All-trans-Retinoic Acid-induced Apoptosis in Human Medulloblastoma: Activation of Caspase-3/Poly(ADP-ribose) Polymerase 1 Pathway

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Abstract

Current treatments for childhood brain tumor medulloblastoma (MB), radiation and chemotherapy, lead to undesirable side effects. Identification of antitumor agents that reduce the toxicity will thus have significant therapeutic value. In this study, we investigated all-trans-retinoic acid (ATRA) as an antitumor agent. Although high concentrations (1–10 μM) of retinoic acid derivatives are generally needed for significant antitumor effects in many cancer cells, we observed that pharmacologically relevant concentrations of ATRA were effective in inhibiting cell death in human MB cells. Using 10-fold lower concentrations (100–500 nM), we found that ATRA inhibits MB (DAOY, D283, D425, and D458) cell proliferation as determined by cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and bromodeoxyuridine incorporation assays. Furthermore, 100 nM ATRA was potent in inhibiting the anchorage-independent growth of the sensitive cell lines (D283, D425, and D458) in soft agar assays. We also demonstrate that the ATRA-induced decrease in cell viability was due to increased cell death by apoptosis, which was accompanied by a 20-fold induction of caspase-3 activity in the most sensitive cell line, D458. By contrast, induction of caspase-3 was only 2-fold in the relatively insensitive DAOY cells. Furthermore, ATRA-induced cell death in D283, D425, and D458 cells was accompanied by activation of caspase-3, a key executioner of apoptosis. We also demonstrate that activated caspase-3 resulted in cleavage of 116-kDa poly(ADP-ribose) polymerase 1 to its signature fragments (85 and 29 kDa). Pretreatment with a specific caspase-3 inhibitor, DEVD-CHO, significantly reduced ATRA-induced apoptotic cell death. Thus, we demonstrate for the first time that low concentrations of ATRA inhibit MB cell proliferation and induce apoptotic cell death in part by activating caspase-3/poly(ADP-ribose) polymerase 1 effector pathway, and we show that retinoic acids and novel retinoids are potential antitumor agents in MB therapy.

Introduction

MBs are the most common malignant brain tumors of childhood with neuronal and glial differentiation that account for more than 20% of pediatric brain tumors. Despite multimodal treatment that includes surgery, radiation, and chemotherapy, tumor recurrence is frequent, and the majority of these patients eventually die from progressive tumor. Adverse neurocognitive effects are common in the long-term survivors. Therefore, identification of antitumor agents with minimal toxicities is beneficial for the long-term survivors. Aberrations in normal differentiation and apoptosis of primitive neuroectodermal cells are implicated in the pathogenesis of neuroblastomas and MBs. Apoptotic cell death is often observed in primary MB tissue. However, the factors that initiate apoptosis and the regulatory pathways, which represent prime targets for therapeutic intervention, are poorly understood. RA derivatives (retinoids) exert antitumor effects by inhibiting cell proliferation and inducing differentiation, cell growth arrest, and apoptosis. The effect of retinoids in neuroblastoma is well documented, and many cell lines are used as model systems to study neuronal differentiation and apoptosis. In contrast, the role of retinoids and their receptors in MBs is poorly understood. The overall aim of our studies is to investigate the therapeutic potential of retinoids and to understand the mechanisms of their action in MB.

Retinoids represent a major class of chemopreventive agents, which exert strong antitumor activity by suppressing in vivo tumor growth. The biological effects of retinoids in target cells are mediated by binding to RARs and RXRs. Both ATRA and 9-cis-RA activate RARs, whereas 9-cis-RA activates RXRs. Both classes of these receptors are sequence-specific, ligand-activated transcription factors. The presence of multiple isoforms of RXR...
and RAR provides potential for complex modulation of signaling by retinoids (10). The spatiotemporal developmental expression pattern and tissue distribution of retinoid receptors suggest that various isoforms of the receptors might play different roles in mediating retinoid signaling (11). RAR-α is expressed ubiquitously in adult tissues, RAR-γ is expressed in skin, and RAR-β is expressed in epithelial cells. Altered retinoid receptor expression has been linked to tumor development (6, 7, 12). RAR-α has been shown to be critical in acute promyelocytic leukemia (PML) because PML-RAR-α contributes to leukemogenesis (13). The expression of tumor suppressor RAR-β is frequently lost in esophageal, lung, and breast cancers (14–16). RAR-β can be induced by retinoids in many cancer cells, including neuroblastomas, and increases in RAR-β correlate with growth-inhibitory effects of retinoids, suggesting that rescue of RAR-β contributes to suppression of tumor growth (17). Exogenous expression of RARs causes increased sensitivity in teratocarcinoma, lung, and breast cancer cells (18–20). Overexpression of RAR-α and RAR-γ in human keratinocyte tumor cells leads to growth inhibition and accumulation of cells in G1 phase of the cell cycle (21).

Despite well-documented effects of retinoids as differentiation-inducing agents in many cancer cells, including neuroblastomas, there are not many studies demonstrating the apoptotic effects of retinoids in target cells. Recent studies demonstrate the activated caspase-3, caspase-7, and PARP-1 cleavage in MB (22), suggesting that apoptotic cell death could play an important role in these tumors. In earlier studies, high concentrations (10 μM) of ATRA were necessary to obtain significant biological effects, and the Fas ligand/Fas system appears to participate in ATRA-induced chemosensitivity of MB cells (23, 24). In view of these observations, we investigated whether retinoids and retinoid receptors play a role in MB. To understand the therapeutic potential, ATRA, a natural retinoid, was selected for these studies. In this report, we present evidence for the first time that low concentrations of ATRA are a potent inducer of apoptosis in selected MB cells. Furthermore, we evaluated the mode of cell death and observed that caspase-3 is an important executioner of apoptosis in MB.

Materials and Methods

**Human MB cell lines (DAOY, D283, D425, and D458) were cultured in Zinc-Option medium containing 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. ATRA was purchased from Sigma, dissolved in ethanol, and kept in the dark at −20°C. MB cell lines growing in mid-log phase were treated with the indicated concentrations of ATRA for different time periods, and the cell lysates were prepared in the buffer as described previously (25). After the protein concentrations were determined by BCA method (Pierce), 100 μg of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. After blocking in 5% nonfat dry milk in Tris buffered saline-triton ×100 (TBST), the blots were incubated with primary and secondary antibodies. The protein bands were visualized with enhanced chemiluminescence reagent. RAR and RXR antibodies were purchased from Santa Cruz Biotechnology. PARP-1 antibodies were from PharMingen.**

**Cell Viability Analysis.** The effect of ATRA on the proliferation of MB cells was determined by MTT method (26) according to the manufacturer’s instructions (Promega). Briefly, 1 × 10^5 cells were seeded in 96-well plates, incubated at 37°C for 24 h, and treated with either vehicle (0.1% ethanol) or the indicated concentrations of ATRA for 3 days. MTT reagent (10 μl/well) was added and incubated at 37°C for 4 h, and the absorbance was measured at 490 nm. Each experiment was repeated three times in triplicate samples. Results were expressed as the percentage of viable cells over control. The results presented are the average of two independent experiments.

**Cell Proliferation Assay.** The ability of ATRA to inhibit DNA synthesis was determined by estimating the amount of BrdUrd incorporation into DNA by a colorimetric immunoassay (Roche). Briefly, cells (2 × 10^5) were cultured in 48-well plates in the presence of different concentrations of ATRA for 3 days. BrdUrd (10 μM, final concentration) was added, and the cells were reincubated for an additional 24 h. After fixing, the cells were incubated with anti-BrdUrd-peroxidase (100 μl/well) for 2 h at room temperature, and the color developed after adding the trimethyl benzidine was measured at 490 nm. The results were expressed as the percentage inhibition of BrdUrd incorporation by ATRA compared with control.

**Anchorage-independent Growth in Soft Agar.** To determine the effect of ATRA on clonal proliferation, soft agar clonogenic assays were performed. After assessing viability, 1 × 10^5 cells were seeded in the presence of the indicated concentrations of ATRA in 35-mm² plates in 0.7% agar. After 2 weeks, the plates were stained with 50 μg/ml MTT, and the colonies were scored. All experiments were performed using triplicates, and each experiment was repeated twice, and the results are the average of two independent experiments.

**Cell Death Detection Assay.** The extent of apoptotic cell death of MB cells (2 × 10^5 cells/well in 48-well plates) exposed to different concentrations (50, 100, 200, and 500 nM) of ATRA was determined by a colorimetric immunoassay using Cell Death Detection ELISA^®plus method (Roche). This assay is based on the quantitation of histone-associated DNA fragments generated in cells undergoing apoptotic cell death. Each experiment was repeated twice times, and the results are the average of two independent experiments. Apoptotic cells were detected with DAPI staining. Briefly, cytosplins of ATRA-treated cells (500 nM, 3 days) were fixed in 4% paraformaldehyde for 8 min, washed with PBS three times, stained with DAPI (1 μg/ml) for 5 min, and observed under a fluorescence microscope.

**Caspase-3 Assays.** Cells (1 × 10^5) were exposed to 200 nM ATRA for 3 days, and caspase-3 activity was determined by a colorimetric method using DEVD-pNA as a substrate (Roche). The cells were harvested in lysis buffer, and 100 μg of protein were incubated with 100 μM DEVD-pNA for 2 h at 37°C and quantitated at 405 nm. Caspase-3 activity was expressed as fold activation over control. To determine the effect of DEVD-CHO, a caspase-3 inhibitor, experiments were performed by pretreating the cells with 50 μM DEVD-CHO for 2 h, followed by exposure to 500 nM ATRA for 20 h. Determining the extent of cell death by Cell Death Detection ELISA^®plus method monitored reversal of caspase-3 function. The results are expressed as enrichment of nucleosomes in the cytoplasm of cells treated
with ATRA. The experiment was repeated two times, and the results presented are the average of triplicates from two independent experiments.

Statistical Evaluation. For all of the experiments, data are expressed as mean $\pm$ SE. One-way ANOVA was used to assess statistical significance between means. $P < 0.05$ was considered to be significant.

Results

Expression of RAR and RXR in MB Cell Lines. The biological effects of retinoids in target cells are mediated by ligand binding to RARs and RXRs. It is not known whether MBs express retinoid receptors. To examine the expression levels of various isoforms of retinoid receptors in MB cell lines (DAOY, D283, D425, and D458) Western blot analysis was performed. As shown in Fig. 1 we observed variations in the levels of retinoid receptors (RAR and RXR) between the four MB cell lines. DAOY cells expressed low levels of RXR-$\alpha$ and RXR-$\beta$. Among the RAR isoforms, the four MB cell lines express RAR-$\alpha$. We could not detect RAR-$\beta$, RAR-$\gamma$, and RXR-$\gamma$. As observed in many other cancer cell lines and tumors, there appears to be a loss of RAR-$\beta$ in MB cell lines also.

ATRA Inhibits the Proliferation of MB Cells. To examine the growth-inhibitory effects, the four MB cell lines were exposed to different concentrations of ATRA (0.1, 0.2, and 0.5 M) for 3 days, and cell viability was determined by MTT assay. Results shown in Fig. 2 suggest that all three concentrations of ATRA caused a dose-dependent decrease in cell viability. For example, 200 nM ATRA results in 32%, 57%, 53%, and 60% inhibition of cell proliferation in DAOY, D283, D425, and D458 cells, respectively. These results are in contrast to previous studies using 5–10 $\mu$M concentrations of RA in combination with cisplatin to achieve significant cell death in Med-3 cells (23, 24).

Effect of ATRA on Anchorage-independent Growth of MB Cells. To determine the in vitro antineoplastic effect, the ability of ATRA to suppress colony formation in soft agar was examined. Because 200 nM ATRA completely suppressed the formation of colonies in D425 and D458 cells, we performed the experiments using 50 and 100 nM ATRA. The number of colonies in control and various treatment groups was counted and is summarized in Fig. 3. From these results, it is evident that 100 nM ATRA caused approximately 90% inhibition of colony formation in D283, D425, and 458 cell lines, indicating that ATRA is a potent inhibitor of clonal proliferation. However, in DAOY cells, 100 nM ATRA caused about 30% inhibition of clonal proliferation.

ATRA Inhibits the Proliferation of MB Cell Lines. To understand whether the decrease in the number of viable cells was due to decreased DNA synthesis leading to decreased cell proliferation, the incorporation of BrdUrd into DNA was determined in the presence of 100, 200, and 500 nM ATRA. As shown in Fig. 4, BrdUrd incorporation results reveal that ATRA
ATRA causes a dose-dependent increase in programmed cell death in MB cells. We consistently observed floating cells (dead cells) in D283, D425, and D458 cells within 24 h after exposure to ATRA. To investigate the mode of cell death, MB cells were treated with 50, 100, 200, and 500 nM ATRA for 3 days, and the extent of DNA fragmentation was quantitated by colorimetric immunoassay. From the results presented in Fig. 5, it was evident that 200 nM ATRA caused 20% cell death in DAOY, whereas in D283, D425, and D458, it caused 49%, 52%, and 81% cell death, respectively. These results suggest that, as observed in MTT and BrdUrd incorporation assays, DAOY cells are relatively resistant, whereas D458 cells were highly sensitive to ATRA. These results indicate that the decrease in the number of viable cells (Fig. 2) could be primarily due to increased apoptosis induced by ATRA. To directly demonstrate apoptosis, MB cells were treated with 500 nM ATRA for 3 days, and the apoptotic nuclei were stained with DAPI. Results shown in Fig. 5C demonstrate apoptosis in D425 and D458 cells. However, under identical conditions, we observed few apoptotic cells in D283 and DAOY cells (data not shown).

ATRA induces the activation of caspase-3 in MB cell lines. Results of cell death assay lead us to hypothesize that ATRA might activate caspase-dependent cell death pathways. In addition, a recent immunohistochemical analysis revealed that the two executioners of apoptosis, caspase-3 and -7, are activated in MB primary tumor tissue (22). These results prompted us to examine whether ATRA-induced cell death was mediated by caspase-3. We treated MB cell lines with 200 nM ATRA for 3 days and determined the caspase-3 activity. Results shown in Fig. 6A demonstrate that ATRA induced caspase-3 activity in all of the four MB cell lines examined. As expected, DAOY cells were relatively resistant (2-fold activation) to ATRA, whereas D458 cells were the most sensitive (20-fold), followed by D425 and D283. To further confirm the contribution of caspase-3 in mediating cell death, we pretreated the MB cell lines with 50 μM caspase-3-inhibitor (DEVD-CHO) for 2 h, followed by exposure to 500 nM ATRA for 20 h. The extent of cell death was determined by cell death immunoassay. We did not perform caspase-3 inhibition studies with DAOY cells because ATRA caused low activation of caspase-3. At the concentrations used in the study, DEVD-CHO did not have any adverse effect in MB cells. Results shown in Fig. 6B indicate that DEVD-CHO partially blocked the cell death observed in presence of ATRA in D283, D425, and D458, supporting the crucial role of caspase-3 in mediating ATRA-induced cell death programs.

Cleavage of PARP-1 in MB cell lines exposed to ATRA. To understand whether caspase-dependent apoptosis affects downstream substrates of caspase-3, we investigated the proteolytic cleavage of PARP-1 in ATRA-treated cells (27). PARP-1 plays an important role in DNA bp excision repair, DNA replication, and transcription (28). Two principal executioner caspases, caspase-3 and -7, are responsible for the processing of PARP-1 to 29- and 85-kDa fragments (29). A recent study identified that caspase-3 and cleaved PARP-1 colocalized in a majority of MB tumors, indicating that caspase-3 may be a major effector caspase leading to cell death in these tumors (22). In view of these observations, we examined whether caspase-3 activated in ATRA-treated cells leads to alteration in the expression/cleavage of PARP-1. The four MB cell lines were treated with 200 nM ATRA for 3 days, and the cleavage of PARP-1 was determined by Western blot analysis. As shown in Fig. 6C, we observed a decrease in the 116-kDa PARP-1 in all of the cell lines. In the most sensitive cell lines, D425 and D458, we observed the generation of PARP-1 cleavage products (85 kDa). In DAOY and D283 cells, we could not detect the 85-kDa fragment due to detection limits in the Western blots. However, we observed a marked decrease in the 116-kDa fragment compared with control cells, indicating that PARP-1 cleavage occurs in ATRA-treated DAOY and D283 cells. Alternatively, it is possible that the decrease in the 116-kDa fragment could be due to a decline in PARP-1 expression in ATRA-treated DAOY and D283 cells. These results suggest that ATRA induced the activation of caspase-3, which in turn cleaves the substrate PARP-1 in D283 and D458 cells. In contrast, 500 nM ATRA had no effect on the cleavage of PARP-1 in selected lung, colon, prostate, and breast cancer; uterine sarcoma; and neuroblastoma cells (data not shown).

Effect of ATRA on the expression of neurofilament-L. To test whether ATRA initiates the synthesis of neuronal markers, the expression of neurofilament-L, a neuron-specific marker, was analyzed by Western blot analysis. It is known that RA-induced morphological differentiation of neuronal cells is accompanied by induction of specific neuronal markers such as MAP2, GAP43, and neurofilaments. Morphological changes and cytoskeletal protein expression are typical hallmarks of neuronal maturation (30). Three of the cell lines (D283, D425, and D458) used in the current study were neuronal in origin. When exposed to low concentrations (10–25 nM) of ATRA for
prolonged periods of time (2 weeks), there was no evidence of morphological differentiation. As shown in Fig. 7, when exposed to 200 nM ATRA for 3 days, we observed increased expression of neurofilament-L (68 kDa) in D283, D425, and D458 cell lines, suggesting that ATRA initiates the synthesis of differentiation markers in MB cells, which are neuronal in origin. We did not observe similar induction of neurofilaments in DAOY cells, possibly due to glial nature of these cells. These results suggest that ATRA might be activating the neuronal markers in the initial stages of drug exposure and that robust activation of apoptotic programs might dominate in D283, D425, and D458 cells that exhibit neuronal phenotype.

Discussion

The present study demonstrates for the first time that caspases mediate, in part, the apoptotic effects of ATRA in MB cells. The cell viability, BrdUrd incorporation, clonogenic proliferation, and caspase-3 activation data presented in this report strongly suggest that ATRA can be further evaluated as an anticancer agent in MB. Expression of retinoid receptors (RAR and RXR) is critical in mediating the effects of retinoids in target cells. We demonstrate for the first time that the four cell lines selected for this study expressed appreciable levels of RXR-\(\alpha\)/H9251, RXR-\(\alpha\)/H9253, and RAR-\(\alpha\)/H9251. The observed biological effects of ATRA are attributable to its binding to RAR-\(\alpha\) because these cells do not express RAR-\(\beta\) and RAR-\(\gamma\), and ATRA does not bind to RXRs. It is possible that these effects are mediated in part by RAR-\(\alpha\) because under the concentrations used in this study, ATRA may not be converted to 9-\textit{cis}-RA, which might activate RXRs. The results of cell death (Fig. 5) and caspase-3 studies (Fig. 6) suggest that differences in the susceptibility of D283, D425, and D458 cell lines to ATRA may be related to differential expression of RAR-\(\alpha\), RXR-\(\alpha\), and RXR-\(\gamma\) or due to activation of receptor-independent mechanisms involving alterations in mitochondrial permeability. DAOY cells, which express low RXR-\(\alpha\) and RXR-\(\gamma\), are relatively less sensitive to
ATRA compared with D283, D425, and D458 cells, which express higher levels of these two receptors and are more sensitive. This is also consistent with low activation of caspase-3 in DAOY cells compared with D458 cells, in which we observed robust caspase-3 activity. In this context, it is important to note that ovarian carcinoma cells that express reduced levels of RAR-α/H9251 and RXR-α/H9251 are relatively resistant to the effects of ATRA (31).

Cell type-specific factors associated with RAR-α/RXR might be different in DAOY cells and contribute to insensitivity. Our results indicate that DAOY cells do not respond like other MB cell lines when treated with ATRA, probably due to glial nature of this cell line. Because many MB-derived cell lines exhibit neuronal and glial characteristics, it is possible that MB cells that are neuronal in phenotype are more sensitive, whereas cells that are glial in phenotype might be relatively resistant to retinoids. In this context, it is important to note that DAOY cells express appreciable levels of glial fibrillary acidic protein and neurofilaments are not induced in this cell line when exposed to differentiation-inducing agents. In contrast D283, D425, and D458 cells do not express glial fibrillary acidic protein but express many markers characteristic of neuronal differentiation including neurofilaments and synaptophysins (32). Additional studies are necessary to understand the mechanisms involved in the relative insensitivity of DAOY cells to ATRA. To achieve appreciable biological effects, 1–10 μM concentrations of retinoids are essential in many cancer cells (23, 33, 34). There are few reports demonstrating the antiproliferative effects of retinoids in MB cell lines using high concentrations (10 μM) of retinoids (23–24). In contrast, our studies for the first time demonstrate that 200–500 nM ATRA induces significant apoptosis in D283, D425, and D458 cells. Our results also indicate that cell lines that are neuronal in origin (D283, D425, and D458) might be more sensitive compared with DAOY cells, which express markers of glial lineage also.

We have provided several lines of evidence indicating that ATRA induces robust apoptosis in part by activating caspase-3, a key executioner of apoptosis. First, we demonstrated a significant dose-dependent increase (Fig. 5) in histone-associated DNA fragmentation (apoptosis) in D283, D425, and D458 cells. The effect of ATRA on the levels of PARP was determined by Western blot analysis. Cells were exposed to 500 nM ATRA for 3 days, and 100 μg of protein were subjected to Western blot analysis and developed with mouse monoclonal antibodies to PARP-1.
Additional studies are needed to delineate the role of retinoid receptors in MB. In addition, it is possible that receptor-independent mechanisms might participate in robust apoptosis induced by low doses of ATRA that may be unique to MB cells of neuronal origin. The cell pathways mediating the effects of ATRA appear to be dependent on the cell line under investigation. For example, a novel retinoid, CD347, activates caspase-3 in leukemic cells, but ATRA failed to exert any effect on caspase-3 (35). In the current study, we provide evidence indicating that ATRA-induced apoptosis in human MB cell lines is mediated in part by caspase pathways. Our observations in the cell culture model system are in agreement with similar colonization studies of activated caspase-3, caspase-7, and cleavage of PARP-1 in primary tumor tissue (22). Taken together, results presented in this report indicate that ATRA-induced activation of caspase-3/PARP-1 network contributes significantly in activating apoptotic programs.

In a clinical setting, the toxic side effects of retinoids can be avoided when target cells are highly responsive to low concentrations of retinoids. In many cell culture studies, ATRA, 9-cis-RA, and N-(4-hydroxyphenyl) retinamide (4HPR) inhibit cell proliferation at 1–10 μM concentrations, depending on the cell line and culture conditions (6, 33, 34). In patients who received 300–400 mg/day ATRA, the plasma concentration was about 0.15 μM (36). Results presented in this study indicate that selected MB cells are sensitive to 0.2 μM ATRA. Therefore, we propose that ATRA or other retinoids might be effective in suppressing MB tumor growth in vivo by caspase-mediated apoptosis. We anticipate that future investigations evaluating the antitumor potential of ATRA or novel retinoids will be necessary in determining their clinical use for MB.

Acknowledgments

We are grateful to Dr. Friedman and Dr. Darell Bigner for the MB cell lines and Dr. Cosenza for other cancer cell lines. We thank Asha Guttapalli for preparing the manuscript.

References


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