Antitumor effects of antiprogestersones on human meningioma cells *in vitro* and *in vivo*

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The presence of the progesterone receptor (PR) in meningioma tissue has been confirmed by previous investigations. Studies have shown that the antiprogestosterone drug, mifepristone, is a potent agent that inhibits the growth of cultured meningioma cells and reduces the size of meningiomas in experimental animal models and humans. However, these studies have not fully examined the relationship between the antitumor effects of an antiprogestosterone agent and the expression of the PR.

The present study examined the antitumor effects of mifepristone and a new potent antiprogestosterone agent, onapristone, and the relationship between the antitumor effects of these antiprogestersones and the expression of PR's in meningiomas *in vitro* and *in vivo* was also investigated. Meningioma tissue surgically removed from 13 patients was used in this study. In the *in vitro* arm of the study, mifepristone and onapristone exhibited cytostatic and cytotoxic effects against cultured meningioma cells, regardless of the presence or absence of PR's; however, three PR-negative meningiomas showed no response to any dose of mifepristone and/or onapristone. In the *in vivo* arm, meningioma cells, embedded in a collagen gel, were implanted into the renal capsules of nude mice. Antiprogestosterone treatment resulted in a marked reduction of the tumor volume regardless of the presence or absence of PR's. No histological changes in the meningioma cells suggestive of necrosis or apoptosis were detected in any of the mice treated with antiprogestersones. These findings suggest that mifepristone and onapristone have an antitumor effect against meningioma cells via the PR's and/or another receptor, such as the glucocorticoid receptor.

**Key Words**  meningioma  antiprogestosterone  progesterone receptor  mifepristone  onapristone  cell culture  nude mice

In normal tissue, progesterone receptors (PR's) are found in the nuclei of uterine epithelia, stroma and smooth muscle, ductal and lobular epithelial cells of the breast, epithelial, stromal, and luteal cells of the ovary, pulmonary parenchymal cells, pituitary parenchymal cells, and the adult meninges. In contrast, in neoplastic tissue the PR has been detected in breast, uterine endometrial, and ovarian carcinomas and intracranial meningiomas.

An antiprogestational agent, mifepristone, with a high affinity for PR's has shown antiproliferative and cytotoxic effects against human breast cancer cell lines *in vitro*; these effects can be prevented by saturation of PR's with progesterone. These findings suggest that mifepristone exerts its antitumor effects against breast cancer cells by a receptor-mediated pathway. The clinical efficacy of mifepristone has been confirmed by previous work in 11 postmenopausal patients with metastatic breast cancer.

The possibility that the growth of meningiomas may be enhanced by female sex hormone levels is supported by higher incidence in women and reports of the exacerbation of its symptoms during pregnancy. In breast cancer, the estrogen receptor is expressed more frequently than the PR, but the opposite is true in meningioma. Previous studies have shown that mifepristone causes growth inhibition in cultured meningioma cells and in meningiomas subcutaneously implanted into nude mice. Clinical trials have also shown that mifepristone is effective in patients with meningioma. However, these studies have not fully examined the relationship between the antitumor effects of mifepristone and the expression of PR's. We examined the antitumor effects of mifepristone and a new potent antiprogestational agent, onapristone, on meningioma cells and correlated the antitumor effects of these antiprogestersones and the expression of PR's in *in vitro* and *in vivo*. To our knowledge, this report is also the first study on the efficacy of onapristone against human meningiomas.
Materials and Methods

Antiprogesterones

The antiprogesterone agents used for this study were mifepristone (RU38486; 17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl)estradiol-3-one-6β) and onapristone (ZK98299: 11β-(4-dimethylamino-phenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13α-methylestradiol-3-one).  

Clinical Specimens

Meningioma tissues were surgically obtained from 13 previously untreated patients. After the initial diagnosis of meningioma by frozen section, additional specimens were fixed in 10% buffered formalin for routine pathological examination. The remaining portion of each specimen was divided into two parts: one was frozen at –80°C for the detection of the PR by immunocytochemistry, and the other was used for primary cell cultures.

Detection of Progesterone Receptor

Human meningioma tissues were cut in sections 5 μm thick at –20°C using a cryostat. The sections were thaw-mounted onto poly-L-lysine-coated glass slides, dried, and fixed in a picric acid-paraformaldehyde solution for 15 minutes at room temperature. They were rinsed several times in phosphate-buffered saline (PBS, pH 7.4) for 15 minutes at 4°C, then immersed in 1% H2O2 to block the endogenous peroxidase activity in the tissues. After a further rinse in PBS, they were treated with 1% normal mouse serum to reduce the nonspecific reaction and incubated in mouse monoclonal antibody (1:40) against human PR for 12 hours at 4°C in a moisture chamber. The antibody was diluted in PBS containing 0.05% Tween-20. The sections were next treated with goat anti-mouse biotinylated antibody and avidin-biotin complex reagent according to the manufacturer’s protocol,† then incubated in PBS containing 0.01% 3,3’-diaminobenzidine and 0.005% H2O2. After the color reaction developed, the sections were counterstained with methyl green, then dehydrated and mounted. Control sections were treated either by incubation without primary antibody or by substituting nonimmune mouse serum for primary antibody.

Primary Culture of Meningioma Cells

Immediately after resection, the meningioma tissues were immersed in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium supplemented with 15% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and minced with a razor blade into small fragments under sterile conditions. The tissue fragments were incubated with shaking at 37°C for 40 minutes in freshly prepared DMEM/Ham’s F-12 medium containing Dispase.‡ A detailed description of the procedures has been published previously. The dissociated cell suspension was filtered through a nylon mesh, and a dispersed single-cell suspension was used for the experiments described below.

In Vitro Study

The meningioma tissues from the 13 patients were used for in vitro study. For the primary culture of the meningioma cells, 0.5 ml of the dispersed single-cell suspension was placed into a 24-well plate precoated with poly-L-lysine at a density of 1 × 103 cells/well. After 3 days of culture, the medium was replaced with DMEM/Ham’s F-12 medium containing 15% FBS pre-treated with dextran-charcoal to remove unbound steroid. Mifepristone and onapristone were dissolved in 95% ethanol and added to the media described above to yield final concentrations of 10−6, 10−5, and 10−4 M. The final concentration of ethanol in the experimental and control media was 0.01%. The cultured meningioma cells were treated with the antiprogesterones at concentrations of 10−6, 10−5, and 10−4 M beginning on Day 3 of culture. All culture media were changed every 3 days.

To examine the specificity of these antiprogesterones on meningioma cells, an established rat pituitary tumor cell line (GH3) and a human glioblastoma cell line (A-172) were used as positive and negative controls, respectively.§

The antitumor effects of the antiprogesterones were determined by colorimetric assay.‡‡ For this, the culture medium was replaced with 500 μl of PBS containing 0.1% sodium succinate and 0.4% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) and incubated for 3 hours at 37°C in a CO2 incubator. After incubation, the MTT solution was aspirated and MTT formazan was dissolved in 500 μl of dimethyl sulfoxide. A 100-μl aliquot of the resultant solution was transferred in duplicate to 96-well microplates and the optical density was measured using a microplate reader with a test wavelength of 570 nm and a reference wavelength of 620 nm. Under the experimental conditions described, the optical density of the MTT assay was linear over a range of 3.9 × 103 to 8.0 × 105 cells/well; all values in every experimental group were within this range.

In Vivo Study

The meningioma tissues obtained from three patients (Cases 2, 3, and 7) were used for the in vivo study. The meningioma cells were plated in 100-mm dishes at an initial density of 2.0 × 106 cells/dish. After reaching confluency, the cultured cells were trypsinized to obtain a dispersed single-cell suspension. The cell suspension was centrifuged, and the resultant cell pellet

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* Mifepristone kindly provided by Dr. D. Martini, Roussel-Uclaf, Romainville, France, and onapristone by Dr. D. Henderson, Schering, Berlin, Germany.
† Reagent obtained from Vector Laboratories, Inc., Burlingame, California.
‡ Dispase obtained from Godo Shuisei Co., Ltd., Tokyo, Japan.
§ Cells supplied by the Japanese Cancer Research Resources Bank, Tokyo, Japan.

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was resuspended at a density of $5 \times 10^7$ cells/ml in a collagen solution on ice. Ten $\mu$l ($5 \times 10^6$ cells) of this solution was dropped onto an uncharged plastic dish, and the dish was inverted and incubated at $37^\circ$C in a CO$_2$ incubator for 30 minutes to convert the collagen solution into a gel. Pieces of the collagen gel were immersed in media and were detached from the bottom of the dish by pipetting.

For implantation of a piece of the collagen gel into the subrenal capsules of nude mice (athymic female BALB/C nu/nu mice aged 4 weeks), the animals were anesthetized intraperitoneally with diluted Nembutal (pentobarbital, 1:10). The surgical procedures were performed according to previously described methods. All meningioma cells embedded in the collagen gel grew successfully. After implantation of the gel into the subrenal capsule, the animals were separated into three groups. Two groups were injected with 0.4 mg of either mifepristone or onapristone dissolved in 0.4 ml sesame oil. The other group received an injection of sesame oil alone and served as a control. In all groups, the antiprogesterone injections were administered subcutaneously every day for 28 days beginning on the day of implantation. At autopsy, performed 28 days after the operation, the tumor region containing the renal tissue was dissected, fixed in 10% buffered formalin, and embedded in paraffin. Serial sections 6 $\mu$m thick were cut, and the tumor area was measured from photographic prints. The tumor volume was calculated based on the tumor area in sections at 60-$\mu$m intervals. To examine the effect of the antiprogesterones on the uterus, ovary, and adrenal glands, these organs were removed and weighed. For histological examination, they were fixed in 10% buffered formalin and embedded in paraffin.

Statistical Analysis

Statistical analysis of differences was performed by the Mann-Whitney U-test followed by the Kruskal-Wallis H test.

Results

Meningioma Specimens and the Existence of PR

In PR-positive meningiomas, the PR's of these cells were localized only in the nuclei by immunocytochemistry using a monoclonal antibody to PR's (Fig. 1A). The percentage of PR-positive nuclei varied among four meningioma specimens (Cases 1 to 4). In contrast, neither the cytoplasm nor the nuclei showed immuno-reactivity in the PR-negative meningioma cells in Cases 5 to 13 (Fig. 1B) or the negative controls. We defined as PR-negative meningiomas, those specimens that showed no specific staining in any of the tumor cells.

Histopathological examination of the 13 tumors showed that nine were a meningothelial type, two were a transitional type, and the other two were a fibroblastic or an angiomatic type (Table 1). All four PR-positive tumors (31%) were meningothelial. However, there was no correlation between the existence of PR's in meningioma tissues and either the tumor location, the maximum diameter of the tumor, or the sex or age of the patients (Table 1).

In Vitro Study

Both antiprogesterones showed an antitumor effect from the 3rd day of treatment, but significant differences were not seen at all concentrations of these antiprogesterones (Fig. 2). A PR-positive meningioma specimen (Case 2) showed profound antitumor effects after 21 days of treatment, especially with mifepristone.

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**FIG. 1.** Immunocytochemical studies for progesterone receptor (PR). ×215, counterstained with methyl green. A: Photomicrograph of a PR-positive meningioma obtained from Case 1, showing the presence of nuclear staining. B: Photomicrograph of a PR-negative meningioma obtained from Case 5, demonstrating the absence of nuclear and cytoplasmic staining.

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Mice obtained from Charles River Japan Co., Ltd., Yokohama, Japan.
Mifepristone

Onapristone

Mifepristone

Onapristone

Mifepristone

Onapristone

Fig. 2. Graphs showing the antitumor effect of antiprogesterones in the in vitro studies. Values show the mean ± standard error of the mean for four culture dishes. The mean values (growth) of optical density in the initial control were designated as 100%. Asterisks: significant difference from the comparable control, p < 0.05. Results are shown for PR-positive meningioma cells from Case 2 (upper) and for PR-negative meningioma cells from Case 7 (center) and Case 12 (lower).

which, at a concentration of $10^{-8}$ M, inhibited cell growth more than 50% as compared with the control group (Fig. 2 upper).

In contrast, the efficacy of antiprogesterones on PR-negative meningiomas varied in different tumor specimens. Contrary to our expectation, the PR-negative meningioma specimen in Case 7 (Fig. 2 center) showed marked antitumor effects after 9 days of treatment. The antitumor effects of mifepristone ($10^{-8}$ and $10^{-10}$ M) or onapristone ($10^{-8}$ and $10^{-10}$ M) on meningioma cells were more than 50% as compared with those of the comparable control. The antiprogesterones failed to show strong antitumor (cytocidal) effects against another meningioma specimen (Case 12, Fig. 2 lower),
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TABLE 1

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Tumor Location</th>
<th>MD (cm)</th>
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</tr>
<tr>
<td>2</td>
<td>32, M</td>
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<td>+</td>
</tr>
<tr>
<td>3</td>
<td>79, M</td>
<td></td>
<td>convexity</td>
<td>4.5</td>
<td>meningothelial</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>50, M</td>
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<td>6</td>
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<td>+</td>
</tr>
<tr>
<td>5</td>
<td>80, M</td>
<td></td>
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<td>5</td>
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<td>3</td>
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<td>9</td>
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<td>meningothelial</td>
<td>-</td>
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<tr>
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<td>tentorial</td>
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<td>-</td>
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<tr>
<td>13</td>
<td>65, M</td>
<td></td>
<td>falcial</td>
<td>6</td>
<td>fibroblastic</td>
<td>-</td>
</tr>
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</table>

* MD = maximum diameter of tumor; PR = progesterone receptor; + = positive; - = negative.

although there were some significant differences (cytostatic effects) from the controls in meningioma cells treated with some concentrations of antiprogesterones.

The antitumor effects of antiprogesterones against the 13 cases in vitro are summarized in Table 2. For comparison, the antitumor effects of antiprogesterones among the different meningioma specimens were evaluated at 9 and 21 days after the start of treatment. The results indicate that these two antiprogesterones have cytostatic and cytotoxic effects against meningioma cells, regardless of the presence or absence of PR. No correlation was observed between the presence or absence of PR's in meningiomas and the antitumor effect of the antiprogesterones (Bhargava's exact probability test). However, three PR-negative meningiomas (Cases 11, 12, and 13) showed little or no response to any concentration of mifepristone and/or onapristone examined.

On the other hand, PR-positive GH3 rat pituitary tumor cells showed marked antitumor effects after 12 days of antiprogesterone treatment, but the PR-negative A-172 human glioblastoma cells failed to show any antitumor effect with the concentration of the antiprogesterones used in this study.

In Vivo Study

The tumors implanted into the subrenal capsules of nude mice were found on histological examination to be located between the renal parenchyma and renal capsule (Fig. 3B). The growing tumors preserved the same histological features as were found in the original tumor specimens, such as whorl formation and elongated cells (Fig. 3A and C).

The groups treated with the antiprogesterone agents exhibited marked reduction in tumor volume, regardless of the presence or absence of PR's (Fig. 4). However, no histological changes suggestive of necrosis or apoptosis were observed in any of the cases treated with antiprogesterones. In addition, no significant difference in tumor volume was observed between the mifepristone- and onapristone-treated mice in each case.

Finally, there were no detectable differences in the weights and histological features of the uteri, ovaries, and adrenal glands in the nude mice treated with mifepristone or onapristone with the doses used in this study.

Discussion

Meningiomas are tumors that arise from the arachnoid cells covering the brain and spinal cord. They are usually benign and are surgically curable in most cases,30,34 but some meningiomas that attach to brain stem or invade the sagittal or transverse sinus totally are difficult to remove. Recurrent meningiomas are usually treated by reoperation or radiation therapy,1,34 and they do not change their basic histological characteristics,36 however, these tumors are not clearly radiosensitive,1,6,13 and reoperation can be fraught with complications.3,37 The demonstration of specific binding proteins for estrogen and progesterone in meningiomas suggests that a trial of hormone therapy should be considered in some patients with recurrence.3,38,39 The present study has shown that the antiprogesterones, mifepristone and onapristone, have antitumor effects on meningiomas in vitro and in vivo. Therefore, these

<table>
<thead>
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<th>Group &amp; Case No.</th>
<th>Antiprogesterones (M)</th>
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<tr>
<td></td>
<td>Mifepristone</td>
</tr>
<tr>
<td></td>
<td>10^-10</td>
</tr>
<tr>
<td>PR-positive, Day 9</td>
<td>+</td>
</tr>
<tr>
<td>PR-negative, Day 9</td>
<td>+</td>
</tr>
<tr>
<td>PR-positive, Day 21</td>
<td>-</td>
</tr>
<tr>
<td>PR-negative, Day 21</td>
<td>-</td>
</tr>
</tbody>
</table>

* PR = progesterone receptor. + = inhibited > 50% vs. cell growth in the control; + = inhibited < 50% vs. cell growth in the control but showed a statistically significant difference from that in the control; - = no inhibition.
antiprogesterone agents may help to reduce the tumor size in patients with recurrent or nonresectable meningiomas.

In this study, seven of nine PR-negative meningiomas responded to antiprogesterones in vitro. The fixation technique and subsequent preparations for frozen section used in this experiment have been shown to permit the best staining for PR's. Immunocytochemical testing for PR's in the meningioma tissues in this study was repeated many times by changing the tissue section used and by varying several experimental conditions (concentration of the primary antibodies and incubation time with primary antibodies), but the results did not vary. The fact that the A-172 cells failed to show any antitumor effect with any concentration of the antiprogesterone agents suggests that mifepristone and onapristone may have antitumor effects on

![Graphs showing the antitumor effect of antiprogesterones on three meningiomas implanted into the subrenal capsule of the nude mouse. The tumor volume is expressed as a percentage of control. The mean values of each tumor volume in controls were 1.314 cu mm (Case 2), 0.877 cu mm (Case 3), and 0.784 cu mm (Case 7). PR = progesterone receptor. Each column depicts the standard error of the mean of the tumor volume. The numbers within columns indicate the number of mice treated for 28 days with either vehicle alone (C), mifepristone (M), or onapristone (O). Asterisks: significant difference from the comparable control, p < 0.05.]}
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cultured PR-negative meningioma via other steroid receptors. In contrast, both antiprogesterones caused an antitumor effect on all PR-positive meningiomas in vitro. These findings suggest that mifepristone and onapristone have antitumor effects on meningioma cells via the PR and/or another steroid receptor. In addition to the estrogen and progesterone receptors, androgen and glucocorticoid receptors have been demonstrated in some meningiomas. Zava, et al., have shown that the proliferation of meningioma cells was promoted by the addition of androgen. Mifepristone and onapristone are not only antiprogesterone agents but also antiguercorticoid agents, therefore, these agents may have an antitumor effect against meningioma cells via glucocorticoid receptors.

Meningiomas clinically become symptomatic at the time of high circulating progesterone levels, suggesting that progesterone stimulates the growth of this tumor via the PR's. We have not been able to determine whether progesterone stimulates or inhibits the cellular proliferation of PR-positive meningiomas in vitro, because of the variability in its effectiveness (unpublished data). Indeed, the effect of progesterone on cultured meningioma cells has been variable. The divergence in the effects of progesterone may in part be due to the disappearance of PR in vitro, although a previous study has shown the continued presence of PR after the primary culture of meningioma cells.

The antitumor effect of mifepristone on meningioma in an in vivo experimental model has been documented by using subcutaneous xenografts of tumor tissue. Although this model allows serial measurements of tumor volume, the tumor does not always grow and, when it does, its growth is slow. We have therefore used the subrenal capsule of the nude mouse as a valuable therapeutic model, with a modification of the method described by Medikour, et al. Our method enabled us to implant an equal number of tumor cells in a collagen matrix which resulted in a 100% tumor take.

The present study has shown that mifepristone and onapristone markedly reduced the tumor volume as compared with the oil vehicle alone, regardless of the presence or absence of PR. Histologically, necrotic changes were detected in the meningioma cells treated with antiprogestones. Previous work has suggested that the antitumor action of mifepristone and onapristone is mediated via the PR's and is associated with the induction of apoptosis in PR-positive MXT-positive mammary tumors in mice. However, the lack of condensed or fragmented nuclei in our tumor cells suggests that the antitumor effects of antiprogestrone on meningioma is not the result of the induction of apoptosis.

We have not yet tested for the presence of other steroid receptors, such as estrogen, androgen, and glucocorticoid receptors, in meningioma tissues. Therefore, we could not exclude the possibility that the observed antitumor effect is due to the blockade of other steroid receptors by the antiprogestones and/or their metabolites in our in vitro and in vivo studies.

In conclusion, although the role of progesterone on the proliferation of meningioma cells and the mechanism of antiprogestrones on the antitumor effect of meningioma cells remain unknown, two antiprogestones, mifepristone and onapristone, may be useful endocrine agents against meningiomas, particularly in cases of recurrent or unresectable meningiomas.

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