Short Communication

Anaplastic and Atypical Meningiomas Express High Levels of Fas and Undergo Apoptosis in Response to Fas Ligation

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In this study we characterized the expression of Fas and Fas ligand in different types of meningiomas and examined the effect of Fas ligation on the death of meningioma cells in culture. Using Western blot analysis, we found that extracts derived from anaplastic and atypical meningiomas expressed high levels of Fas, whereas benign meningiomas did not express detectable levels of this protein. All of the meningiomas examined expressed low levels of Fas ligand. Cultures of anaplastic meningiomas also expressed Fas and treatment of these cells with anti-Fas antibody induced cell death. The results of this study indicate that Fas is preferentially expressed in atypical and anaplastic meningiomas and suggest that it is involved in the increased apoptosis observed in these tumors. (Am J Pathol 2001, 159:1193–1197)

Meningiomas are tumors that arise from the leptomeningeal covering of the brain and the spinal cord, accounting for 15 to 20% of all central nervous system (CNS) tumors.¹ According to the current World Health Organization histological grading system,² meningiomas are classified as typical, atypical, or anaplastic. Most meningiomas are slow-growing and are generally considered benign tumors (grade I, typical meningiomas). Approximately 10% of cases are classified as grade II (M2, atypical) or anaplastic (grade III, M3) meningiomas. The atypical and anaplastic tumors exhibit a more aggressive clinical behavior and higher recurrence rate following therapy when compared with typical meningiomas.³ Atypical and malignant meningiomas are histologically characterized by frequent mitoses, increased cellularity,

high nuclear to cytoplasmic ratio, uninterrupted, patternless or sheet-like growth, and foci of necrosis.² In addition, anaplastic meningiomas exhibit an increased apoptosis index^{4,5} and express higher levels of p53 and Bax and lower levels of Bcl2.^{6,7}

Fas-APO1 (CD95), is a 48-kd transmembrane cysteine-rich glycoprotein that belongs to the tumor necrosis factor receptor superfamily.⁸ The amino acid sequence of the extracellular domain of the various members of this family is relatively conserved. Fas also contains a conserved cytoplasmic motif called "death domain."⁹ Fas-mediated apoptosis is one of the major mechanisms of programmed cell death and it plays important roles in various processes such as the physiological turnover of lymphoid cells^{10,11} and the maintenance of liver homeostasis.¹²

Activation of Fas by the Fas ligand (FasL) or by crosslinking with anti-Fas antibodies leads to interaction of the receptor with the Fas-associated death domain (FADD) adaptor protein which recruits caspase 8 and leads to the activation of a caspase cascade and eventually, cell apoptosis.^{13,14} In the present study we examined the expression of Fas and FasL in typical and malignant meningiomas and the apoptotic response of meningioma cells to activation with Fas.

Materials and Methods

Tumor Samples

Tumors were classified according to the World Health Organization criteria into the various subtypes of the benign, atypical, and anaplastic meningiomas. All of the tumors examined in this study were intracranial meningiomas. Histopathological diagnoses were made according to the World Health Organization guidelines and eval-

Supported by the Committee for the Advancement of Research of Bar-Ilan University.

Accepted for publication June 13, 2001.

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uated in formalin-fixed paraffin-embedded hematoxylin/eosin-stained tissue slices. Tumors were collected from 24 patients operated on at Hadassah University Hospital. Fresh tissue was frozen immediately following surgery in liquid nitrogen and stored at -70°C until processing. The mean age of patients with benign and atypical/malignant meningiomas were 52 and 58, respectively. Sample collection and processing were performed according to the regulations of the committee on research involving human subjects of the Hadassah Medical Organization Institutional Review Board (IRB).

Primary Meningioma Cultures

Primary cultures were obtained from freshly resected tissues within one hour of surgical removal. Samples were first washed in phosphate-buffered saline (PBS) and then minced into small pieces in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and were further pipetted to obtain maximal cell dispersion. Cells were plated in 25 cm^2 tissue culture flasks and were grown for 7 to 10 days. Medium was changed every 3 to 4 days and cultures were split using 0.25% trypsin.

Immunocytochemical Staining

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde for 15 minutes at room temperature and processed for staining as previously described.¹⁵ Before staining, cells were treated with blocking solution (10% BSA in PBS) for 30 minutes followed by permeabilization with 1% saponin in PBS. Cells were incubated with polyclonal anti-Fas or anti-FasL antibodies for 2 hours at RT in a humid chamber. Cells were then washed three times and incubated with donkey anti-rabbit antibody conjugated to horseradish peroxidase. To determine nonspecific staining, adjacent cells were incubated with the second antibody only or with control isotype-matched antibody.

Cell Homogenates

Tissue was homogenized and cells were scraped with a rubber policeman and centrifuged at 1400 rpm for 10 minutes. The supernatants were aspirated and the cell pellets were resuspended in 100 μL of lysis buffer (25 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 0.5% Na deoxycholate; 2% NP-40; 0.2% SDS; 1 mmol/L phenylmethylsulfonyl fluoride (PMSF); 50 $\mu\text{g}/\text{mL}$ aprotinin; 50 $\mu\text{mol}/\text{L}$ leupeptin; 0.5 mmol/L Na_3VO_4) on ice for 15 minutes. The cell lysates were centrifuged for 15 minutes at 14,000 rpm in an Eppendorf microcentrifuge, supernatants were removed, and 2X sample buffer was added.

Immunoblot Analysis

Lysates (30 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis (10%) and were transferred to nitrocellulose membranes. To determine the

protein content of individual lanes, membranes were stained with 0.1% Ponceau S in 5% acetic acid. The Ponceau S staining was removed by several washes with PBS (pH 7.4); the membranes were blocked with 5% dry milk in PBS and subsequently stained with primary antibody. Specific reactive bands were detected using a goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham, Pharmacia Biotech).

Measurement of Apoptosis

Cell apoptosis was measured using MTT assay and by ELISA using anti-histone1 antibodies. Cells ($1 \times 10^6/\text{mL}$) were plated in 6-well plates and treated with monoclonal anti-human Fas antibody (200 ng/mL; Upstate Biotechnology, Lake Placid, NY) for 24 hours. For anti-histone1 ELISA, fragmented DNA was extracted from the cells and was incubated in 96-well plates coated with anti-histone1 antibodies for 2 hours. Plates were then washed and incubated with anti-DNA antibodies conjugated to peroxidase for an additional 2 hours. Substrate solution was added and absorbance was measured at 405 nm. For MTT assay, cells were plated in a 96-well plate and treated with anti-Fas antibody for 24 hours. MTT reagent was added to the cells and the cultures were incubated for 4 hours at 37°C . The reaction was stopped by addition of acidified Triton buffer [0.1 mol/L HCl, 10% (v/v) Triton X-100; 50 L/well] and absorbance was measured at a wavelength of 595 nm.

Results

Expression of Fas and FasL in Different Types of Meningiomas

The expression of Fas and FasL was determined in anaplastic, atypical, and benign tumors using Western blot analysis. Figure 1A shows the expression of Fas in 12 tumors consisting of six anaplastic and six atypical meningiomas. All tumors expressed high levels of Fas without significant differences between the anaplastic and the atypical tumors. In contrast, 11 benign tumors that were examined expressed undetectable or very low levels of Fas, whereas one benign tumor expressed relatively high levels of Fas (Figure 1A). This tumor is currently being characterized with regard to the expression of other apoptotic markers. Variable levels of FasL were found in the atypical/anaplastic and the benign tumors (Figure 1B).

Expression of Fas in Primary Cultures Derived from Malignant Meningiomas

To further characterize the expression of Fas expressed on meningiomas we generated primary cultures from benign and anaplastic meningiomas (tumors 19 and 4, respectively) and examined the expression of both Fas

Figure 1. Expression of Fas and FasL in meningioma tumors. Frozen atypical, anaplastic, or benign tumors were homogenized and subjected to SDS-PAGE and Western blot analysis. The membranes were probed with an antibody against Fas (**A**) or FasL (**B**) followed by a secondary antibody conjugated to horseradish peroxidase. **Lanes 1–6**, anaplastic meningiomas (mean age, 58); **lanes 7–12**, atypical meningiomas (mean age, 52); **lanes 13–24**, benign meningiomas (mean age, 55). The results are representative of four similar experiments.

and FasL in these cells. Cells were allowed to grow for 3 to 7 days, were then plated on glass coverslips and stained with either anti-Fas or anti-FasL antibody. Similar to the results obtained with the Western blot of the tumor samples, we found that around 90% of the cultured anaplastic meningioma cells exhibited strong immunostaining of Fas in the cytosol and in the plasma membrane (Figure 2B). In contrast, we did not find any detectable staining of Fas in cultures derived from benign meningiomas (Figure 2C). Cultures derived from additional anaplastic tumors (1 and 2) and benign tumors (23 and 24) exhibited a similar pattern of Fas expression (data not shown). Very weak immunostaining of FasL was observed in the benign and anaplastic meningioma cultures (data not shown).

Ligation of Fas with Anti-Fas Antibody Induces Meningioma Cell Death

To examine the function of the Fas expressed on the anaplastic meningioma cells, we measured the induction of meningioma cell death in response to ligation of Fas. Cultures of anaplastic meningioma expressing high levels of Fas (tumor 4) were stimulated with anti-human Fas antibody and cell death was determined by anti-histone1 enzyme-linked immunosorbent assay and by a 3-(4,5 dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide assay kit.

Anaplastic meningioma cells exhibited a low degree of apoptosis. Treatment of the cells with anti-Fas antibody resulted in a strong death response, which was observed following 24 hours of treatment (Figure 3A and B). The treated cells became rounded and detached from the dish and exhibited a decrease in MTT staining (Figure 3A) and an increase in the level of fragmented DNA as detected by anti-histone1 ELISA (Figure 3B). In contrast, cultures derived from benign meningiomas (tumor 19) did not undergo cell apoptosis in response to Fas ligation.

Figure 2. Immunocytochemical staining of Fas in benign and malignant meningioma cultures. Cultures derived from anaplastic (tumor 4) (**B**) or benign (tumor 19) (**C**) tumors were stained with anti-Fas antibody followed by anti-rabbit antibody conjugated to peroxidase, or only with the secondary antibody (**A**). Cells were visualized with light inverted microscope ($\times 400$). Cells shown are representative of three independent experiments.

Similar results were obtained with additional anaplastic (tumors 1 and 2) and benign tumors (23 and 24).

Discussion

In this study we examined the expression of Fas and FasL in benign (M1) and atypical/anaplastic (M2/M3) meningiomas and the effect of Fas ligation on meningioma cell death. We demonstrated for the first time the expression of Fas protein in atypical and anaplastic meningiomas. In

Figure 3. Effects of anti-Fas antibody on the apoptosis of cultured meningioma cells. Anaplastic (tumor 4) and benign (tumor 19) meningioma cells were treated with monoclonal anti-human Fas antibody (200 ng/ml) for 24 hours. Apoptosis was measured by the MTT assay (**A**) and by ELISA using anti-histone1 antibodies (**B**). For measurement of MTT, cells were plated in a 96-well plates and treated with anti-Fas antibody for 24 hours. MTT reagent was added to the cells for 4 hours at 37°C and absorbance was measured at a wavelength of 595 nm. Fragmented DNA was extracted from the cells and was incubated in 96-well plates coated with anti-histone1 antibodies for 2 hours followed by incubation with anti-DNA antibodies conjugated to peroxidase for an additional 2 hours. Absorbance was measured at 405 nm. Results represent the means $\pm$ SD of three separate experiments. * $P < 0.001$.

contrast, significantly lower expression of Fas was observed in benign meningiomas. The expression of Fas has been reported in a variety of tissues including skin,¹⁶ liver,¹⁴ thymus,¹⁷ and heart,¹⁶ whereas only low expression of Fas has been documented in the central nervous system.¹⁸ Expression of Fas appeared also to vary in transformed and non-transformed cells and in tumors with various degrees of malignancy.^{19,20,21} Indeed, in the CNS, the majority of neoplastically-transformed cells of neuroectodermal origin, such as medulloblastoma, express Fas, whereas normal cells do not.²² Similarly, the expression of Fas in glial tumors is increased with the degree of malignancy.^{23,24}

The significance of the increased expression of Fas in malignant meningiomas is still not clear, however it may suggest a role for Fas in the progression from benign to malignant tumors. Alternatively, Fas expression may reflect increasing anaplasia and cell proliferation associated with the progression of meningioma.

Fas-mediated apoptosis is one of the major mechanisms of programmed cell death.²⁵ Induction of cell death in response to Fas ligation has been demonstrated in various Fas-positive cells.^{26,27} In contrast, some cells expressing Fas are resistant to apoptosis induced by

activation of Fas due to an intrinsic defect in the intracellular signaling pathways activated following Fas ligation, the synthesis of cytoprotective proteins, or due to the presence of a soluble form of Fas.^{28,29} Our results suggest that the Fas protein expressed on the malignant meningiomas was functional since cultures derived from these tumors were stained positively for Fas and underwent cell death in response to anti-Fas antibody.

Interestingly, anaplastic meningiomas have been reported to exhibit a high apoptosis index,^{4,5} and apoptosis is thought to be associated with atypical or malignant changes in these tumors.³⁰ The increased level of apoptosis in atypical and anaplastic meningiomas is associated with increased expression of the pro-apoptotic protein Bax and decreased expression of the anti-apoptotic protein Bcl2 in these cells.^{6,7} Based on our results indicating high Fas expression in anaplastic and atypical meningiomas, it is possible that cell death observed in meningiomas could also result from tumor infiltration by cytotoxic T lymphocytes expressing FasL. Alternatively, apoptosis induced by the Fas/FasL may occur if under specific conditions there is a strong up-regulation of FasL on meningioma cells.

The major prognostic questions for meningiomas are prediction of recurrence, and for higher-grade tumors prediction of survival. Unfortunately, the assignment of tumor grade is somewhat subjective. Thus, there have been numerous attempts to correlate specific histological features with recurrence rate and prognosis, and these have produced conflicting data.^{31,5} Therefore, the possible specific identification of high-grade meningiomas using the Fas receptor may provide diagnostic and prognostic tools.

Clinically, malignant meningiomas may be difficult to cure surgically due to the technical difficulty of initial radical resection and a relatively high rate of recurrence. Radiotherapy and radiosurgery, which are the major non-surgical therapies, are potentially associated with high morbidity.³² With the lack of current effective chemotherapy against anaplastic meningiomas, the Fas/FasL pathway may serve as a possible candidate for the development of novel therapeutic approaches for the treatment of this aggressive form of meningioma.

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