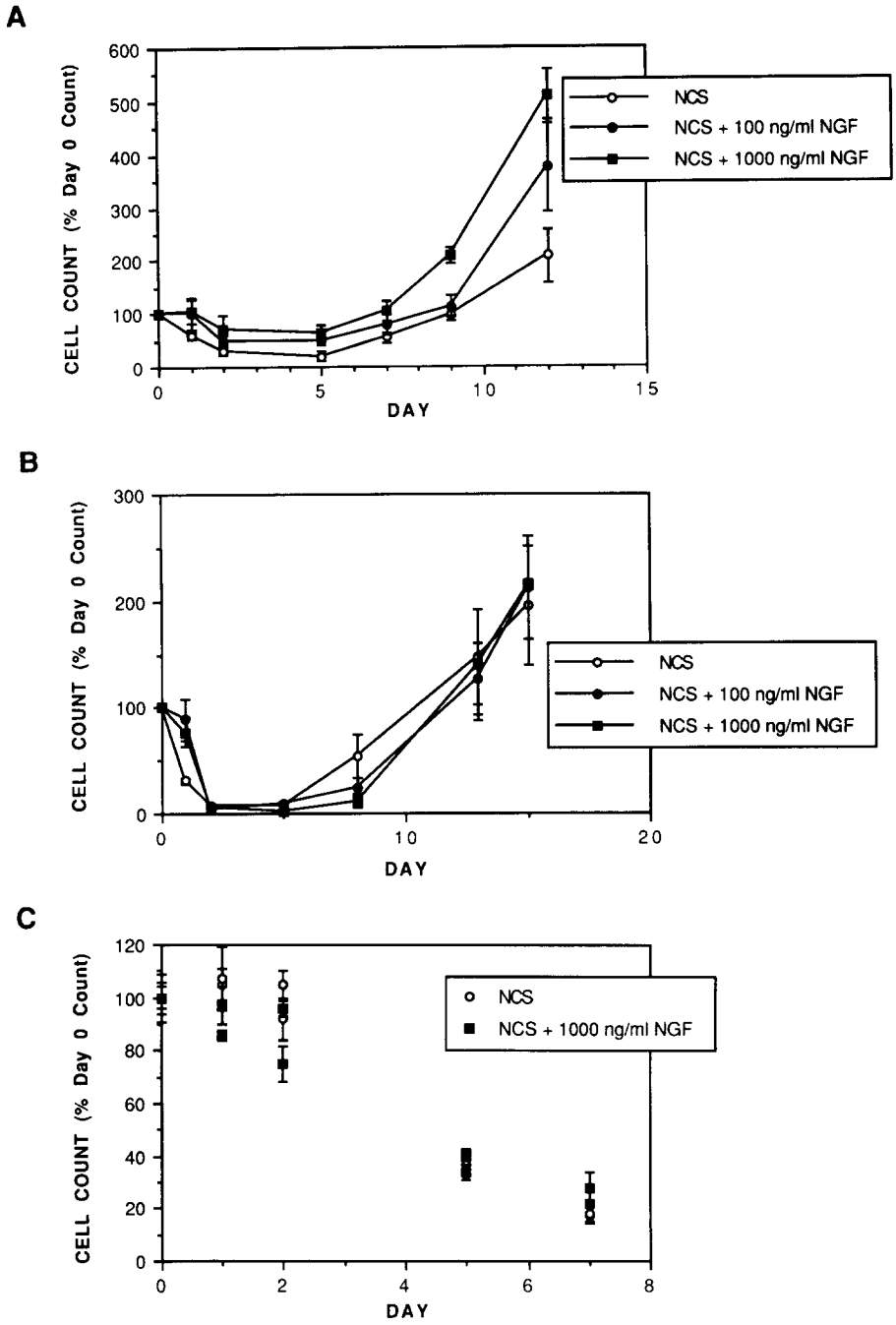



Fig. 1. Effects of NGF upon the decrement in neuroblastoma cell culture growth produced by neocarzinostatin (NCS) treatment. Key: (○) neocarzinostatin treatment (1 hr, 37°) alone; (●) neocarzinostatin treatment plus NGF (100 ng/mL) exposure for the specified time period(s); (■) neocarzinostatin treatment plus NGF (1000 ng/mL) exposure for the specified time period(s). The results shown at each point represent the mean cell count for three high power fields (N = 3). Error bars represent the standard error of the mean (SEM). (A) Sister cultures of SK-N-SH human neuroblastoma cells were treated with neocarzinostatin (0.017 μg/mL) on day 0 and (closed symbols) exposed to NGF on days −1 through 12. The results are those of one representative experiment out of nine performed. The cell count on day 12 for neocarzinostatin + 1000 ng/mL NGF was significantly higher than that for neocarzinostatin alone ($P < 0.005$, one-tailed Student's $t$-test). (B) Sister cultures of SK-N-SH human neuroblastoma cells were treated with neocarzinostatin (0.017 μg/mL) on day 0 and (closed symbols) exposed to NGF only during the 1 hr of neocarzinostatin treatment. The results are those of one representative experiment out of two performed. (C) Sister cultures of C1300 cells were treated with neocarzinostatin (0.4 μg/mL; produces equivalent percent of cells in culture with "differentiated" morphology to that produced by 0.017 μg/mL in SK-N-SH cells) on day 0, and (closed squares) 1000 ng/mL NGF on days −1 through 7. The results of two such experiments are shown.

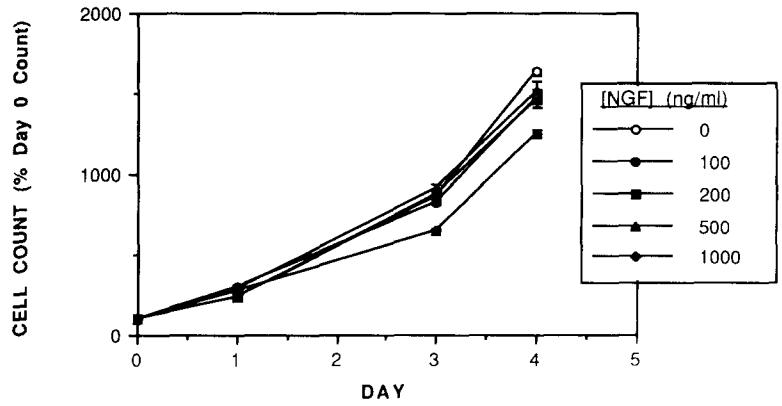



Fig. 2. Effects of continuous exposure to NGF upon SK-N-SH human neuroblastoma cell culture growth. Sister cultures were exposed to NGF from the day of initial plating (day −1) through day 4. The results shown at each point are the mean cell count for three high power fields (N = 3). The error bars represent the SEM.

was dependent upon the concentration of NGF. Continuous treatment with NGF alone (100, 200, 500 or 1000 ng/mL) resulted in cell culture growth that did not differ significantly from the growth seen in untreated cultures (Fig. 2).

This effect was not the result of a direct chemical interaction between NGF and neocarzinostatin. Maintenance of NGF in the culture medium only during the 1-hr treatment with neocarzinostatin had no effect upon cell culture survival or subsequent growth (Fig. 1B). Furthermore, exposure to NGF before, during, and after neocarzinostatin treatment had no effect upon neocarzinostatin-induced cell death in the murine NB41A3 cell line (Fig. 1C), which, unlike SK-N-SH cells, is morphologically unresponsive to NGF alone [8]. This implies that it is the NGF-neuroblastoma cell interaction that governs modulation of the cellular response to neocarzinostatin exposure, rather than a direct interaction between neocarzinostatin and NGF.

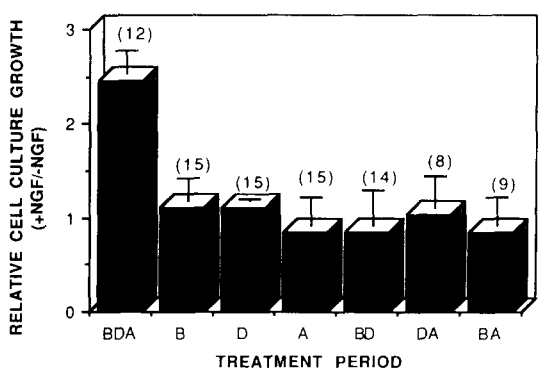



Fig. 3. Effects of various periods of exposure to NGF upon SK-N-SH human neuroblastoma cell culture growth after treatment with neocarzinostatin. Neocarzinostatin treatment (0.017 μg/mL; 1 hr; 37°) was performed on day 0. NGF (1000 ng/mL) exposure was maintained for 24 hr before (B), during (D), and/or for the duration of the experiment after (A) neocarzinostatin treatment. The relative cell culture growth is defined as the cell count (% day 0 count) in the presence of NGF divided by the cell count for a sister culture maintained in the absence of NGF. The relative cell culture growth shown was determined on the final day of the experiment, indicated by the number in parentheses for each study. In each case a representative experiment (three determinations) is shown. The number of experiments performed under each of the conditions was: BDA- 9, B- 3, D- 2, A- 7, BD- 2, DA- 2, BA- 2. Error bars represent the SEM. The relative culture growth for NGF treatment BDA was significantly larger than that for each of the other treatment paradigms ($P < 0.01$, one-tailed Student's $t$-test).

We have subsequently attempted to define the "critical period" of NGF exposure which results in attenuation of the response to neocarzinostatin treatment in SK-N-SH cells. As shown in Fig. 3, NGF must be present before, during, and after neocarzinostatin exposure to be effective in this regard. Omission of NGF at one or more of these stages resulted in a neocarzinostatin effect upon cell culture survival and growth that did not differ significantly from the effect obtained with neocarzinostatin alone. This implies that the effects of NGF which modulate the response of the cell to neocarzinostatin are reversible, and must begin prior to neocarzinostatin exposure.

*Effects of neocarzinostatin alone and in combination with NGF upon the morphology of surviving cells.* We have demonstrated previously that neocarzinostatin produces profound changes in the morphology of murine NB41A3 neuroblastoma cells [4]. We therefore studied the effects of neocarzinostatin upon the morphology of human neuroblastoma cells in culture.

Four days after a 1-hr exposure to neocarzinostatin (0.017 or 0.033 μg/mL), SK-N-SH cells that survived the initial 24 hr after treatment adopted a new morphology. As shown in Fig. 4B, the somata elongated by a factor of between 5 and 7, and the cells extended long, thick processes. This morphological change was stable for the lifetime of the cell.

While continuous exposure to NGF (100 or 1000 ng/mL) alone also produced process outgrowth, the processes so produced were thinner, shorter, and more heavily branched than those induced by neocarzinostatin. Furthermore, the somata of cells treated with NGF were slightly smaller

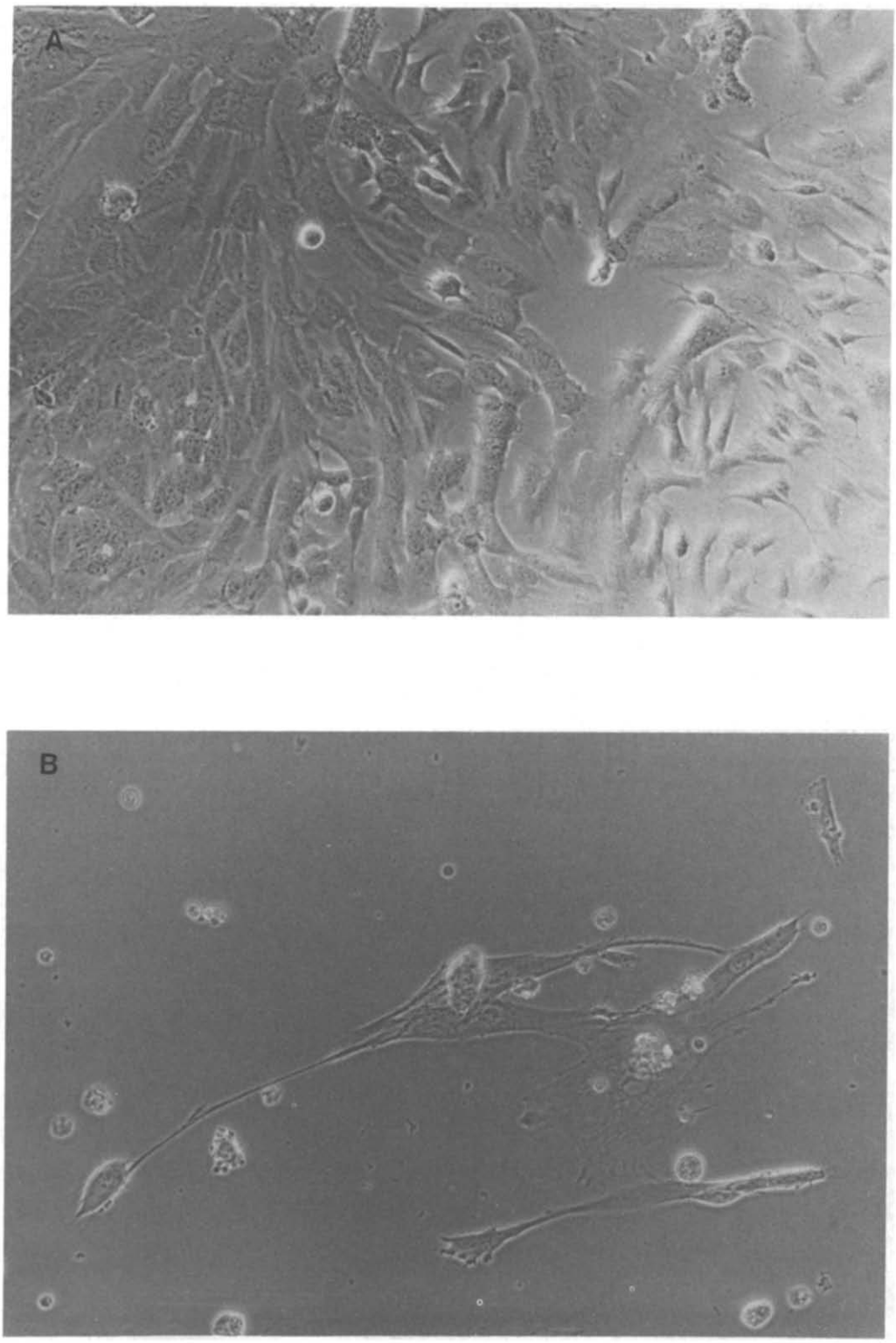
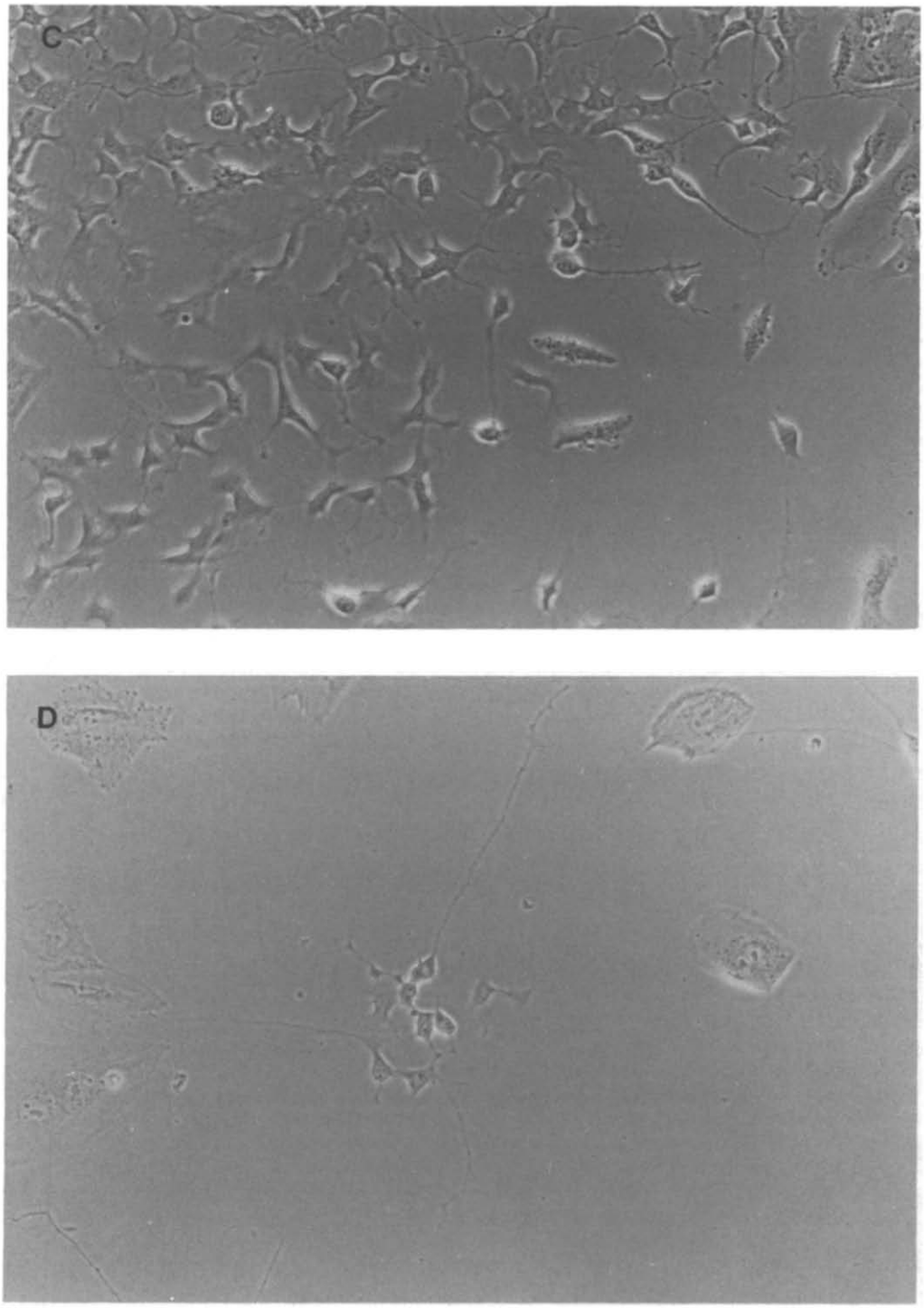



Fig. 4. Photomicrographs of sister cultures of SK-N-SH cells (A) under control conditions, or treated with (B) 0.017 μg/mL neocarzinostatin on day 0, (C) 1000 ng/mL NGF on days −1 through the end of experiment, or (D) 0.017 μg/mL neocarzinostatin on day 0 plus 1000 ng/mL NGF on days −1 through end of experiment. In all cases, the magnification was 200× and cells were photographed on day 5. In panel (A) both the epithelioid (left side) and neuronoid (right side) morphologies of SK-N-SH cells are illustrated. These two morphological subtypes arise spontaneously in cultures of SK-N-SH cells [16].




and rounder than those of untreated cells (see Fig. 4C).

Continuous exposure to NGF (100 or 1000 ng/mL) for 24 hr before neocarzinostatin treatment, during neocarzinostatin treatment, and after neocarzinostatin treatment largely prevented the morphological changes induced by neocarzinostatin (Fig. 4D). Cells that had undergone NGF-induced process outgrowth (small cells with slender processes) did not exhibit somatic enlargement or a change in process morphology in response to neocarzinostatin exposure, although some of these cells did develop longer processes of the slender type than were observed with NGF treatment alone. The occasional

cell that did not extend processes in response to NGF and exhibited somatic enlargement in response to neocarzinostatin did not extend processes after neocarzinostatin exposure, implying that NGF may have altered the responsiveness of even these cells to neocarzinostatin. Furthermore, these cells tended to adopt an epitheloid morphology, rather than the elongated morphology of cells treated with neocarzinostatin alone. This phenomenon, shown in Fig. 4A, has been observed to occur spontaneously in SK-N-SH cells [16], further suggesting that, like their small rounded counterparts, these cells, too, are unresponsive to neocarzinostatin treatment.

As was the case for cell growth, attenuation of the morphological response of SK-N-SH cells to neocarzinostatin required continuous exposure to NGF. Omission of NGF from the culture medium during any of the three exposure periods resulted in morphological changes that resembled those seen with neocarzinostatin alone (data not shown).

## DISCUSSION

Neocarzinostatin is a DNA strand-breaking agent whose chemical mechanism is well characterized [17]. We have shown that NGF protects SK-N-SH cells from the effects of neocarzinostatin. That this is true interference with the actions of neocarzinostatin, and not simply the result of an NGF-produced increase in the mitotic rate of subpopulation of cells unaffected by neocarzinostatin, is shown by the lack of effect of NGF alone on the rate of cell culture growth. The apparent protection could indicate that NGF-induced signal transduction or the cellular events that follow it preclude entry of neocarzinostatin into the cell or nucleus, prevent the cleavage of DNA by neocarzinostatin, or block the expression of morphological and physiological characteristics related to neocarzinostatin treatment. Protection from neocarzinostatin required that NGF be present before, during, and after neocarzinostatin treatment, implying that the protection afforded by NGF has a latency necessitating pretreatment, and is reversible. Neocarzinostatin is most likely taken up by the cells and can exert its effects once NGF is removed, even after neocarzinostatin is washed out of the medium.

A multitude of pharmacological agents have been shown to give rise to morphological differentiation of neuroblastoma cells in culture [2–4]. There is, however, mounting evidence that the biochemical effects of these drugs upon neuroblastoma cells are not identical [18, 19]. In addition, it is clear that morphological criteria, such as neurite outgrowth, are not sufficient to define a cell as "differentiated," or to equate the actions of two neurite outgrowth-eliciting drugs [20]. This raises the question of the relationship and resemblance of the effects of so-called "differentiation-inducing" drugs to those produced by known endogenous mediators of neural crest cell maturation.

The most well-characterized endogenous mediator of neural crest cell maturation is NGF. In recent years, the pathway from cellular binding to NGF to the induction of neurite outgrowth and biochemical maturation has begun to be worked out. The binding of NGF to the high-affinity NGF receptor appears to give rise to the phosphorylation of that receptor. Present evidence supports the notion that the high-affinity NGF receptor is the same as the protein $p140^{prototrk}$, which, upon phosphorylation, becomes $p140^{trk}$, setting in motion a cellular signalling cascade [21–23]. Phosphorylation of $p140^{trk}$ gives rise to sequential phosphorylation of $pp60^{c\text{-}src}$ followed by $p21^{c\text{-}ras}$ [24]. Furthermore, there is evidence that activation of the high-affinity NGF receptor produces tyrosine phosphorylation of phospholipase C-γl, leading to hydrolysis of inositol lipids [25].

Conflicting reports have emerged regarding the effects of NGF upon the morphology of SK-N-SH cells [11, 26]. These differences are almost certainly related to the substratum on which the cells are plated [26] and the passage number in which the cells are used [12]. All of the studies described herein were performed on cells used in passage numbers 35–42, and plated on tissue culture plastic without an added substratum.

The present studies indicate that, although both NGF and neocarzinostatin elicit process outgrowth in SK-N-SH human neuroblastoma cells, the morphology of the processes produced differs depending upon which agent is used. The processes produced in response to NGF were short and slender; those produced in response to neocarzinostatin were longer and thicker. Furthermore, neocarzinostatin produced a change in the morphology of the soma, cessation of cell culture growth, and, in some cases, cell death. None of these phenomena were observed with NGF treatment. The morphology induced by neocarzinostatin resembles that of Schwann cells, known neural crest derivatives. However, definitively determining whether these neocarzinostatin-altered cells most resemble Schwann cells or neurons will require immunohistochemically probing them for specific markers of each lineage [27].

From a therapeutic standpoint, endogenous NGF may protect normal neural crest elements from the deleterious effects of neocarzinostatin, while neural crest tumors lacking NGF receptors or functional NGF-mediated signalling pathways [9] may still be susceptible to its antineoplastic activity. In addition, metastatic neural crest tumor cells in sites remote from normal neural crest may not be subjected to as high a local NGF concentration as are those in or near sympathetic chain or adrenal gland. This may afford a measure of selectivity in the treatment of metastatic neural crest tumors. Further studies are needed to define the relationship of these local NGF concentrations to those used in our *in vitro* system.

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