Induction of Apoptosis in Primary Meningioma Cultures by Fenretinide

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Abstract
Fenretinide, a synthetic retinoid that induces apoptosis in tumor cells in vitro, is being evaluated in clinical trials as a chemotherapeutic agent against several malignancies. Due to its ease of administration, long-term tolerability, and low incidence of long-term side effects, we explored its potential as a therapeutic agent against meningiomas by examining its efficacy in vitro against such cells in primary culture. Cells, cultured from freshly resected benign, atypical, or malignant meningiomas, were exposed to fenretinide (10 μmol/L). Treatment effects were assessed using flow cytometry, Western blot analysis, semiquantitative reverse transcription-PCR for retinoid receptor expression, and changes in insulin-like growth factor-1 (IGF-1)-induced proliferation. Fenretinide induced apoptosis in the three grades of meningioma primary cells tested, as shown by the appearance of a sub-G1 fraction in flow cytometric analysis and by the detection of polyadenosyl ribonucleotidyl phosphorylase cleavage indicating caspase activation. Fenretinide treatment also increased levels of the death receptor DR5 and caused mitochondrial membrane depolarization. The levels of the retinoid receptors, retinoic acid receptor α and retinoid X receptor γ, were upregulated in response to fenretinide, suggestive of ligand-induced receptor up-regulation. IGF-1-induced proliferation in the meningioma cells was abolished by fenretinide. We conclude that fenretinide induces apoptosis in all three histologic subtypes of meningioma and exerts diverse cellular effects, including DR5 up-regulation, modulation of retinoid receptor levels, and inhibition of IGF-1-induced proliferation. These results provide preliminary evidence that fenretinide has activity against meningiomas and suggest that further studies are warranted to explore its potential as a therapeutic agent against meningiomas.

Introduction
Meningiomas constitute ~20% of all intracranial primary brain tumors and are more frequent in females (1). Most meningiomas are effectively addressed by surgical resection. Unresectable, residual tumors after partial resection, and recurrent meningiomas are treated with radiation therapy. However, patients who are not candidates for surgery or radiotherapy or whose tumors recur after such treatments have limited therapeutic options. Chemotherapy has no proven benefit in controlling these tumors; immunotherapy, hormonal therapy, and agents such as hydroxyurea have also been tried, with uncertain benefits (2). Therapeutic strategies based on meningioma biology are currently lacking (3). Because the indolent nature of many meningiomas leads to long survival of such patients who have them, the agents used for treatment need to be easily given, well tolerated, and suitable for long-term therapy.

Retinoids are vitamin A derivatives that in their natural forms exert diverse biological effects in humans and show activity against malignancies. Direct inhibitory effects include inhibition of growth and enhancement of cell differentiation; indirect effects include angiogenesis inhibition (4). Synthetic retinoids such as all-trans retinoic acid have also been used for chemoprevention and treatment of specific malignancies like acute promyelocytic leukemia. Fenretinide is a related synthetic retinoid, which inhibits growth by inducing apoptosis in various tumor cell lines. It is well tolerated during long-term oral administration, as shown in chemoprevention studies (5–7) and in recent phase I trials against solid tumors (8–10). Fenretinide induces apoptosis in vitro in several solid tumors by retinoid receptor–dependent and retinoid receptor–independent pathways and in part due to the generation of free radicals and activation of the caspase pathway (11, 12). The current study aimed to determine the in vitro efficacy of fenretinide against meningiomas and to elucidate its mechanism of action.

Materials and Methods

Primary Meningioma Cell Culture. All tissue samples were obtained at the University of Texas M.D. Anderson Cancer Center under a protocol approved by the Institutional Review Board. Primary cultures of meningiomas were established as previously described. (13) Briefly, tumor tissue was collected at the time of resection from patients with benign, atypical, or malignant meningioma (Table 1) as determined by neuropathologic review using WHO 2000 criteria. (14) Tumor fragments were immediately transferred to the laboratory for further processing, carefully minced into small pieces, and treated with 0.4% trypsin/EDTA to dissociate the cells. The cells were washed with PBS supplemented with 200 units/ml penicillin and 200 μg/ml streptomycin and were transferred to 100-mm2 tissue culture dishes containing DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown in a humidified incubator at 37°C with 5% CO2 for 5 days, with periodic medium changes and were allowed to reach confluence. Low-passage cells (<3 passages) were used for subsequent experiments.

Western Blot Analysis and Antibodies. Cleavage of polyadenosyl ribonucleotidyl phosphorylase (PARP) and levels of expression of DR5 were studied by Western blot analysis at the indicated times after exposure to drug (Fig. 2). For PARP cleavage studies, the cell pellet was treated with a reducing buffer containing 6 mol/L urea to improve detection. Briefly, 20 μg of protein sample were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Inc., Keene, NH). The membrane was blocked using Blot-to-Tween [5% nonfat dry milk, 0.05% Tween, 1% Triton X-100, 0.9% NaCl, and 50 mmol/L Tris (pH 7.5)]. Immunoblotting was done using the following antibodies at the dilutions indicated: rabbit anti-human DR5 polyclonal IgG, 1:500 (Alexis Biochemicals, San Diego, CA); and mouse anti-human PARP monoclonal IgG, 1:500 (PharMingen, San Diego, CA). Horseradish peroxidase–conjugated donkey anti-rabbit and goat anti-mouse antibodies (Amersham Co., Arlington Heights, IL) were used as secondary antibodies. Actin levels were determined as a loading control using a mouse

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antihuman β-actin monoclonal antibody (Amersham). The blots were developed using the enhanced chemiluminescence chemiluminescent detection reagent (Amersham Biosciences, Piscataway, NJ).

Flow Cytometric Analysis. Cells were plated at a density of $10^5$ cells per dish and exposed to fenretinide (10 μmol/L) for various periods (24, 72, and 96 hours). The harvested cells were fixed in 70% ice-cold ethanol, treated with propidium iodide (50 mg/mL) and RNase (20 mg/mL), and subjected to flow cytometry using a EPICS II flowcytometer (Coulter Co., Hialeah, FL) equipped with an air-cooled argon ion laser, and emitting at a wavelength of 488 nm at 15 mW. A minimum of 10,000 events was analyzed per sample using the Coulter cytologic program. The cells, sorted according to their DNA content into various phases of the cell cycle were, were analyzed for changes in the cell cycle distribution other than appearance of a sub-G1 fraction were seen in response to fenretinide treatment.

Meningioma cultures (M17, benign; M2, atypical; M31 and M33, malignant) treated with fenretinide were analyzed using flow cytometry. No significant changes in the cell cycle distribution other than appearance of a sub-G1 fraction were seen in response to fenretinide treatment.

Real-time Quantitative Reverse Transcription-PCR. Total RNA was extracted from primary meningioma cultures according to the manufacturer’s instructions using the TRizol reagent (Invitrogen, Carlsbad, CA). Aliquots of each RNA (100 ng) were reverse transcribed in quadruplicate (including a no reverse transcriptase control) in 10 μL total volume with a RT master mix consisting of 400 nmol/L assay-specific reverse primer, 500 μmol/L deoxynucleotides, Superscript II buffer, DTT, and 10 units Superscript II reverse transcriptase (Life Technologies, Rockville, MD) at 50°C for 30 minutes, followed by 72°C for 10 minutes. Specific quantitative assays for retinoic acid receptor γ (RARγ) and retinoid X receptor α (RXRα) were developed using Primer Express software (Applied Biosystems, Foster City, CA) following the recommended guidelines based on sequences from Genbank. The forward and reverse primers and probe used for each target were as follows respectively: hRARγ: (855+) TGCATTATCAAGATCGTGAG, (928–) GTGATCTGTGACGAATGCTG and (880+)FAM CCAACGCGTTGCTGCTTAC; hRXRα: (1278+) GGCTTACCTGCAACGACAAGTA, (1341–) CAGCGGAGCAAGAGCTTA and (1321–)FAM-CGAACCTTCCGCGCTCTCTG. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log range and a no template control. Subsequently, 40 μL of PCR mix containing 1× PCR buffer, 400 nmol/L specific forward and reverse primers, 3 mmol/L MgCl₂, 200 μmol/L deoxynucleotides, 1.25 units Taq DNA polymerase (Life Technologies), and 100 nmol/L fluorogenic probe were added to each 10-μL reverse transcription reaction. Amplification was done by use of the ABI Prism 7700 sequence detection system (Applied Biosystems) at 95°C for 1 minute, followed by 40 cycles of 40 amplifications cycles.

Table 1. Designation of grades of malignancy of primary meningioma cultures

<table>
<thead>
<tr>
<th>Grade</th>
<th>Primary culture</th>
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<tr>
<td>Benign</td>
<td>M46, M17</td>
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<tr>
<td>Atypical</td>
<td>M2, M68, M71</td>
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<tr>
<td>Malignant</td>
<td>M6, M31, M33</td>
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Figure 1. Fenretinide induces apoptosis in primary meningioma cultures. A. primary cultures derived from benign (M46 and M17), atypical (M2), and malignant (M6) meningiomas ($10^5$ cells per dish) were exposed to fenretinide (10 μmol/L) for 72 hours and analyzed by flow cytometry for induction of apoptosis. Columns, derived from at least three independent experiments; bars, SE. A significant increase in the sub-G1 fraction was seen in fenretinide-treated cells. B. Cell cycle profiles of meningioma cultures (M17, benign; M2, atypical; M31 and M33, malignant) treated with fenretinide were analyzed using flow cytometry. No significant changes in the cell cycle distribution other than appearance of a sub-G1 fraction were seen in response to fenretinide treatment.
a 12-second step at 95°C and a 1-minute step at 60°C. The resulting data were analyzed using SDS software (Applied Biosystems) with TAMRA as the reference dye. Synthetic DNA oligos used as standards (sDNA) encompassed exactly the entire 5’ to 3’ amplicon for the assay (Biosource International, Camarillo, CA). The amount of RNA added to a reverse transcription-PCR from each sample was more accurately determined by measuring the β-actin transcript levels in each sample. The final data were normalized to β-actin and are presented as the molecules of transcript/molecules of β-actin × 100 (% β-actin).

Results

Fenretinide Induces Apoptosis in Meningiomas. Fenretinide is being evaluated in clinical trials against several different malignant tumors, but preclinical or clinical data are not available on its activity against meningiomas. Given that fenretinide is well tolerated during long-term administration and could potentially be a suitable treatment for meningiomas, we studied its efficacy against meningioma cells in vitro. Most studies in which fenretinide showed in vitro activity against malignancies used established tumor cell lines, which may not mirror activity against the original tumor due to changes from acquired genetic alterations. In this study, we used early-passage cells in primary culture derived from freshly resected specimens of human meningioma. Exposure of these cells to fenretinide resulted in the appearance of a sub-G1 fraction of cells seen at 72 hours, indicating post-treatment induction of apoptosis (Fig. 1). To determine if this effect was related to the histologic grade of the tumor, fenretinide was assessed in primary cultures derived from low-grade, atypical, and malignant meningiomas. Apoptosis was induced regardless of degree of malignancy, suggesting that fenretinide uses common pathways for inducing apoptosis, which remain intact irrespective of tumor grade. To ascertain whether the effects seen with fenretinide were specific for this retinoid, primary meningioma cells (M6, M2, and M46) were treated with

![Figure 2. Effect of 13 cis-retinoic acid and ATRA on primary meningioma cultures.](image)

![Figure 3. Effect of fenretinide treatment on levels of the DR5 death receptor.](image)
13 cis-retinoic acid and all-trans retinoic acid. In contrast to fenretinide, these retinoids did not induce apoptosis in the meningioma cells suggesting that the effect was specific to fenretinide (Fig. 2).

**Fenretinide Up-regulates DR5 Levels and Induces Cleavage of PARP.** Previous studies have shown that retinoids can up-regulate the expression of death receptors, activating the caspase-mediated direct apoptotic pathway (15, 16). The principal tumor necrosis factor–related apoptosis inducing ligand receptor, DR5, is a death receptor involved in inducing apoptosis in response to various endogenous and exogenous signals. We used Western blot analysis to determine the effect of fenretinide treatment on DR5 protein expression and found that DR5 levels were up-regulated in all primary meningioma cell cultures tested regardless of tumor grade (Fig. 3). These data suggest that death receptor activation could be one mechanism by which fenretinide induces apoptosis in meningiomas. PARP cleavage is one downstream effect of death receptor activation and is mediated by caspase activation. Fenretinide treatment resulted in PARP cleavage in all grades of meningiomas, further confirming that the induction of apoptosis was the primary means of cell death caused by this agent (Fig. 4).

**Meningioma Cells Exhibit Mitochondrial Membrane Depolarization in Response to Fenretinide.** Fenretinide activates the mitochondrial apoptotic pathway in some cell types by triggering generation of reactive oxygen species, which induces the release of cytochrome c, and subsequently activates caspase-9. The depolarization of the mitochondrial membrane is an early feature of such activation. To determine if fenretinide activates this reactive oxygen species–driven pathway in primary meningioma cells, we measured the relative uptake of the mitochondrial stains, CMXRos and MTG. CMXRos is lipophilic and is concentrated in active mitochondria, whereas MTG preferentially accumulates in mitochondria regardless of mitochondrial membrane potential. Double staining with both dyes results in labeling all cells by MTG and selectively labeling of nonddepolarized mitochondria (live cells) by CMXRos, which form a separate population detected by flow cytometry (Fig. 5). Fenretinide treatment resulted in the loss of mitochondrial membrane potential in primary meningioma cells, with the degree of depolarization being unrelated to the grades of meningioma cells suggesting that the degree of malignancy does not influence this effect.

**Fenretinide Induces RARγ and RXRa Expression.** Fenretinide has been shown to preferentially transduce signals via RAR-γ and induces the receptor expression (17). To determine if fenretinide-mediated signals in meningioma cultures resulted in increased expression of RARγ and its receptor partner, RXRa, as a downstream effect, we assessed the levels of mRNA for the two receptors by real-time quantitative reverse transcription-PCR, normalizing the results to β-actin mRNA as the control. The M46, M6, and M2 primary meningioma cultures tested in this experiment showed expression of mRNA for both retinoid receptors. After exposure to fenretinide for 48 hours, the levels of expression of RAR-γ and RXRa were elevated at the same drug concentration that apoptosis had been induced (Fig. 6). It is thus possible that the receptor-dependent effects of this retinoid are relevant to its ability to induce apoptosis, as suggested in previous reports of fenretinide activity in other cell types (15).

**Fenretinide Inhibits IGF-I-Induced Proliferation in Primary Meningioma Cultures.** IGF-I has been implicated as a potent proliferative signal for malignant cells. We have previously shown...
that IGF-I can induce proliferation in primary meningioma cultures (13). To determine if fenretinide could directly alter the effects of IGF-I on these cells, we examined changes in the proliferative rate of meningioma cultures exposed to both agents. Treatment of meningioma cultures with IGF-I for 24 hours resulted in a dose-dependent increase in cell proliferation as measured by the XTT proliferation assay (Fig. 7). At the 24-hour time point, fenretinide-treated cells showed a proliferation rate similar to control cells. However, when treated concurrently with both agents, fenretinide-treated cultures failed to undergo an increase in proliferation in response to IGF-I.

Discussion

About 15% to 20% of meningiomas exhibit a more aggressive clinical course (2). Such tumors are more likely to recur after surgical removal and may require adjuvant therapy. As a preliminary assessment of the potential of fenretinide as a therapeutic agent in this setting, we assessed its in vitro activity against primary meningioma cells derived from various histologic grades of the tumor and studied the signaling pathways activated. Data from phase I trials of fenretinide have shown that serum drug concentrations of up to 10 μmol/L are clinically achievable, which provides the rationale for the concentrations of the agent used in this study.

When exposed to fenretinide, meningioma cells became growth inhibited, rounded in morphology, detached from the culture flask, and subsequently showed membrane blebbing and nuclear condensation (data not shown) suggestive of apoptosis. As previously reported in other neoplastic cells, further investigation confirmed that fenretinide-induced meningioma cell death was mediated through induction of apoptosis, as seen in morphologic, flow cytometric, and biochemical studies. That the induction of apoptosis was independent of grade of malignancy suggested that the biological pathways for fenretinide-induced apoptosis are intact in these cells and are conserved across tumor grades. Fenretinide-induced apoptosis was mediated by caspases, as shown by PARP cleavage, a downstream indicator of caspase activation. Fenretinide also caused depolarization of mitochondrial membrane, an effect that is believed to be related to free radical generation and to the subsequent recruitment of the intrinsic apoptotic pathway. To determine if the effects of fenretinide on primary meningiomas cells were shared by other retinoids, we assessed the effects of 13 cis-retinoic acid and all-trans retinoic acid on three representative primary meningioma cultures (i.e., benign, atypical, and malignant grades). Neither retinoid induced apoptosis suggesting that the effect of fenretinide on meningiomas was specific to this agent. These results are consistent with demonstrations of fenretinide's effects in other cell types, as reported by other investigators (18–20).

The induction of DR5, the principal receptor for tumor necrosis factor–related apoptosis inducing ligand, is of particular interest because it suggests that the death receptor-dependent extrinsic apoptotic pathway could also be recruited by fenretinide in meningioma cells. Sun et al. previously showed that apoptosis induced by the synthetic retinoid, CD437, is associated with an increased expression of the death receptors, DR4, DR5, and Fas in a p53-dependent manner, and that this effect was selective for malignant cells. In a related cDNA microarray experiment using malignant glioma cells, we found that treatment with 10 μmol/L fenretinide resulted in a greater than threefold up-regulation of p53 compared with the control.3 The p53 status of the primary meningioma cells examined in this study is not yet known, nor is it known if the observed induction of DR5 expression is p53 dependent in meningioma cells; these effects are being examined in ongoing studies. Our results suggest that treating primary meningioma cultures that have been exposed to fenretinide may be more sensitive to tumor necrosis factor–related apoptosis inducing ligand due to the increased DR5 protein expression.

3 VK Puduvalli, unpublished data.
Retinoid ligands interact with RAR/RXR heterodimers that in turn assemble the transcriptional machinery responsible for transactivating retinoid-responsive genes. Retinoid receptors are critical in mediating the pleiotropic biological effects of natural and synthetic retinoids, although their role in retinoid-induced apoptosis is less clear (21, 22). Inhibition of RAR receptors only partially abrogates fenretinide-induced apoptosis in some cell types. The retinoic acid-resistant HL60 myeloid leukemia cells that have a mutant RARα are sensitive to fenretinide and efficiently undergo apoptosis, suggesting that this receptor might not be critical to fenretinide's activity (23). Overexpression of RARγ induces terminal differentiation of the human embryonal carcinoma cell line, NT2/D1 in cooperation with RXRα (24, 25). In a derivative retinoid-resistant cell line, NT2/D1-R1, resistance is conferred by suppression of RARγ and overcome by overexpression of RARγ. Fanjul et al. reported that RARγ is preferentially induced by fenretinide and plays an important role in fenretinide's receptor-dependent effects (17). These findings provided the rationale for our experiments to assess the effects of fenretinide on RARγ mRNA expression and on its heterodimeric partner RXRα. Quantitative measurement of mRNA levels by quantitative reverse transcription-PCR showed increased RARγ and RXRα mRNA levels compared with untreated control cells, supporting the finding that these receptors are transcriptionally activated by fenretinide.

A few in vitro studies have shown that IGF-I and its binding proteins induce proliferation of meningioma cells (26). Some investigators reported that meningiomas strongly express IGF-I mRNA and protein in contrast to normal pachymeningeal tissue, which has undetectable levels. This finding suggests that an autocrine/paracrine loop involving IGF-I could be active in meningiomas but not in non-neoplastic meningeal cells (27, 28). Others have argued that an autocrine mechanism cannot account for the action of IGF-I in meningioma growth and theorize that inhibiting circulating IGF-I decreases meningioma growth and even caused tumor regression in vivo (29). Treatment of primary meningioma cells with IGF-I increases their proliferation rate (13) suggesting circulating IGF-I may potentially have a direct growth promoting effect on meningiomas. This finding is relevant to fenretinide therapy because sustained reduction in circulating IGF-I levels has been noted in patients receiving long-term fenretinide therapy in breast chemoprevention trials (30). This effect persisted after 2 years of fenretinide therapy with IGF-I level reverting to baseline only after the therapy was discontinued. Results from the current study confirm that exogenous IGF-I can act as a proliferation factor in primary meningioma cells. In addition, the proliferative effect of IGF-I was abolished by concurrent treatment with fenretinide. Thus, fenretinide may potentially inhibit the IGF-I-mediated growth signal in meningiomas in two different ways, first by reducing the levels of circulating IGF-I and second by inhibiting the direct proliferative signal of this factor upon meningioma cells. This study is the first to show that fenretinide inhibits proliferation and induces apoptosis in meningiomas. Additional studies are needed to elucidate the signaling pathways activated by fenretinide in this tumor type particularly with reference to the effects of IGF-I on meningioma cells. Fenretinide is well tolerated during long-term administration and is currently being tested as a therapeutic agent against several malignancies. The demonstration of in vitro activity against meningiomas provides a rationale for exploring the possibility of clinical trials in patients with recurrent or unresectable meningiomas who have no other viable options for therapy.

Acknowledgments


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Figure 7. IGF-1-induced proliferation of meningioma cells is abrogated by fenretinide. Malignant meningioma cultures (M6) were plated in triplicate in 96-well plates at a density of 3 × 10⁶ per well and treated with IGF-1 alone, fenretinide alone, or a combination of the two at the indicated concentrations. The cells were harvested and the viable cell fraction assessed using the XTT assay. A dose-dependent increase in the cells is seen with the addition of IGF-1 compared with untreated control cells. The addition of fenretinide abolished the proliferative effect of IGF-1 on the cells.
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