Differential Effects of N-(4-Hydroxyphenyl)retinamide and Retinoic Acid on Neuroblastoma Cells: Apoptosis versus Differentiation

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ABSTRACT

Retinoids exert various important biological effects in the control of normal growth, differentiation, and fetal development. While retinoic acid (RA) has entered clinical trials as a differentiation-promoting agent, it is only recently that the synthetic retinoid N-(4-hydroxyphenyl)retinamide (HPR) has been shown to be of potential clinical interest in cancer chemoprevention and treatment. Since thus far no data exist on the effects of HPR on neural crest cell-derived tumors, we have examined its in vitro effects on neuroblastoma (NB) cell lines and found that at relevant pharmacological concentrations it induces a dose-dependent growth inhibition. The antiproliferative effects of HPR were, in six of six cell lines tested, drastically more potent that those induced by an equimolar dose of RA. Time course growth analysis showed that HPR at 3 × 10^-6 M induces a very rapid (24—72 h) fall in thymidine uptake (>90%), whereas at 3 × 10^-7 M it exhibits cytostatic effects.

In contrast to RA, HPR did not show morphological changes typical of NB cell maturation nor did it induce the expression of any cytoskeletal protein associated with neuronal differentiation.

DNA flow cytometric analysis revealed that HPR did not induce an arrest in a specific phase of the cell cycle while triggering apoptosis. This phenomenon was evidenced both by the visualization of "DNA ladders" on gel electrophoresis and by a quantitative assay for evaluating programmed cell death based upon the labeling of DNA breaks with tritiated thymidine. With the latter method, apoptotic cells were detectable as early as 3—6 h after treatment of NB cells with 10^-5 M HPR, while more than 50% of cells were apoptotic by 24—72 h following exposure to 3 × 10^-6 M HPR. In contrast, RA induced a low rate of apoptosis in NB cells only after 3—5 days.

Time lapse photomicroscopy showed that NB cells treated with HPR underwent a death process highly reminiscent of apoptosis, with progressive condensation of the cytoplasm around the nucleus and intense cell shrinkage. The cells then rounded up and detached from the plate. Furthermore, propidium iodide staining of the DNA showed that a high proportion of cells treated with HPR displayed a small and brightly staining nucleus; chromatin appeared aggregated into dense masses in the nuclear periphery, a typical feature of apoptotic cells.

In conclusion, our study demonstrates that contrary to the differentiation-promoting activity of RA, HPR dramatically suppresses NB cell growth by inducing programmed cell death. Since the peak HPR plasma concentration in patients treated with the drug is superimposable to the dose inducing apoptosis in vitro (i.e., 1—3 × 10^-4 M), our results may support the hypothesis of the use of HPR in the treatment of advanced neuroblastoma.

INTRODUCTION

In both vertebrates and invertebrates, apoptosis, also called programmed cell death, is an active process depending on the expression of a specific set of genes which play a key role in normal development and oncogenesis (1—3). Since it has recently been suggested that anticancer agents exert part of their biological effects by triggering apoptotic cell death (4, 5), the induction of apoptosis in tumor cells has become a therapeutic objective.

In the last decade, apoptosis has been intensively studied in many laboratories, but only recently have the relationships among apoptosis, cell proliferation, and terminal differentiation been faced, mainly in immune and hematological model systems (6—8).

NB is the most common extracranial malignant solid tumor of childhood arising from the sympathetic nervous system (9). Although NB cells are presumably derived from a common transformed progenitor (10), they usually express multiple phenotypes, including the neuronal, neurilemmal, epithelioid, and melanocytic ones (10, 11). All phenotypes may interconvert in vitro either spontaneously or depending on tissue culture conditions (12, 13). A variety of biological agents and drugs, particularly retinoids, have been reported to induce differentiation of NB cells in vitro (14, 15). Therefore, NB cell lines are suitable models for investigation of the mechanisms of neuronal death and its relation to differentiation (16—18).

Retinoids are a class of natural or synthetic compounds structurally related to vitamin A that exert various relevant biological effects in the control of cell proliferation, differentiation, and fetal development. The recent reports of dramatic antitumor effects of retinoids, especially RA, have raised interest in further elucidating the potential therapeutic role of retinoids in cancer patients (see Ref. 19 for review). However, systemic administration has been limited by their high toxicity (19, 20). Among retinoids, HPR, a synthetic derivative of RA, has shown mild toxicity (21) and seems very promising in animal models, to prevent carcinogen-induced mammary cancer (22) and to inhibit metastatic spreading of prostate adenocarcinoma (23). Moreover, HPR displays antiproliferative effects in vitro against human breast carcinoma cells (24) and induces apoptosis in hemopoietic cell lines (25). On the basis of these findings and since no data exist thus far on the effects of HPR on neural crest cell-derived tumors, we have evaluated its in vitro effects on human NB cell lines in comparison with those of RA. We found that at relevant pharmacological concentrations (26) HPR is a potent and rapid inducer of programmed cell death, while RA, besides being an attractive differentiation-promoting agent, is able to induce a modest and late form of apoptosis.

MATERIALS AND METHODS

Chemicals. All-trans-retinoic acid (Sigma Chemical Co., St. Louis, MO) and HPR (kindly supplied by Johnson Pharmaceutical Research Institute, Spring House, PA) were dissolved in absolute ethanol at the concentration of 10^-5 M and stored in aliquots at −20°C for a maximum of 1 week.

Cell Cultures. Human NB cell lines LAN-5, SK-N-BE2(c), and IMR-32 were gifts of R. Seeger (27), J. L. Biedler (28), and J. J. Tumilowicz (29), respectively. GI-LI-N, GI-ME-N, and GI-CA-N NB cell lines were established in our laboratory as described (30—32). Cells were maintained in the logarithmic growth phase using Minimal Essential Medium supplemented with 5% fetal bovine serum.

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2 To whom requests for reprints should be addressed.
pellets were fixed with 70% ethanol overnight. The cells were washed twice aliquots of 5 × 10^6 cells were centrifuged at 1200 rpm for 10 min, and the cells were resuspended in 0.5 ml of 1% SDS. Both lysates and pellets were then counted using a modification of the technique described by Nicoletti et al. (37). Briefly, the cells were washed with fresh medium containing the inducing agents or their solvent control every other day up to the time indicated in each experiment.

Cell Morphology. LAN-5 cells (5 × 10^5) and SK-N-BE2(c) cells (1 × 10^5) were seeded into T-25 flasks with 5 ml of culture medium and treated with the inducers as described above. Since day 1 of treatment, 200 cells/culture from at least 3 different random areas of the flasks were examined daily with a phase contrast microscope (Olympus IMT-2).

Assay for Inhibition of Cell Growth. NB cells were plated into T-25 flasks and treated as described. At different time points, the cells were detached with trypsin solution and counted with a hemocytometer, using a Turk dye solution.

Assay for Inhibition of [3H]Thymidine Incorporation. All NB cell lines were plated in sextuplicate wells of flat-bottomed microtiter plates (Costar, Cambridge, MA) and treated as described. At various intervals after the induction was added, the cultures were seeded with 0.5 μCi [methyl-3H]thymidine/well (specific activity, 5 Ci/mmol; Amersham, Buckinghamshire, United Kingdom), incubated for 19 h at 37°C, trypsinized, and harvested on strips of fiberglass filter paper with the use of a multiple automated sample harvester (Flow), and the radioactivity associated with individual samples was measured in a liquid scintillation counter (Tri-Carb 4530; Packard Instruments Company, Downers Grove, IL).

Immunofluorescence Analysis. Cytoskeletal proteins were detected as reported previously (33), using mAbs with the following specificities: M_1, 200,000 neurofilaments and their phosphorylated forms MAP2, MAP5, and MAP6 (Sigma-Aldrich, Milan, Italy). Briefly, the cells were seeded at 25 × 10^3/ml into multiwell slides and incubated as described. At the defined times the plates were fixed in methanol/acetic acid (1/4, v/v) for 10 min at 20°C and incubated for 1 h at 37°C in a humid chamber with 200 μl of the appropriately diluted mAbs. After three washing with PBS for 3 min at room temperature, a second incubation with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma-Aldrich) was performed. The cells were washed twice, observed under a microscope (Leitz Orthoplan; Leitz GmbH, Wetzlar, Germany) equipped with a UV 100-W mercury bulb, and evaluated as described elsewhere (34).

The exposure time of an automatic camera has been quantified at a magnitude of ×250 on fields of comparable numbers of cells labeled with different antibodies and used as the indirect index of fluorescence intensity. The following score was used (exposure time): infinite; (−) 60 s; (−+) 40 s; (+) 20 s; (+++) 10 s; less than 10 s.

Quantitative Evaluation of Apoptosis. The percentage of apoptotic cells was evaluated by a modification of the JAM test (35) and of the quantitative assay described by Wright et al. (36). Briefly, NB cells were treated with HPR and RA, both at 3 × 10^-7 M, for 3 days in 24-well tissue culture plates. During the last 16 h the cells were labeled with [3H]thymidine (specific activity, 100 Ci/mmol; 5 μCi/1 × 10^6 cells). After removal of medium, the cells were rinsed three times with complete medium and then treated for further 6 h. At the end of the incubation, supernatants were removed for scintillation counting and the cells were lysed by the addition of 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5–8.0, 10 mM EDTA-0.2% Triton X-100). Intact chromatin was separated from fragmented DNA by centrifugation at 13,000 × g for 20 min. The pellets were resuspended in 0.5 ml of 1% SDS. Both lysates and pellets were then counted in a scintillation counter. The percentage of DNA fragmentation was calculated as

\[
\text{% of DNA release} = \frac{\text{cpm supernatant} + \text{cpm lysate}}{\text{cpm supernatant} + \text{cpm lysate} + \text{cpm pellet}} \times 100
\]

Flow Cytometry. Detection of apoptotic/hypodiploid cells were performed using a modification of the technique described by Nicoletti et al. (37). Briefly, aliquots of 5 × 10^5 cells were centrifuged at 1200 rpm for 10 min, and the pellets were fixed with 70% ethanol overnight. The cells were washed twice with PBS and then resuspended in 1 ml of a solution containing 3.4 mM sodium citrate, 20 μg/ml propidium iodide (Sigma), and 100 μg/ml RNase A (ICN, Costa Mesa, CA) and stored in the dark for 30 min. Cells were analyzed on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA).

DNA Fragmentation Assay. Cells were treated for 3 days as described, washed with ice-cold PBS, centrifuged at 1200 rpm for 10 min, and resuspended in lysis buffer consisting of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, 150 μg/ml RNase A, and 100 μg/ml proteinase K (Sigma). After incubation at 37°C for 16 h, samples were extracted twice with phenol:chloroform (v/v) and then precipitated overnight at −20°C by adding 1:10 (v/v) sodium acetate (3 M, pH 5.2) and 2 volumes of absolute ethanol. DNA was then pelleted by centrifugation at 13,000 × g for 10 min, washed with 1 volume of 70% ethanol, and dried under vacuum. DNA was resuspended with 10 mM Tris-HCl-1 mM EDTA buffer (pH 8.0) and supplemented with loading buffer (50% glycerol-0.05% bromophenol blue-0.05% xylene cyanole FF). Electrophoresis was performed for 3 h at 40 V in 1.2% agarose slab gels containing ethidium bromide at a final concentration of 0.1 μg/ml in 40 mM Tris-40 mM acetic acid-1 mM EDTA buffer (pH 8.0). A DNA 123 ladder (Gibco-BRL, Gaithersburg, MD) was applied to each gel to provide molecular size markers. DNA banding was evidenced with a UV transilluminator.

Morphological Evaluation of Apoptosis. Detection of apoptotic nuclei was performed according to the method of Jacobson et al. (38). Briefly, after the treatment period, the cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature, followed by a 10-min incubation with 5% acetic acid-95% ethanol at −20°C, and then stained with propidium iodide (0.05 μg/ml in PBS) containing 100 μg/ml RNase A for 30 min at 37°C. The cells were mounted in permanent aqueous mounting medium (Ortho Diagnostic Systems, Milan, Italy) and examined with a Leitz Orthoplan fluorescence microscope. In some experiments the morphology of apoptotic cells was evaluated by phase-contrast microscopy of May-Grünwald-Giems-stained cells.

RESULTS

Cell Proliferation

The effect of HPR on NB cell growth was tested at doses between 10^-5 and 10^-7 M, which span broadly the peak plasma concentration achieved in patients undergoing chemopreventive trials (26), and compared to that obtained following treatment with RA. Cell proliferation was dramatically inhibited by HPR treatment in six of six NB cell lines tested except SK-N-BE2(c) (90% inhibition). For the present study we therefore selected this dose.
INDUCTION OF APOPTOSIS IN NEUROBLASTS BY UPR

Throughout the subsequent experiments. Although RA suppressed NB cell growth in a dose-dependent manner, its effect was equal to or less evident than that of HPR (Fig. 1). To examine the kinetics of growth inhibition induced by HPR and RA treatment, we performed time course analysis. Growth curves in the presence of HPR or RA (both at 3 × 10⁻⁶ M) are depicted in Fig. 2. HPR induced a fast inhibition of [³H]thymidine uptake, with about 95% block by 24 h in cell growth of GI-LI-N and IMR-32 cells and by 3 days in the other NB cell lines. In contrast, RA was less inhibitory, reaching about 75% reduction of cell proliferation only after 7 days of treatment. It is noteworthy that the partially RA-resistant NB cell line GI-ME-N was highly sensitive to HPR. Similar results were obtained by cell counts (data not shown).

Neuronal Maturation

Since the ability of RA to inhibit NB cell growth is generally associated with induction of neuronal differentiation we tested the capability of HPR to induce morphological changes and cytoskeletal protein expression, typical hallmarks of neuronal maturation, in our NB cell lines.

Morphological Differentiation. Phenotype characteristics of NB cell lines were evaluated by microscopic inspection of overall morphology after 5 days of treatment. In all NB cell lines, represented in Fig. 3 by SK-N-BE2(c) (Fig. 3, A-C) and LAN-5 (Fig. 3, D-F) cells, treatment with RA resulted in a marked differentiation toward the neuronal phenotype, inducing the cells to be more polar, with longer neurites interconnected with frequent varicosities (Fig. 3, C and F). In contrast, NB cells treated with HPR rounded up, became smaller in diameter, and eventually detached from the dish (Fig. 3, B and E). These morphological features are consistent with the occurrence of programmed cell death (39, 40) rather than of cell differentiation.

Cytoskeletal Protein Modulation. Cytoskeletal proteins expressed by treated and control LAN-5 cells were assessed by immunofluorescence assay of permeabilized cells and are summarized in

Fig. 2. Time course of the effects of HPR and RA on [³H]thymidine uptake of six NB cell lines. Points, means of four different experiments each performed in sextuplicate; bars, SD. ○, control cells; □, 3 × 10⁻⁶ M RA; Δ, 3 × 10⁻⁶ M HPR.

Fig. 3. Morphological evaluation of SK-N-BE2(c) (A–C) and LAN-5 (D–F) cell lines after 5 days of treatment with HPR or RA. Cells were examined with a phase contrast microscope: A, C, D, F, × 20; B, E, × 30. A, D, control cells; B, E, cells cultured in the presence of 3 × 10⁻⁶ M HPR; C, F, 3 × 10⁻⁶ M RA.
Table 1. NFPs are specific markers of neurons and neuroblasts (41, 42). The high molecular weight NFPs and their phosphorylated forms were up-modulated by treatment with RA with a shift in fluorescence localization from a diffuse somatic to an intense perinuclear and neuritic pattern. Microtubule-associated proteins copolymerize with brain tubulin. As for NFPs, treatment of LAN-5 cells with RA induced a marked increase in MAP5, MAP2, and MAP (τ) with a sharp immunolocalization in the perinuclear and neuritic compartments. On the contrary, treatment of NB cells with HPR did not induce any modulation of cytoskeletal proteins, further indicating the inability of HPR to trigger neuronal maturation.

Neuronal Cell Death

Morphological Assessment. The morphology of cells treated with HPR was consistent with their having died by programmed cell death rather than by necrosis and/or terminal differentiation (39, 40). Indeed, phase contrast microscopy of May-Grünwald-Giemsa-stained NB cells treated with HPR showed progressive condensation of cytoplasm around the nucleus and intense cell shrinkage (Fig. 4, C and D). The cells eventually rounded up and detached from the dish. Fluorescence microscopy after propidium iodide staining showed that a relevant number of cells treated with HPR displayed a small, brightly staining, and highly condensed nucleus. Chromatin was aggregated into dense masses at the periphery of the nucleus and, in some cases, fragmented (Fig. 4, G and H). Conversely, treatment of NB cells with RA for 3 days showed that only a few cells acquire these apoptotic features (Fig. 4, B and F).

DNA Fragmentation. During apoptosis, loss of membrane integrity is typically preceded by chromatin condensation and internucleosomal cleavage of genomic DNA (39). All NB cell lines treated with $3 \times 10^{-6}$ M HPR for 3 days contained large amounts of low molecular weight DNA, which produced a characteristic "ladder" on agarose gels (Fig. 5). The degraded DNA was present in oligomers that were multiples of approximately 180–200 base pairs suggesting internucleosomal cleavage. With this method we did not observe DNA fragmentation in RA-treated cells (data not shown).

Furthermore, using a quantitative assay of DNA fragmentation we detected a dramatic induction of apoptosis in all NB cells treated with $3 \times 10^{-6}$ M HPR for 3 days (ranging from 45 ± 5% (SD) to about 100%, depending on the cell line), while RA treatment induced a smaller rate of cells to become apoptotic (Table 2). Using $10^{-5}$ M HPR, apoptotic cells were detectable as early as 3–6 h after treatment (data not shown).
**Table 1** Effects of RA and HPR on cytoskeletal proteins of LAN-5 human neuroblastoma cells assessed by immunofluorescence analysis after 5 days of treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NF (Mr 200,000)</th>
<th>NF (Mr 200,000)-P</th>
<th>MAP2</th>
<th>MAP5</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAN-5 cells</td>
<td>73 ± 5 a</td>
<td>40 ± 6</td>
<td>51 ± 7</td>
<td>20 ± 6</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>++ b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RA (3 × 10⁻⁶ M)</td>
<td>90 ± 6</td>
<td>85 ± 7</td>
<td>80 ± 8</td>
<td>96 ± 4</td>
<td>81 ± 6</td>
</tr>
<tr>
<td>P, N</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HPR (3 × 10⁻⁶ M)</td>
<td>70 ± 6</td>
<td>38 ± 5</td>
<td>50 ± 6</td>
<td>24 ± 7</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>S</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>P</td>
<td>S, P</td>
<td>S, P</td>
<td>S, P</td>
<td>S, P</td>
<td>S</td>
</tr>
</tbody>
</table>

a Percentage of positive cells. Mean ± SD of three independent experiments.  
b Fluorescence intensity: +, weakly positive; ++, moderately positive; ++++, strongly positive.  
c Fluorescence localization: S, somatic; P, perinuclear; N, neuritic.

**DISCUSSION**

This study shows that the synthetic retinoid HPR, at pharmacologically relevant doses, strongly and rapidly inhibits neuroblastoma cell growth through the induction of programmed cell death. On the contrary, our findings indicate that RA induces growth arrest of NB...
cell lines by triggering neuronal cell maturation and later on by partially inducing apoptosis.

NB is a peculiar tumor in its behavior inasmuch as it can occasionally undergo in vivo spontaneous or chemically induced maturation or regression (43, 44). One mechanism of tumor regression probably involves maturation of neoplastic cells into terminally differentiated, nonproliferating, ganglion-like cells (45). A second mechanism, tightly associated with differentiation and reported even for nonneuronal models (17, 18, 46, 47), could be the induction of programmed cell death. The process of apoptosis plays a critical role in the development of the nervous system where a large number of newly generated neuronal cells die by apoptosis, thus ensuring that the appropriate number of mature neurons is selected and eventually survives (48). Therefore, programmed cell death can be viewed as a normal process by which tissues and organs regulate their own development and maintenance, and perturbations of this equilibrium have been considered as likely causes of tumor occurrence or progression (5, 49). In this light, the attempt to restore the normal rate of apoptosis in neoplastic tissues may represent a novel antitumor strategy.

Several reports have shown that retinoids have antitumor properties. Besides the well known differentiation-inducing ability of RA (14, 15, 20, 22), the proven antitumor activity of HPR (21, 26, 50) and its mild toxicity (21) prompted us to investigate the effects of HPR in human NB cells in comparison with those of RA. Thus far, the effects of HPR on NB cell lines have not been investigated. Regarding antiproliferative activity, HPR was shown to inhibit colony formation of human tumors (51), to suppress mammary tumor virus-infected tumor cells (52), and to induce growth arrest of hematopoietic cell lines (25). Moreover, studies on HL-60 cells demonstrated that HPR has little differentiation-promoting activity as compared to RA (53). We have obtained similar results even in the neuronal model. Our findings clearly indicate that HPR did not induce any morphological changes or cytoskeletal protein modulation typical of neuronal maturation in NB cells, although it completely blocked cell proliferation. Indeed, changes in the type and amount of intermediate filaments have been shown to accompany neuronal cell differentiation (41, 42). Our findings demonstrate that RA, but not HPR, is able to induce the high molecular weight neurofilament proteins, and particularly their phosphorylated forms, which are typically expressed in mature neurons (42, 54).

Moreover, data on chromaffin (55) and NB cells (33) induced to differentiate indicated that MAPs are key regulators of neurite outgrowth. The enhanced expression of these cytoskeletal proteins by RA treatment (but not by HPR treatment) confirms the ability of this biological agent to induce true differentiation of NB cells.

These results suggest that the HPR-induced growth arrest of NB cell lines occurs through mechanisms distinct from those induced by RA and is uncoupled from the differentiation process.

Herein we show that HPR causes a rapid and drastic induction of programmed cell death in the neuronal model. More that 90% of cells died within 5–7 days in 6 of 6 NB cell lines tested after exposure to a pharmacologically relevant dose of HPR, 3 × 10−6 M. The possibility of an specific toxic effect of HPR was ruled out since some of the typical features of apoptosis were fulfilled. The dying cells usually undergo a characteristic sequence of morphological changes that

Table 2 Effects of HPR and RA on NB cell death evaluated by [3H]thymidine labeling of fragmented DNA after 3 days of treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>HPR (3 × 10−6 M)</th>
<th>RA (3 × 10−6 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAN-5</td>
<td>7.5 ± 2.5</td>
<td>58 ± 6.5</td>
<td>11.5 ± 3.5</td>
</tr>
<tr>
<td>GI-LI-N</td>
<td>8.5 ± 1.5</td>
<td>95 ± 5</td>
<td>15 ± 2.5</td>
</tr>
<tr>
<td>GI-ME-N</td>
<td>4 ± 1.5</td>
<td>69 ± 3.5</td>
<td>10 ± 2.5</td>
</tr>
<tr>
<td>GI-CA-N</td>
<td>7 ± 2</td>
<td>66 ± 3</td>
<td>10.5 ± 2.5</td>
</tr>
<tr>
<td>IMR-32</td>
<td>9 ± 2.5</td>
<td>93 ± 7</td>
<td>15.5 ± 2.5</td>
</tr>
<tr>
<td>SK-N-BE2(c)</td>
<td>3.5 ± 1.5</td>
<td>45 ± 5</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

a Mean ± SD of three different experiments.
include cytoplasmic and nuclear condensation; the chromatin is often cleaved by endonucleases into oligonucleosome-sized fragments (4, 39, 46). Moreover, the ability of HPR to cause apoptosis of NB cells was confirmed by flow cytometry, showing a reduction in the frequency of cycling cells and the appearance of a discrete population of cells containing hypodiploid DNA, in analogy with that already shown in hemopoietic cells treated with HPR (25) or with dexamethasone (56). However, this pattern does not seem the rule to evoke programmed cell death, since a multiple cell cycle access to the apoptotic death program has been demonstrated (57).

It has been reported recently that RA can induce programmed cell death in the neuronal model, depending on the phenotype of the NB cell line. RA would act on the substrate adherent (S) and intermediate (I) phenotypes but not on the neuroblastic-like (N) phenotype (16). Although our results seem to confirm the ability of RA to induce a moderate increase of apoptosis in NB cells, this event appears to be secondary to the induction of terminal differentiation and is unrelated to phenotype. Indeed, we did not observe apoptosis in RA-treated NB cells at times when we already detected neuronal differentiation (before 1–2 days), starting to reveal the apoptotic process after at least 3 days of treatment. Moreover, this moderate RA-induced apoptotic process was observed in all the NB cell lines tested, in spite of their different phenotypes. These findings are in line with those already reported in both the neuronal model (18) and the myeloid system (58).

Fig. 7. Time-dependent induction of hypodiploid DNA in GI-LI-N cells treated with 3 × 10^{-6} M HPR. A, histograms of total DNA contents; the percentages of cells with hypodiploid DNA are shown. B, bivariate dot-plot evaluation according to the level of forward light scattering: gate 1, cycling cells; gate 2, cells containing hypodiploid DNA. C, univariate DNA distributions of cells gated to gate 1. Data are representative of one experiment of three.

Fig. 6. DNA histograms of NB cells cultured for 3 days with HPR or RA, stained with propidium iodide solution, and analyzed using flow cytometry as described in "Materials and Methods." Histograms are representative of one of four independent experiments. The percentage of cells with DNA contents lower than G1 is showed in each histogram.
The cellular response to RA is mainly due to changes in gene expression, resulting from its binding to and activation of specific nuclear receptors (59). Thus far, the mechanism of action of HPR is completely unknown. The differential effects of HPR and RA on the NB model confirm the results obtained in myeloid and lymphoid models, indicating distinct pathways of action for these drugs.

In conclusion, our findings that HPR induces complete growth arrest of NB cells by triggered programmed cell death at pharmacologically relevant concentrations, together with the information regarding its proven mild toxicity (21), the in vitro synergistic antitumor effect of its combination with 5-fluorouracil (60), the strong induction of apoptosis in the hemopoietic system (25), and the cancer chemopreventive effects in animals (21, 50), should provide the basis for clinical testing of HPR in neuroblastoma patients.

ACKNOWLEDGMENTS

We thank T. Carlucci for secretarial assistance. 4-HPR was kindly provided by R. W. Johnson Pharmaceutical Research Institute of Spring House, PA.

ADDITION

Since the submission of this paper, Mariotti et al. (61) have shown that HPR can induce apoptosis at a pharmacologically relevant level in NB cells. Although they did not compare in detail the effects of HPR and RA (the current study), the results of both studies highlight a new drug (HPR) that may have preventive effects in animals (21, 50), should provide the basis for a one year follow-up study of breast cancer patients. Cancer Res., 49: 6149–6152, 1989.


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