Apoptosis of N-Type Neuroblastoma Cells After Differentiation With 9-cis-Retinoic Acid and Subsequent Washout

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Background: The overall survival rate for patients with neuroblastoma has improved over the past two decades, but long-term survival for the subgroup of patients with high-risk disease remains low. In recent years, there has been interest in the potential clinical use of drugs able to induce differentiation of neuroblastoma cells. Since 9-cis-retinoic acid induces better and more sustained differentiation of neuroblastoma in vitro than other retinoid acid isomers, this may be a more appropriate retinoid for use in neuroblastoma therapy. Purpose: The purpose of this work was to compare the long-term effects of all-trans- and 9-cis-retinoic acid on neuroblastoma differentiation using an N-type (neuroblastic) cell line, SH SY 5Y, as an in vitro model. In addition, we wanted to find out whether 9-cis-retinoic acid would induce programmed cell death (apoptosis) in these N-type neuroblastoma cells and to determine whether the effects of either 9-cis- or all-trans-retinoic acid are dependent on their continued presence in the culture medium. Methods: SH SY 5Y cells were incubated in either the continued presence of all-trans- or 9-cis-retinoic acid or for 5 days with retinoic acid followed by culture in the absence of retinoid for up to 13 days. Morphologic changes were observed using phase-contrast and scanning electron microscopy. Apoptosis was determined by flow cytometry of propidium iodide-stained cells and by using terminal deoxynucleotidyl transferase to end-label DNA fragments in situ in apoptotic cells. Results: Culture of SH SY 5Y cells with all-trans- or 9-cis retinoic acid for 5 days induced morphologic differentiation and inhibited cell growth. These effects were maintained in the continuous presence of each retinoic acid isomer but were more profound in cells treated with 9-cis-retinoic acid. The differentiation of cells treated with all-trans-retinoic acid was reversible once retinoic acid was removed from the medium. Conversely, apoptosis was induced in cells treated with 9-cis-retinoic acid for 5 days and cultured for 9 days (4 days after washout) but not in cells cultured in the continuous presence of 9-cis-retinoic acid. This effect was specific to 9-cis-retinoic acid. Conclusions: Previous studies have demonstrated differential responses to all-trans-retinoic acid in N- and S-type (substrate-adherent or Schwann-like) neuroblastoma cells: Apoptosis is induced in S-type cells, whereas differentiation occurs in N-type cells. The present results show that, unlike all-trans-retinoic acid, 9-cis-retinoic acid induces both differentiation and apoptosis in N-type SH SY 5Y neuroblastoma cells. However, apoptosis was dependent on removal of 9-cis-retinoic acid from the culture medium. Implications: Since both differentiation and apoptosis are involved in tumor regression, 9-cis-retinoic acid may be a more appropriate retinoid for clinical trials in neuroblastoma. The dependence of apoptosis on treatment and subsequent removal of 9-cis-retinoic acid implies that drug scheduling may be an important parameter affecting therapeutic efficacy.

Notes

1Editor’s note: SEER is a set of geographically defined, population-based central tumor registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Each registry annually submits its cases to the NCI on a computer tape. These computer tapes are then edited by the NCI and made available for analysis.

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See “Notes” following “References.”
While the overall survival rate for patients with neuroblastoma has improved over the past two decades, long-term survival for the subgroup with high-risk disease remains low. Neuroblastoma is a heterogeneous disease, with a spectrum from the primitive neuroblast to the well-differentiated ganglion cell occurring in the same tumor (1). In some cases, tumors may differentiate into the more benign ganglioneuroma or undergo spontaneous regression via programmed cell death (apoptosis) (2,3). In vitro, at least three morphologically distinct cell phenotypes, termed neuroblastic (N type), substrate-adherent (S type), and intermediate (I type), have been recognized in neuroblastoma cell lines. These cell types, likely to represent the glial, Schwannian, melanocytic, and neuroblastic phenotypes present in neuroblastoma tumors in vivo (4), undergo either apoptosis or differentiation in response to all-trans-retinoic acid: S-type cells (glial, Schwannian, and melanocytic) undergo apoptosis (5,6), whereas N-type cells differentiate into a more mature phenotype (7).

In recent years, there has been considerable focus on the potential clinical use of drugs, including retinoic acid, which are able to induce differentiation of neuroblastoma in vitro. However, despite the ability of all-trans-retinoic acid to induce differentiation or apoptosis of neuroblastoma cells in vitro, clinical trials of 13-cis- and all-trans-retinoic acid in patients with neuroblastoma have been disappointing (8-10). This may be explained by poor pharmacokinetics (9) and an inability of all-trans-retinoic acid to induce apoptosis of N-type cells (6). Furthermore, N-type cells differentiated with all-trans-retinoic acid may be more resistant to cytotoxic drugs (11), and this will be an important factor limiting disease remission if the differentiated phenotype is reversible. Since both differentiation and apoptosis are important mechanisms of tumor regression, an important future direction toward effective therapy for neuroblastoma may be to include agents that can specifically activate both processes.

The effects of all-trans-retinoic acid are mediated by retinoic acid receptors (RARs), ligand-dependent transcriptional regulators that act as heterodimers with ligand-independent auxiliary factors referred to as retinoid X receptors (RXRs) (12,13). RXRs also bind 9-cis-retinoic acid with high affinity (14) and regulate gene transcription as 9-cis-retinoic acid-dependent RXR homodimers (15) or as ligand-dependent heterodimers with other nuclear receptors (16,17). Since 9-cis-retinoic acid induces better and more sustained differentiation of neuroblastoma in vitro (18-20) and may be an effective retinoid for the treatment of other cancers (21,22), this may be a more appropriate retinoid for use in neuroblastoma therapy. However, for treatment with retinoic acid to be effective, it is important that the differentiated phenotype is maintained. The purpose of this study was to compare the long-term effects of all-trans- and 9-cis-retinoic acid on neuroblastoma differentiation using an N-type neuroblastoma cell line, SH SY 5Y, as an in vitro model. In addition, we wanted to find out whether 9-cis-retinoic acid would induce apoptosis in these N-type neuroblastoma cells and to determine whether the effects of either 9-cis- or all-trans-retinoic acid are dependent on their continued presence in the culture medium.

Materials and Methods
Cell Culture and Morphologic Studies of SH SY 5Y Cells
To study morphologic responses to retinoic acid isomers, 0.4 × 10^4 SH SY 5Y cells (23) were seeded into 24-well tissue culture plates (Costar UK, High Wycombe, U.K.) in 2 mL of Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum (Sera-Lab, Crawley, U.K.) (culture medium), and incubated overnight at 37°C in a humidified atmosphere of 5% CO2 in air. All-trans-retinoic acid (Sigma Chemical Co., Poole, U.K.) or 9-cis-retinoic acid (Hoffmann-La Roche Inc., Basel, Switzerland) was then added in ethanol to a final concentration of 10^-6 M. An equal volume of ethanol (<0.1% of culture volume) was used to treat control cells. The culture medium was changed every 2 days and was replaced with fresh medium containing the appropriate concentrations of retinoic acid. Cultures were treated continuously with all-trans- or 9-cis-retinoic acid for up to 13 days or were treated with either retinoic acid isomer for 5 days and then cultured in the absence of any retinoid for up to an additional 8 days. The cells were photographed after 3, 5, 9, 11, and 13 days. Neurite lengths, expressed as relative units, were measured using Vernier calipers as described previously (18) and were analyzed using nonparametric statistics (Mood median test and Kruskal–Wallis test, Minibit Statistical Package, Minibit Inc., State College, PA). For scanning electron microscopy, SH SY 5Y cells were seeded in tissue culture-treated Trans-wells (Costar UK) in 12-well plates (Costar UK) at a density of 1.3 × 10^5/mL and cultured for 13 days in the presence of all-trans- or 9-cis-retinoic acid for either 13 days continuously or for 5 days followed by culture in the absence of retinoic acid isomers as above. Cells were fixed with 2% glutaraldehyde in Sorenson’s phosphate buffer and prepared for scanning electron microscopy (24) by serial dehydration through ethanol and critical-point drying in a Tousimis/Samgrdi-780 critical-point drying apparatus (Balzers, Milton Keynes, U.K.), using liquid CO2 as the transition fluid. Specimens were then mounted on stubs, allowed to dry, coated with 12 nm of gold using a Polaron Cool Stage Sputter Coater (Fisons Apparatus Ltd., Uckfield, U.K.), and then scanned using a Cambridge S240 microscope (Leo Instruments Ltd., Cambridge, U.K.) at settings of 8 kV and a working distance of 12 mm.

Evaluation of Apoptosis by Flow Cytometry
SH SY 5Y cells were seeded into 25-cm² tissue culture flasks (Costar UK) at a density such that at least 1 × 10^6 cells were available from each flask at the time of harvest. Cells were initially allowed to attach overnight and were then treated with 10^-6 M all-trans- or 9-cis-retinoic acid in ethanol or an equal volume of control ethanol. The culture medium was changed every 2 days, and at each change, the existing medium was harvested, centrifuged at 800g for 5 minutes at 21°C, and pelleted material, consisting of cells and apoptotic bodies, was resuspended in fresh culture medium plus retinoid or control ethanol as appropriate. At final time points, the medium was harvested, and apoptotic bodies and nonadherent cells were collected by centrifugation at 800g for 5 minutes at 21°C and pooled with cells recovered from the flasks by trypsinization (25). Apoptosis was evaluated by flow cytometry of propidium iodide-stained cells (26) by use of a FACSScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells suspended in phosphate-buffered saline (PBS; ICN-Flow, High Wycombe, U.K.) were fixed by adding an equal volume of cold (−20°C) methanol:acetone (4:1 vol/vol) and were stored at 4°C. Fluorescence, resulting from excitation at 488 nm with a 15 mW Argon laser, was monitored at 570 nm. Events were triggered by forward-scatter signal and gated for forward-scatter height and angle and side-scatter angle to avoid aggregates. Five thousand events were evaluated by use of the Lysis II program.

Identification of Apoptosis In Situ via Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate–Biotin Nick End Labeling (TUNEL)
SH SY 5Y cells were treated with 9-cis- or all-trans-retinoic acid at 10^-6 M or with ethanol as a control either continuously for 9 days (as above) or for 5 days followed by 4 days’ culture in the absence of retinoic acid isomers. At day 9, cells were trypsinized, washed once in PBS, and approximately 5000 cells in a 10-μL drop were placed onto slides precoated with 0.01% aqueous solution of poly-I-lysine (3 × 10^6 molecular weight, Sigma) and air-dried. Programmed cell death was identified by specific labeling of nuclear DNA fragmentation according to the method of Gavrieli et al. (27). Briefly, cells were fixed in 4% buffered paraformaldehyde, and endogenous peroxidase was removed with 2% H2O2. The cells were washed in distilled water and then incubated with terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reagents.
nucleotidyl transferase and biotinylated deoxyuridine triphosphate in the appropriate buffer in a humidified atmosphere at 37 °C for 60 minutes. The reaction was then terminated, and the slides were incubated with 2% aqueous solution of bovine serum albumin (Sigma) for 30 minutes at room temperature, rinsed in distilled water, and immersed in PBS for 5 minutes. Incorporated biotin was then detected using avidin–peroxidase, diluted 1:20 in water, and followed by development with 3-amino-9-ethylcarbazol for 15 minutes at 37 °C. Cells were counterstained with hematoxylin–eosin (27).

Results

Morphologic Effects of 9-cis- and all-trans-Retinoic Acid on SH SY 5Y Cells

Although we have previously demonstrated that 9-cis-retinoic acid induces morphologic differentiation over 4 days more effectively than all-trans-retinoic acid (18,19), it is not known if these effects are reversible. After 3-5 days of treatment with retinoic acid, SH SY 5Y cells appeared differentiated, as evidenced by the extension of neurite processes, with 9-cis-retinoic acid producing a greater effect (Fig. 1). Median neurite lengths for control, all-trans-, and 9-cis-retinoic acid-treated cells after 5 days were (sample size and 95% confidence interval [CI]) 4.7 (n = 55; 95% CI = 3.7-6.2), 5.4 (n = 45; 95% CI = 4.7-6.5), and 11.2 (n = 47; 95% CI = 8.2-14.6) relative units, respectively (Mood median test; P < .01). Removal of all-trans-retinoic acid from the culture medium resulted in the cells reverting to their original morphology within 2-4 days and regaining their proliferative capacity (Fig. 1; day 9). However, in 9-cis-treated cultures, cell processes or neurites also became shorter, taking on a clubbed appearance after removal of 9-cis-retinoic acid (Fig. 1; day 9). By 4-6 days after 9-cis-retinoic acid washout, the cell bodies lacked neurite processes and appeared contracted (Fig. 2, A and C). Control cells were similar but had shorter neurites and a larger number of small-cell surface processes (Fig. 2, E). Cells treated with all-trans-retinoic acid and then cultured in the absence of retinoic acid were morphologically similar to control cells (Fig. 2, D). Conversely, cells treated with 9-cis-retinoic acid before washout were smaller, had lost their prominent neurites, and had fewer cell surface processes and intercellular connections (Fig. 2, B and F). The loss of specialized surface structures such as microvilli, neurites, and contact regions, together with a reduction in cell volume in 9-cis-retinoic acid washout cultures, are consistent with the morphologic features of apoptotic cells (28).

Flow Cytometry of SH SY 5Y Cells Treated With all-trans- or 9-cis-Retinoic Acid

To determine whether treatment with 9-cis-retinoic acid followed by washout and continued incubation in the absence of retinoic acid results in apoptosis, ethanol, all-trans-, and 9-cis-retinoic acid (10⁻⁶ M)-treated cells were analyzed by flow cytometry at 3 and 5 days after treatment and at 4 days after washout of the retinoid (day 9). At 3 and 5 days, no apoptosis was apparent in the cell cultures (Fig. 3, A-F). This was also the case in the ethanol and all-trans retinoic acid-treated cultures after 9 days (4 days after washout; Fig. 3, G and H). Conversely, apoptotic cells accounted for 35%-45% of

Fig. 1. Morphology of SH SY 5Y neuroblastoma cells treated with ethanol (control), all-trans-, or 9-cis-retinoic acid (10⁻⁶ M) for 5 days followed by subsequent culture in the absence of retinoic acid for an additional 8 days. Time in days is from the start of the experiment; thus, day 9 is 4 days after retinoic acid washout and day 13 is 8 days after washout. The time of retinoic acid removal is indicated by the arrow. Bar (top right panel) = 10 μm.
Fig. 2. Scanning electron microscopy of SH SY 5Y cells after 13 days of culture. Samples were scanned at settings of 8 kV and a working distance of 12 mm. Cells were either cultured in the continued presence of $10^{-6} \text{M} \ 9$-cis-retinoic acid (A) or $10^{-6} \text{M} \ \text{all-trans-retinoic} \ \text{acid}$ (C) or with ethanol as the vehicle control (E). In addition, some cells were treated for 5 days with either $10^{-6} \text{M} \ 9$-cis-retinoic acid (B and F) or $10^{-6} \text{M} \ \text{all-trans-retinoic acid}$ (D) and cultured for an additional 8 days in the absence of either retinoid. Scale bars are as indicated in each panel.

Fig. 3. Flow cytometry of SH SY 5Y cells treated with ethanol (A, D, G), all-trans-retinoic acid (B, E, H), or 9-cis-retinoic acid (C, F, I) at $10^{-6} \text{M}$ for 5 days and then cultured in the absence of retinoic acid for an additional 4 days. The cell cultures were analyzed by flow cytometry (see “Materials and Methods” section) to estimate the degree of apoptosis at days 3, 5 (the day of washout), and 9. Apoptotic bodies were detectable only in cultures treated with 9-cis-retinoic acid for 5 days and then cultured in the absence of retinoid. The peak of apoptotic cells is marked with an arrow in (I).
the cells in the 9-cis-retinoic acid-treated cultures at 9 days (4 days after washout; Fig. 3, I). Apoptosis was not apparent with continuous culture of cells in the presence of 9-cis- or all-trans-retinoic acid (data not shown) and was thus specific to the washout experimental design. These data support the view that 9-cis-retinoic acid induces differentiation of neuroblastoma in vitro and that the subsequent survival of these cells is dependent on the continued presence of 9-cis-retinoic acid.

**DNA Fragmentation in SH SY 5Y Cells Treated With Either all-trans- or 9-cis-Retinoic Acid for 9 Days (TUNEL Analysis)**

To confirm the flow cytometry results showing that 9-cis-retinoic acid induced apoptosis, SH SY 5Y cells were treated with $10^{-6}$ M all-trans- or 9-cis-retinoic acid either continuously for 9 days or for 5 days followed by an additional 4 days in the absence of any retinoid. TUNEL analysis confirmed the flow cytometry data, whereby treatment of SH SY 5Y cells with 9-cis-retinoic acid with subsequent washout resulted in the induction of apoptosis as shown by the TUNEL-positive cells in Fig. 4, B and D. Apoptotic cells were not apparent in cultures treated continuously with either 9-cis-retinoic acid (Fig. 4, A and C) or all-trans-retinoic acid (Fig. 4, E) or indeed in those treated with all-trans-retinoic acid washed out after 5 days (Fig. 4, F).

**Discussion**

These experiments demonstrate that 9-cis- and all-trans-retinoic acid differ in their ability to induce morphologic differentiation and apoptosis of SH SY 5Y neuroblastoma cells. Differentiation was maintained in the continuous presence of either retinoid, with 9-cis-retinoic acid inducing a more pronounced increase in neurite length. This supports previous data for SH SY 5Y and LA N-5 human neuroblastoma cells in which 9-cis-retinoic acid was more effective than all-trans-retinoic acid at promoting morphologic differentiation at concentrations greater than $10^{-7}$ M (18,20). After removal of the retinoids from the culture medium, different effects were observed, depending on which retinoic acid isomer was used. In contrast to all-trans-retinoic acid, cells treated with 9-cis-retinoic acid for 5 days before further culture in the absence of retinoic acid developed morphologic changes consistent with apoptosis (28); this was confirmed by flow cytometry and TUNEL analysis.

The fact that no apoptosis was observed in cells treated continuously with either all-trans- or 9-cis-retinoic acid or following washout of all-trans-retinoic acid raises interesting questions concerning the mechanism of apoptosis that occurs after washout of 9-cis-retinoic acid. It should not be too surprising that 9-cis- and all-trans-retinoic acid have different biologic effects, given that all-trans-retinoic acid binds only to RARs, whereas 9-cis-retinoic acid binds to both RARs and RXRs; this may explain why in HL60 promyelocytic leukemia cells, ligand activation of RARs is sufficient to induce differentiation, but ligand activation of RXRs is essential for the induction of apoptosis (29).

Under normal conditions, cell death, survival, and growth are in a state of equilibrium (30). Specific neurotrophic factors are known to be responsible for maintaining the viability of neuronal cells. For example, insulin-like growth factors (IGFs) are survival factors for neuroblastoma cells (31,32) and are induced by all-trans-retinoic acid in these cells (33-36). All-trans-retinoic acid also stimulates nerve growth factor (NGF)-dependent survival in sympathetic neurons (37). In addition to growth factors and their receptors, the induction of other intracellular proteins may be linked to maintaining cell survival. The Bcl-2 protein at high levels prevents cell death in response to a variety of stimuli (38). Bcl-2 is involved in the regulation of neural differentiation (39), and the retinoic acid-induced differ-
entiated phenotype of SH SY 5Y cells has been associated with high expression of Bcl-2 (11,40). In contrast to N-type neuroblastoma cells, all-trans-retinoic acid induces apoptosis in S-type neuroblastoma cells (6) that express low levels of Bcl-2 (40). Thus, the survival of neuroblastoma cells differentiated with all-trans-retinoic acid may be dependent on the expression of autocrine factors and their receptors, such as IGF, and/or on the continued expression of proteins such as Bcl-2.

The reason why, in this context, 9-cis-retinoic acid induced apoptosis of N-type SH SY 5Y cells after washout is not clear. It is possible that Bcl-2 expression decreases after withdrawal of 9-cis-retinoic acid, allowing cells to enter a programmed cell death pathway, and this hypothesis will need to be addressed experimentally. Gene-induction experiments using a short-term (6-hour) treatment of SH SY 5Y cells have shown that 9-cis-retinoic acid-induced expression of the RAR-β and cellular retinoic acid-binding protein II (CRABP II) genes is rapidly reduced following washout; this is in marked contrast to all-trans-retinoic acid, in which induced expression of RAR-β and CRABP II is sustained for at least 24 hours after the removal of this isomer from the culture medium (41). If this is also true for Bcl-2 after longer-term (5-day) treatment with 9-cis-retinoic acid, this could explain the apoptosis of SH SY 5Y cells after washout in the context of the induction of Bcl-2 and/or autocrine survival factors. The rapid reduction in 9-cis-retinoic acid-induced RAR-β and CRABP II expression after washout is one argument to support the view that the effects of 9-cis-retinoic acid in these cells are mediated by RXRs, perhaps acting as RXR homodimers (41) or via 9-cis-dependent heterodimers between RXRs and other nuclear receptors, such as LXXRs (17) or NGFI-β (16). This raises the question whether 9-cis-retinoic acid induces qualitatively different patterns of gene expression to all-trans-retinoic acid, or if the washout effects result from differences in the kinetics of ligand-receptor interactions. We are currently addressing these hypotheses to elucidate the mechanism of 9-cis-retinoic acid-induced apoptosis.

Since retinoids can induce both differentiation and apoptosis of N- and S-type neuroblastoma cells, respectively, in vitro, these compounds may play an important role in future treatment protocols. However, the fact that differentiated N-type cells apparently are more resistant to cytotoxic drugs (11) is an important factor limiting the success of therapy with all-trans- or 13-cis-retinoic acid. The results from this study indicate that this problem could be overcome by the use of 9-cis-retinoic acid in an intermittent treatment schedule, thus promoting the apoptosis of cells differentiated by exposure to 9-cis-retinoic acid following withdrawal of the drug. Since phase I trials of 9-cis-retinoic acid in the treatment of adult cancers have now been reported (22,42), these data suggest that 9-cis-retinoic acid may be the most appropriate retinoid for clinical trials in the treatment of children with minimal residual neuroblastoma following chemotherapy. It may also have potential for inducing regression in stage IVs neuroblastoma and more localized disease, where current treatment is a short course of chemotherapy. The dependence of apoptosis on treatment and the subsequent removal of 9-cis-retinoic acid implies that drug scheduling in clinical trials may be an important parameter affecting therapeutic efficacy.

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