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Patricia Will · Kathleen A. Guger · Nina Felice Schor

Effects of neocarzinostatin upon the development of tumors from murine neuroblastoma cells

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Abstract The use of differentiation-inducing agents has been proposed for the purging of bone marrow and for the treatment of minimal residual disease prior to autologous bone marrow transplantation in patients with metastatic neuroblastoma. The present studies examine the effects of the enediyne differentiation inducer neocarzinostatin (NCS) on tumor development from subcutaneous implants of murine (Neuro-2A) neuroblastoma cells. Prior in vitro treatment with NCS results in a concentration- and drug exposure time-dependent decrease in the incidence of tumors from subcutaneously implanted cells. In vivo treatment results in a dose-dependent decrease in the rate of tumor growth. These results imply that enediynes such as NCS may be useful in ex vivo purging regimens and in in vivo treatment of microscopic residual disease in patients with neuroblastoma.

Key words Neuroblastoma · Differentiation · Enediyne

Introduction

Neuroblastoma is the single most common solid tumor of childhood. It arises from primitive cells of the sympathetic nervous system. Disseminated neuroblastoma is particularly resistant to conventional therapy [1]. Novel means of approaching disseminated neuroblastoma have included the use of differentiation-inducing agents, that render the tumor cells nonproliferative and morphologically similar to mature sympathetic neurons. This approach to therapy has particularly been proposed for the purging of bone marrow ex vivo and for the treatment of minimal residual disease in vivo prior to autologous bone marrow transplantation [14]. Proposal of drugs as purging agents is contingent upon the demonstration of their efficacy at levels that produce tolerable toxicity both in vitro and in vivo. The present studies examine the in vitro concentration- and drug exposure time-response characteristics of the enediyne differentiation inducer neocarzinostatin (NCS) for murine C1300 (Neuro-2A) neuroblastoma. In addition, they explore the in vivo dose-response characteristics of NCS in the A/J mouse-subcutaneous C1300 neuroblastoma model.

NCS is an antimitotic natural product that consists of an active enediyne chromophore noncovalently linked to a protein of 10 kDa molecular weight. NCS induces both single- and double-stranded breaks in cellular DNA, particularly in A-T-rich regions [4]. Although early clinical studies have demonstrated toxicity of NCS in the therapeutic range, more recent reports have suggested that this toxicity may be obviated by coupling the protein to inert polymers [6], by using NCS adjunctively with 6-mercaptodopamine [12], or by modifying the NCS chromophore structure to produce other less toxic enediynes [7]. We have previously shown that NCS is a differentiation-inducing agent for C1300 neuroblastoma cells in culture and that these "differentiated" cells cease to divide and go on to die within 2 weeks of treatment despite replenishment of the medium [11]. Unlike the case for other differentiation-inducing agents, a single 1-h exposure to NCS is sufficient to commit neuroblastoma cells to cease dividing and undergo morphological change at 2–4 days after treatment. However, the behavior of NCS-treated cells in vivo is not known. We therefore studied the effects of in vitro and in vivo treatment with NCS upon C1300 cell tumorigenicity in an A/J mouse-subcutaneous C1300 model. Implantation of cells treated with NCS in vitro mimics the situation for ex vivo purging regimens, whereas in vivo treatment the day after implantation of native cells mimics the situation for minimal residual disease.

P. Will · K. A. Guger · N. F. Schor
Departments of Pediatrics, Neurology, and Pharmacology, University of Pittsburgh, Pittsburgh, PA 15213, USA

N. F. Schor (✉)
Division of Child Neurology, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213, USA

Materials and methods

Chemicals

NCS was obtained as a sterile solution (0.5 mg/ml) in 0.015 *M* sodium acetate buffer (pH 5.0) from Dr. William Bradner (Bristol Myers-Squibb, Wallingford, Conn.). Dilutions of the drug into medium were made up fresh immediately preceding each experiment.

Animals

Male A/J mice (5–7 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, Me.). Mice were housed five per cage and were given free access to food and water throughout the experiments.

Cell culture

Neuro-2A murine neuroblastoma cells were obtained from the American Type Culture Collection (Bethesda, Md.). Stock cultures were maintained as adherent cultures in plastic tissue-culture flasks and fed with minimal essential medium (MEM) supplemented with 10% fetal calf serum (Gibco, Grand Island, N.Y.). Cultures were incubated at 37 °C in an atmosphere containing 5% CO_2. For each experiment, cells were harvested from stock cultures with trypsin, subcultured into tissue-culture flasks at half-confluence (0.5×10^7 cells/75-cm^2 flask) in complete medium, and permitted to incubate overnight before drug treatment.

NCS treatment of Neuro-2A cells in culture

Previous studies from our laboratory have examined the effects of NCS upon cells of the C1300-derived neuroblastoma line NB41A3 [11]. For the present studies, we used Neuro-2A cells rather than NB41A3 cells because of the greater propensity of cells of the former line to form subcutaneous tumors (Nylander and Schor, unpublished observations). Neuro-2A cells in culture undergo morphological changes after NCS treatment identical to those seen with NB41A3 cells. However, we have presented evidence in preliminary form that in Neuro-2A cells, the concentration of NCS required to produce maximal morphological change and ultimate apoptosis is 0.05 μg/ml (i.e., approximately 1 order of magnitude less than that required for NB41A3 cells [5]). These findings are detailed in Results (see below).

NCS was added to the medium bathing Neuro-2A cells as a stock solution (5 μg/ml) in complete medium. For dose-response studies, treatment consisted of exposure to final concentrations of NCS of 0, 0.02, 0.03, 0.04, and 0.05 μg/ml, respectively, for 1 h at 37 °C. For exposure time-response studies, treatment consisted of exposure to a final concentration of NCS of 0.025 μg/ml for 0, 15, 30, and 60 min, respectively, at 37 °C. After incubation, cells for injection were washed twice with serum-free MEM, harvested with trypsin, and taken up into a volume of phosphate-buffered saline (PBS) such that the final cell concentration was $1-2\times10^6$ cells/ml.

Establishment of subcutaneous tumors in A/J mice

At 2 days (48–58 h) after treatment with NCS or incubation under control conditions, Neuro-2A cells were used for the establishment of subcutaneous tumors in A/J mice. Treatment was performed on adherent cells, and 2 days later, the cells were removed from the surface of tissue-culture flasks using trypsin and then suspended in PBS. Cells were counted by hemacytometry, and the appropriate number of cells (as determined by preliminary studies to characterize this model; see Results) were injected subcutaneously into the right flank of each mouse.

In vivo treatment of A/J mice with NCS

At 24 h following the subcutaneous implantation of Neuro-2A cells, A/J mice were treated with NCS by intraperitoneal injection. In two separate experiments, mice received 0, 1, 2, 3, 4, or 5 mg/kg NCS as a single dose or 0.5 mg/kg NCS daily for 0, 1, 2, or 3 days. Systemic toxicity was estimated by computing the activity score for each group as previously described elsewhere [10]. Briefly, the activity level of each mouse at each time point was graded as follows: 3, normal activity; 2, ambulatory but lethargic; 1, nonambulatory but alive; and 0, dead. Statistical analysis of the values obtained for each treatment group was performed by comparison of such data using the Mann-Whitney *U*-test for nonparametric data [15].

Determination of tumor incidence, growth rate, and final weight

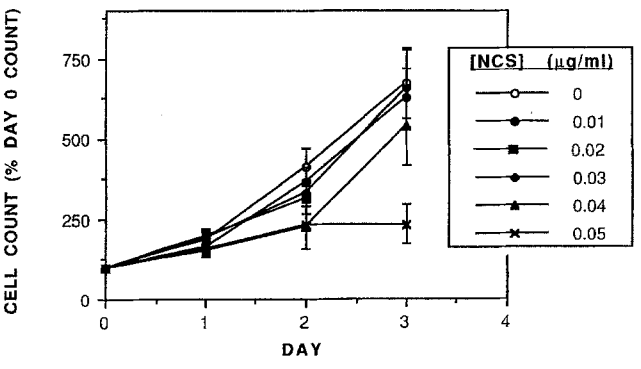
Tumor volume was estimated daily by external measurement and calculation of the product of the largest diameter and the square of the smallest diameter [3]. Volume versus time was plotted on a log scale, and a "best fit" line was determined for each growth curve. The slopes of curves obtained from control and treated tumors, respectively, were determined by linear regression analysis and compared statistically by *f*-test, with $P<0.05$ being accepted as significant [16].

Tumor-bearing mice were euthanized on day 30 after injection. Tumors were excised and their wet weights, determined. Mean tumor weights from control and treated tumors, respectively, were compared statistically using Student's *t*-test, with $P<0.05$ being accepted as significant. The relationships between tumor incidence and drug dose, drug concentration, drug dosing frequency, and drug exposure time, respectively, were compared with the expected relationship for the logistic regression model [8]. For computation of the dosing regimen that reduced tumor incidence by 50% (ED_{50}) for use in this test, the fraction of mice in the simultaneously injected control group that remained tumor-free was multiplied by the total number of mice in each experimental group, and this number was subtracted from the actual number of tumor-free mice in each respective experimental group.

Tumor histology

Where indicated in Results (see below), excised tumors were immersed in 10% formalin for 24 h, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were compared qualitatively and scored for percentage of differentiation as previously described elsewhere [11,12] by an observer blinded to the treatment history of the cells of origin of the tumor.

Fig. 1 NCS concentration dependence of suppression of Neuro-2A cell culture growth. Cells were treated with NCS for 1 h at 37 °C and the number of cells per high-power field was determined daily as previously described [11]. Each point on each day represents the mean value for three high power-fields, and the error bars signify the standard error of the meanl



Results

Characterization of the A/J mouse-Neuro-2A tumor model

Cells of the Neuro-2A subclone of the C1300 murine neuroblastoma line were treated in vitro (1 h, 37 °C) with various concentrations of NCS. Fig. 1 shows that, as is the case for another C1300-derived subclone, NB41A3 [11], there is a concentration-dependent decrease in the growth rate of cell cultures treated with NCS. Furthermore, the qualitative effect of NCS upon cell morphology is the same for Neuro-2A cells as that reported for NB41A3 cells (data not shown). However, as is shown in the Fig. 1, the concentrations required for growth suppression of a given magnitude are approximately 1 order of magnitude lower for Neuro-2A cells than for NB41A3 cells.

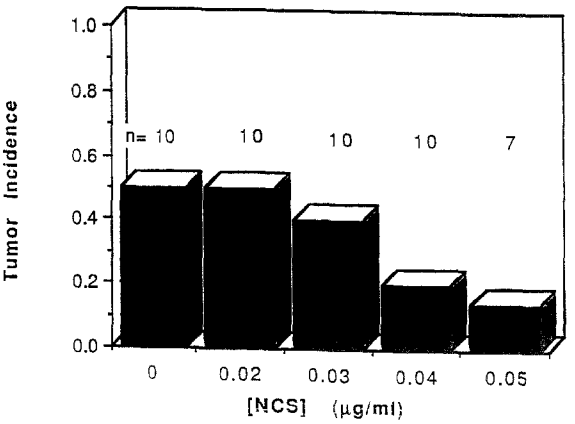


Fig. 2 NCS concentration dependence of tumor incidence with in vitro-treated Neuro 2A cells. Neuro-2A cells were pretreated with NCS at 0, 0.02, 0.03, 0.04, or 0.05 µg/ml for 1 h at 37 °C; 2 days later (day 1), each A/J mouse received a subcutaneous injection of 10^6 viable cells into the right flank. Tumor incidence was determined by necropsy on day 30. The relationship between the NCS concentration and the tumor incidence is consistent with the logistic regression mode

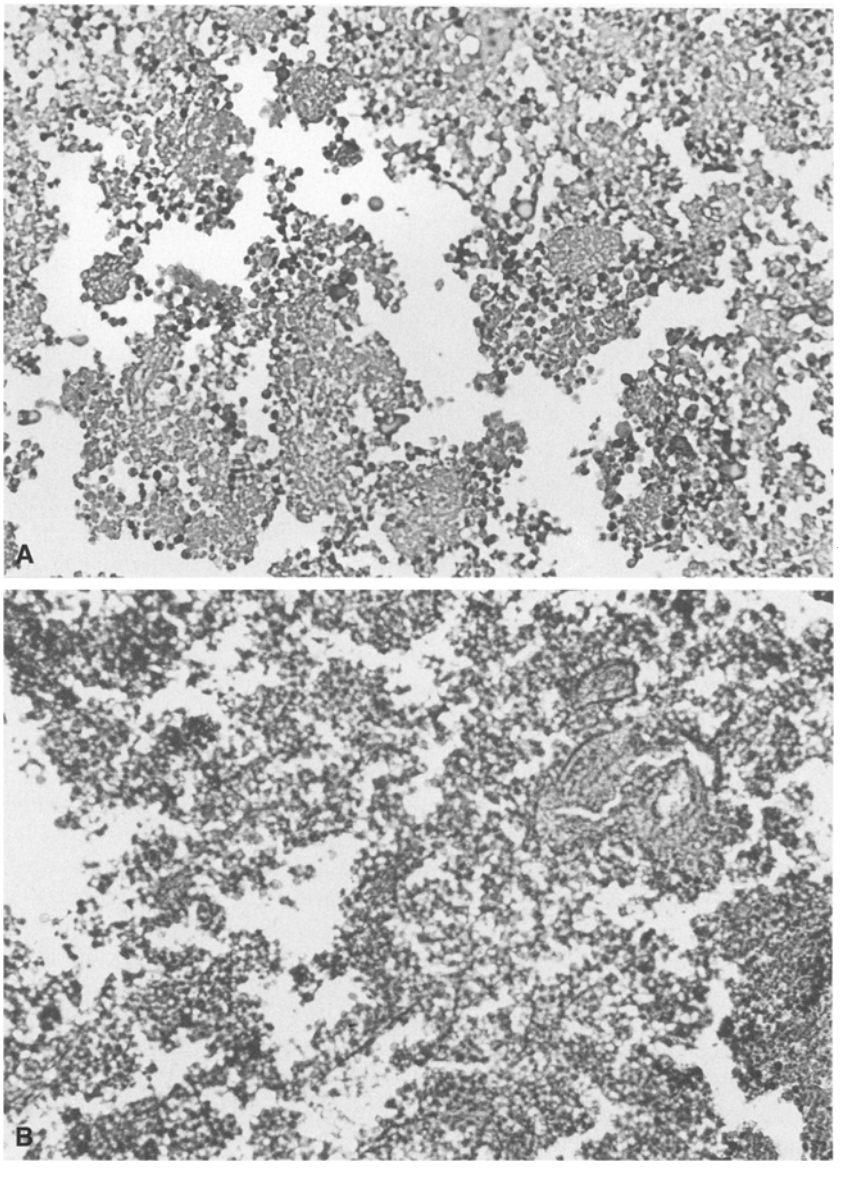


Fig. 3 A, B Representative histological sections through subcutaneous tumors that developed from **A** native Neuro-2A cells and **B** Neuro-2A cells that had been treated with NCS at 0.025 µg/ml 2 days prior to subcutaneous implantation. Sections were paraffin-imbedded and stained with hematoxylin and eosin. Tumors that developed from cells in NCS-exposed cultures were histologically indistinguishable from those that arose from native cells

An initial set of studies was performed to determine the optimal trypsin exposure time for cell harvesting and the optimal cell inoculum for the performance of studies of NCS effects upon tumor characteristics. Incubation of adherent cells with trypsin for between 3 and 5 min gave optimal viable cell recovery, with >95% trypan blue exclusion and a 50%–80% tumor "take" rate being observed with the subcutaneous injection of 10^6 Neuro-2A cells. Incubation times of less than 3 min resulted in fewer than 50% of the cells being detached from the plastic flask surface. Incubation times of greater than 5 min resulted in clumping of cells, making suspension for injection difficult. All subsequent studies were performed with cells that were harvested with a 4-min trypsin exposure time.

The relationship between the number of Neuro-2A cells injected and the incidence of tumors at 30 days was determined for A/J mice. Injection of 10^5, 10^6, or 10^7 cells/mouse gave rise to tumors in 80% (4/5) of the mice injected by day 30. Injection of 10^4 cells/mouse gave rise to tumors in 20% (1/5) of the mice injected in the same time frame. For all subsequent studies, each mouse was injected with 10^6 cells so as to avoid the inflection point of the cell dose-tumor incidence curve and such that the initial number of cells injected would not be limiting in terms of tumor development.

Concentration dependence of the effects of NCS upon Neuro-2A tumors

To determine the effects of varying the concentration of NCS to which Neuro-2A cells are exposed upon the tumors that develop after subcutaneous implantation of these cells, Neuro-2A cells were treated with NCS at 0, 0.02, 0.03, 0.04, or 0.05 μg/ml for 1 h at 37 °C. In two separate experiments, mice injected with control cells (no NCS) developed tumors by day 30 in 50%–80% of cases. In both experiments, there was an inverse correlation between the NCS concentration and the incidence of tumors at 30 days. This relationship was consistent with the statistical model posed by the logistic regression test. As the pooled data shown in Fig. 2 illustrate, mice injected with cells treated with 0.02, 0.03, 0.04, and 0.05 μg/ml developed tumors in 5 of 10, 4 of 10, 2 of 10, and 1 of 7 cases, respectively.

Increasing the in vitro concentration of NCS had no significant effect upon the tumor growth rate, final tumor wet weight, or tumor histology. As is illustrated in Fig. 3, tumors that developed from treated cells did not demonstrate "differentiated" neuron-like as morphology as previously defined [11] and were indistinguishable from those that developed from control cells. Sections from tumors that developed in the mice were remarkable for monotonous sheets of small round cells with scant cytoplasm and for occasional areas of necrosis and hemorrhage. In no case were cell processes, a prominent feature of NCS-treated Neuro-2A cells in culture, seen, most likely implying that those cells that underwent NCS-induced differentiation were rendered nontumorigenic.

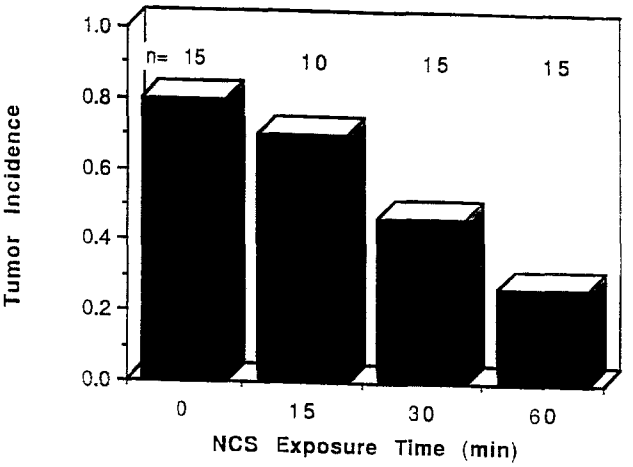


Fig. 4 NCS exposure time dependence of tumor incidence with in vitro-treated Neuro 2A cells. Neuro-2A cells were pretreated with NCS at 0.025 μg/ml for 0, 0.25, 0.5, or 1 h at 37 °C; 2 days later (day 1), each A/J mouse received a subcutaneous injection of 10^6 viable cells into the right flank. Tumor incidence was determined by necropsy on day 30. The relationship between the NCS exposure time and the tumor incidence is consistent with the logistic regression model

Exposure time dependence of the effects of NCS upon Neuro-2A tumors

We also determined the effect of varying the length of time during which Neuro-2A cells are exposed to NCS upon tumor incidence, growth, and histology. In three separate experiments, treatment of Neuro-2A cells with NCS at 0.025 μg/ml for 0, 15, 30, and 60 min, respectively, resulted in an inverse correlation between drug exposure time and tumor incidence (Fig. 4). The relationship between tumor incidence and drug exposure time was consistent with the statistical model put forth in the logistic regression test. Once again, NCS treatment of the cells had no effect upon the rate of increase in estimated tumor volume, final tumor wet weight, or tumor histology. Sheets of small round cells dominated sections of these tumors as well.

Treatment of subcutaneous microscopic neuroblastoma with NCS

Because microscopic residual disease is a major cause of treatment failure in neuroblastoma patients who undergo bone marrow transplantation, it becomes relevant to study the ability of NCS to prevent and/or reduce the magnitude of tumors arising from microscopic niduses of neoplastic cells. We tested the effects of intraperitoneal administration of NCS beginning on day 2 upon the incidence and growth of tumors arising from Neuro-2A cells injected into the subcutaneous tissue on day 1.

As is shown in Fig. 5A, when mice were treated with NCS at a constant daily dose of 0.5 mg/kg, there was a treatment duration-dependent decrease in the mass of tumors that developed from the implanted cells. The treatment was nontoxic (activity score of 3 throughout)

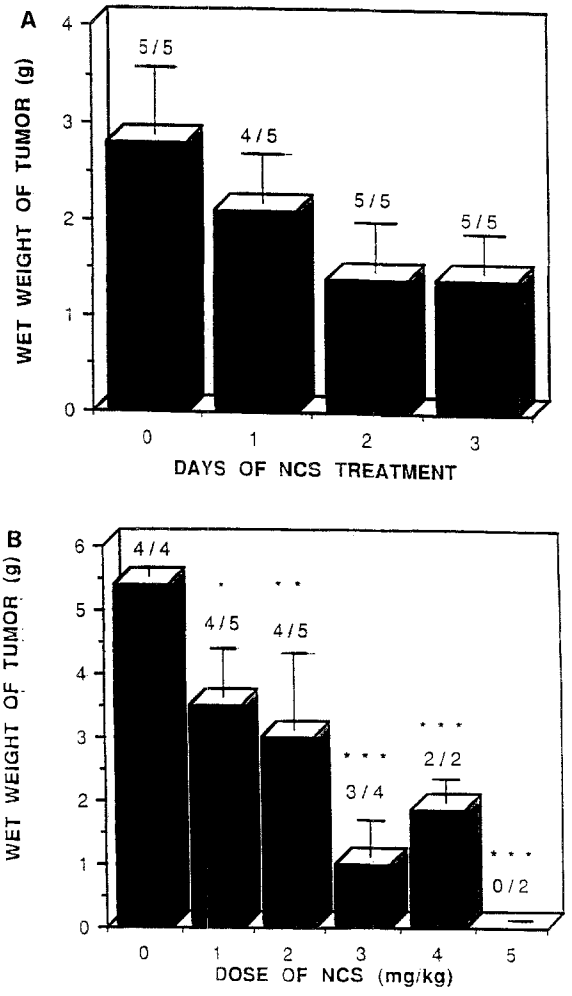


Fig. 5 A, B NCS dose dependence of tumor mass with in vivo treatment of A/J mice subcutaneously implanted with Neuro-2A cells. **A** A/J mice were subcutaneously injected with 10^6 Neuro-2A cells on day 1 and were treated with NCS (0.5 mg/kg by intraperitoneal injection) for 0, 1, 2, or 3 days beginning on day 2. Tumor mass was determined by necropsy on day 30. *Numbers above the bars* give the tumor incidence in each group. Although there is a trend toward decreased tumor mass with increasing frequency of initial treatment, this trend does not attain statistical significance ($P > 0.05$, Student's *t*-test). **B** A/J mice were subcutaneously injected with 10^6 Neuro-2A cells on day 0 and were treated with NCS (0, 1, 2, 3, 4, or 5 mg/kg by intraperitoneal injection) given as a single dose on day 2. Tumor incidence was determined by necropsy on day 30. *Numbers above the bars* give the tumor incidence in each group. *Asterisks* indicate the degree of statistical significance of the difference between the control group tumor mass and that of each of the experimental groups in turn as follows: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.0005$

when given for up to 3 days. However, the differences in tumor weight observed between the control group and each of the experimental groups in turn did not quite achieve statistical significance ($P > 0.05$, Student's *t*-test).

Fig. 5B illustrates the dose-dependent decrease in the mass of tumors that developed in mice treated with a single dose of NCS on day 2. The differences observed between the control group and each of the experimental groups in turn were highly statistically significant (see Fig. 5B). Unlike the case for cells treated in vitro with subsequent implantation, in vivo NCS treatment had no independent effect upon the incidence of tumors; that is, until the tumor mass was reduced to the lower limit of detectability, the tumor incidence remained comparable with that seen in the untreated population. Drug toxicity became apparent at 4 mg/kg (3/5 mice died prior to expected tumor development, i.e., before day 20). An additional group of 5 mice treated with 7.5 mg/kg succumbed to presumed NCS toxicity within 24 h of treatment.

Discussion

Disseminated neuroblastoma represents a major therapeutic problem in pediatric oncology. Despite maximal conventional surgery, radiation therapy, and chemotherapy, children with metastatic neuroblastoma have only a 5%–10% 5-year survival rate [1]. Bone marrow transplantation has been proposed as a means of dealing with this problem. The early results of this approach appear promising. However, both residual tumor in the transplanted marrow and minimal residual disease in the host have been cited as major impediments to the success of autologous bone marrow transplantation [14]. The use of differentiation-inducing agents has been proposed for bone marrow purging and as an adjunct to conventional chemotherapy for residual disease. Many of the agents that induce differentiation in neuroblastoma cells in culture need to be present for long periods of time to have this effect [9, 13]. We have previously shown that the antimitotic natural product NCS induces morphological differentiation in murine and human neuroblastoma cells several days after a single 1-h exposure to the drug [2, 11]. The effectiveness of this drug in a "pulsed" exposure regimen makes it attractive for both ex vivo and in vivo treatment of neuroblastoma. The present studies demonstrate that NCS attenuates tumor development after in vitro treatment and tumor growth after in vivo treatment of C1300 (Neuro-2A) neuroblastoma cells.

In the case of in vitro treatment, there is a concentration- and drug exposure time-dependent decrease in the tumorigenicity of Neuro-2A cells exposed to NCS. The observation that the incidence, but not the mass or growth rate, of tumors decreases implies that the drug influences the "take rate" rather than the doubling time of these cells or the time to recurrence of tumor in vivo. This in turn implies that for any given cell, the outcome of NCS treatment involves an all-or-none process. A cell either is or is not capable of proliferating to form a tumor. The relationship of the incidence to the drug concentration and exposure time derives from the dependence of the number of tumorigenic cells upon these parameters and the stochastic nature of the tumor formation process; that is, the number of tumorigenic cells that remain determines the likelihood that one of these cells will form a clonal tumor.

In the case of in vivo treatment, there is a dose-dependent decrease in the size of tumors that develop

without an independent change in tumor incidence. This implies that by the time of NCS administration, the binary decision as to whether or not a tumor will form has been made. By this time the microscopic tumor consists of a group of cells, some of which will cease to proliferate upon NCS treatment and some of which will continue to replicate. Thus, although the effects of NCS are all-or-none for any given cell, its effects upon a preexistent tumor are graded.

The present studies also imply that NCS might be efficacious in ex vivo purging regimens and in in vivo treatment of microscopic residual disease, especially since our previous studies have demonstrated similar antimitotic and morphological effects of NCS upon human neuroblastoma cell lines [2, 12]. Clinical phase I and phase II trials of NCS were thwarted by the development of anaphylaxis in patients treated with this drug. Obviation of this ideosyncratic response to NCS is potentially achievable by coupling the antigenic protein moiety to an inert polymer [6], by using NCS analogues that contain more chemically stable organic components [7], or by using NCS in regimens such as the bone marrow purging regimen, where the agent is washed out of the bone marrow prior to transplantation. The irreversible nature of the changes produced by a brief exposure of the cells to NCS makes this a possibility. The dose-related toxicity of NCS has included bone marrow toxicity. This effect of the drug might be avoided by its adjunctive use with agents such as 6-mercaptodopamine, which are hypothesized to increase selectively the activation of NCS in neural crest cells, thereby decreasing the dose needed for efficacy [12], or by the use of other less toxic congeners of NCS such as enediyne-5 [7]. Preliminary studies in our laboratory indicate that the latter compound is equipotent to NCS in producing morphological differentiation in murine and human neuroblastoma cells (Nylander and Schor, unpublished observations) and that it affords a favorable 5- to 10-fold differential toxicity for human neuroblastoma cell lines versus human myeloid precursor cells (Hartsell et al, unpublished observations). Direct demonstration of the utility of NCS and other enediynes for bone marrow purging and/or treatment of minimal residual disease must await studies using human tissue.

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