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Apoptosis of T lymphocytes invading glioblastomas multiforme: a possible tumor defense mechanism

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Abstract

Object—The goal of this study was to investigate whether apoptosis occurs in T lymphocytes that invade Fas ligand (FasL)–expressing glioblastomas multiforme (GBMs) and if its induction could be mediated by Fas.

Methods—Apoptotic T lymphocytes were detected in GBMs by using detection of cell-type markers combined with active caspase-3 immunohistochemical analysis, a recently introduced apoptosis-specific in situ ligation assay, as well as by examining morphological criteria. Apoptotic T cells expressed Fas and were localized in the vicinity or in direct contact with FasL-expressing tumor cells. The T lymphocytes were undergoing apoptosis in spite of Bcl-2 expression. Expression of Bax was also detected in dying T cells, which can explain the absence of the protective effect of Bcl-2, because Bax inhibits Bcl-2 death–repressor activity.

Conclusions—On the basis of the data presented in this paper, the authors suggest that GBM cells that express FasL can induce apoptosis in invading immune cells. This phenomenon may play an important role in these tumors' maintenance of immune privilege and evasion of immune attacks. Awareness of this phenomenon should be helpful for the development of novel strategies for treatment of malignant gliomas.

Keywords

glioblastoma multiforme; apoptosis; T lymphocyte; Fas

The lack of an effective treatment for malignant glioma is an enigma in clinical neuroscience. Normally, the development of tumors is prevented by body defenses such as activation of tumor suppressor genes to induce apoptotic cell death¹¹ or attack of lymphocytes against neoplastic cells.¹⁷ Tumor prognosis in patients with malignant gliomas, however, is not improved, even when multiple T lymphocytes cross the blood–brain barrier and make contact with tumor cells.¹³ This suggests that mechanisms have developed in GBM cells to avoid immune system–mediated destruction.

Recently it was reported that in some tumors, including hepatocellular carcinoma, melanoma, and colon cancer, an immune evasion strategy has been developed that is based on FasL–mediated destruction of invading lymphocytes.^{9,12,15} Various immune privileged sites, such

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as the eye or testis, rely on FasL expression for maintenance of their immune privileged positions because FasL expression induces apoptosis in entering activated immune cells. Similarly, in the tumors mentioned earlier, invading T lymphocytes that express Fas are stimulated to apoptosis by tumor cells that express FasL.

Expression of FasL has recently been demonstrated in GBMs.⁸ It is therefore possible that these tumors may induce apoptosis in invading immune cells, thus maintaining an immune privilege and evading immune attack.

In this paper, we demonstrate that apoptotic T lymphocytes are indeed present in GBMs. On the basis of our data, we suggest that the death of T lymphocytes in these tumors is mediated by the Fas system. We conclude that the malignant GBM can induce apoptosis in invading T lymphocytes and that this, in part, could account for the aggressive growth of this tumor.

Materials and Methods

Tumor Specimens

Seven formalin-fixed, paraffin-embedded astrocytomas were reviewed by a neuropathologist and classified as Grade IV/IV tumors (GBMs) according to the criteria established by the World Health Organization.

Immunohistochemical Analysis

Six-micrometer-thick sections of tumor were deparaffinized with xylene, rehydrated in graded concentrations of alcohol, and washed in water. Microwave-induced antigen retrieval was performed in 0.01 M sodium citrate (pH 6) for 15 minutes to allow determination of caspase-3, Bcl-2, and Bax. Cleaved caspase-3 (17 kD) polyclonal antibody (concentration 1:100); polyclonal Bcl-2 antibody (concentration 1:500), and monoclonal Bax antibody (concentration 1:500); the T-lymphocyte markers monoclonal CD3- ϵ (concentration 1:25) and CD3- ζ (concentration 1:25); and polyclonal FasL (concentration 1:25) and Fas (concentration 1:25) antibodies were used in the experiments. The CD3- ϵ is a highly specific marker for T lymphocytes; it participates in the transition of T cells from a dormant to an activated state. To avoid false-positive results in the detection of tumor-invading T lymphocytes, additional staining was performed using the lymphocyte marker CD3- ζ . The signal was visualized using biotinylated goat anti-rabbit antibody (concentration 1:500) and a tyramide signal amplification kit (TSA-direct kit) for caspase-3, Bcl-2, and Bax. The lymphocyte markers, Fas, and FasL were visualized using streptavidin-fluorescein isothiocyanate or streptavidin-Texas Red conjugates (concentration 1:500). Sections were counterstained with DAPI at a concentration of 1 μ g/ml. Negative control experiments, in which no primary antibody was added, were performed during every staining procedure to ensure a low background. Positive control experiments, in which primary antibody specificity was tested, had been performed by the manufacturers of the antibodies and results were supplied with the antibodies.

In the dual staining procedures, antibodies were used sequentially in pairs: active caspase-3 and the lymphocyte marker CD3- ϵ ; CD3- ϵ and Fas; Fas and FasL; Bcl-2 and CD3- ϵ ; and Bax and CD3- ϵ . All sections were additionally counterstained with DAPI for visualization of nuclear structure. In experiments in which triple staining was used, in situ ligation was followed by CD3- ζ and Fas detection. In this case, DAPI counterstaining was not used.

In Situ Ligation

We have recently introduced a new approach for apoptosis detection in tissue sections: the in situ ligation assay.^{4,5} The assay is used to label selectively a single type of apoptotic DNA damage, namely, full double-strand breaks with 3'OH and 5'PO₄ oligonucleotide end groups.

The assay utilizes DNA ligase, which attaches the hairpin-shaped oligoprobe only to cellular DNA with full double-strand breaks, thus eliminating the possibility of labeling-nicked or single-strand DNA present in necrotic and transiently damaged cells.^{4,5} Vast areas of necrosis are one of the most characteristic features of GBMs.¹ This encouraged us to use the in situ ligation assay, as opposed to the less specific terminal transferase-mediated technique (the TUNEL assay), for apoptosis detection in these tumors. Analysis of a mixture of apoptotic and necrotic cell death in malignant gliomas using the TUNEL assay is problematic, because TUNEL does not discriminate between apoptotic and necrotic cell death.^{3,7} The in situ ligation technique, however, is more specific for apoptosis.⁵

The probes with 3' single A overhangs and blunt-ended probes were both used in experiments, and produced identical staining patterns. Blunt-ended probes produced a brighter signal and were used to generate the illustrations in this paper. The sequences of the probes were the following: 3' A overhang hairpin, 5' GCGCTA-GACCTGC5*GCTGGTCTAGCGCA 3'; and blunt-ended hairpin, 5' GCGCTAGACCTGC5*GCTGGTCTAGCGC 3' (in which 5* represents biotin-triethyleneglycol spacer).

Sections were deparaffinized with xylene, rehydrated in graded concentrations of alcohol, washed in water, and treated with protein-ase K (50 mg/ml) for 15 minutes. Sections were rinsed with water and then were incubated for 15 minutes in 80 ml of ligation buffer without the probe (66 mM Tris HCl [pH 7.5], 5 mM MgCl₂, 0.1 mM dithioerythritol, 1 mM adenosine triphosphate, and 15% polyethylene glycol 8000) to ensure even saturation. The buffer was aspirated; the full ligation mix containing ligation buffer with the hairpin probe (35 mg/ml) and T4 DNA ligase (250 U/ml) was applied to the sections. In a mock-control reaction solution, an equal volume of 50% glycerol in water was substituted for the T4 DNA ligase. Sections were incubated in a humidified box for 16 hours at 23°C; after which they were briefly washed in water. The signal was visualized using Alexa Flour 350-streptavidin conjugate (1:300) in 0.1 M sodium bicarbonate buffer. In double- and triple-staining procedures, in situ ligation was performed first, followed by antibody staining as previously described (see *Immunohistochemical Analysis*). The signal was acquired with the aid of a fluorescent microscope, which was equipped with a digital camera system containing a round, thermoelectrically cooled camera head, charge-coupled device with a 1300 × 1030 imaging array cooled by a Peltier device.

Counting Apoptotic Lymphocytes

To form an initial estimate of the significance of lymphocyte apoptosis, we performed manual counting of apoptotic lymphocytes in seven Grade IV gliomas. The sections were double stained for active caspase-3 and both CD-3ε and CD-3ζ T-cell markers. We used a 20× objective and manually counted all T cells in each field of view as well as double-stained apoptotic T cells. Six fields of view per tumor were analyzed (42 fields of view total) using the fluorescent microscope equipped with a digital camera. Data are presented as means ± standard errors of the means.

Sources of Supplies and Equipment

The caspase-3 antibody was purchased from New England Bio-labs (Beverly, MA), and the Bcl-2 and Bax antibodies from Phar-Mingen (Los Angeles, CA). The T-lymphocyte markers CD3-ε and CD3-ζ, and the FasL and Fas antibodies were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The TSA-direct kit was obtained from Perkin-Elmer Life Science Products (Charlotte, NC). Molecular Probes (Eugene, OR) provided the streptavidin-fluorescein isothiocyanate and the streptavidin-Texas Red conjugates. The biotin-triethyleneglycol spacer was purchased from Glen Research (Sterling, VA).

The fluorescent microscope (model IX-70) was manufactured by Olympus Instruments (San Francisco, CA), and the digital camera system (MicroMax camera with RTE/CCD-1300-Y/HS array) was manufactured by Princeton Instruments, Roper Scientific USA (Trenton, NJ).

Results

Detection of Apoptotic Lymphocytes in GBMs

Costaining with caspase-3 and CD3- ϵ antibodies revealed apoptotic T lymphocytes in GBMs. Figure 1A shows numerous apoptotic T lymphocytes coexpressing active caspase-3 and CD3- ϵ .

The CD3- ζ antibody also revealed T cells displaying an apoptotic structure in malignant gliomas (Fig. 1B). The characteristic apoptotic-type chromatin condensation and apoptotic body formation (*arrow* in Fig. 1B) by a T lymphocyte is clearly seen.

Using 14 sections derived from seven GBMs, we performed manual counting of apoptotic lymphocytes. We counted cells that had been double stained to detect active caspase-3 and CD-3. In 42 fields of view (using a 20 $\times$ objective), we counted on average 18.2 ± 2 lymphocytes per field; of these 2.3 ± 0.5 displayed active caspase-3 and were undergoing apoptosis. Thus, approximately 10% of T lymphocytes in Grade IV gliomas were apoptotic.

Expression of the Fas Receptor by Apoptotic Lymphocytes in Gliomas

To investigate the causes of T cell apoptosis, we used in situ ligation, detection of the T-cell marker CD3- ϵ , and staining for Fas in a triple-staining format. We performed Fas/FasL codetection with additional counterstaining of cellular DNA by DAPI. We did not use a specific lymphocyte marker because we had already assigned the green, red, and blue fluorophores for visualization of Fas, FasL, and chromatin. Nevertheless, FasL/Fas-coexpressing apoptotic immune cells with characteristic round nuclei and high nucleus/cytoplasm ratios were easily identifiable in a Fas-negative tumor sample.

Triple staining, in which in situ ligation and CD3- ϵ and Fas detection were combined, revealed apoptotic T lymphocytes expressing Fas in GBMs (Fig. 1B). A triple-stained T cell is shown in Fig. 1B *lower right*; the in situ ligation signal produced the blue fluorescence; the CD3- ϵ marker, the red fluorescence; and the Fas receptor, the green fluorescence.

Coexpression of FasL and Fas in Lymphocytes and Expression of FasL in Glioma Cells

FasL/Fas-coexpressing lymphocytes were also present in the tumors and, frequently, were located close to or in direct contact with FasL-expressing GBM cells (Fig. 1C). Figure 1C shows an Fas-expressing (green) immune cell that also coexpresses FasL (red) in direct interaction with an FasL-expressing tumor cell (red). Apoptotic DNA fragmentation is evidenced by chromatin condensation on the nuclear membrane (*far upper right inset* in Fig. 1C) and is clearly seen in a Fas-expressing cell. Some of the cells in the figure appear to be undergoing apoptosis without expression of either Fas or FasL. This probably indicates the participation of additional mechanisms of apoptosis induction in these cells that are independent from the Fas system.

Expression of Bcl-2 and Bax in Apoptotic Lymphocytes

It was previously demonstrated that the inability of Bcl-2 to confer protection is a characteristic feature of Fas/FasL-induced apoptosis in activated T lymphocytes.¹⁶ Thus detection of Bcl-2 in apoptotic T lymphocytes could support a Fas-related mechanism of T-cell death in GBMs. Based on this notion we performed codetection of apoptosis, lymphocyte marker, and Bcl-2.

The lack of Bcl-2 protection of lymphocytes from FasL-induced apoptosis occurs in conjunction with expression of Bax.¹⁸ Bax has been shown to be responsible for the neutralization of Bcl-2 in Fas-mediated cell death.¹⁸ Although both are homologous proteins, Bcl-2 and Bax display opposing effects on apoptosis, with Bax promoting cell death following apoptotic stimulus.¹⁸ Detection of Bax in apoptotic T cells in GBMs would be consistent with participation of the Fas system in the induction of T-cell death in these tumors. We performed immunohistochemical studies designed to detect Bax in apoptotic T cells in GBMs.

Expression of Bcl-2 was seen in apoptotic T lymphocytes that were infiltrating GBMs (Fig. 1D). A Bcl-2/T-cell marker-coexpressing cell is visualized in direct contact with a GBM cell. Figure 1E, which shows a CD3-ε/Bax-coexpressing apoptotic cell, demonstrates that Bax was detected in apoptotic T lymphocytes.

Discussion

It has been proposed and recently demonstrated that, when activated immune cells expressing Fas enter tumors, they are killed by tumor cells that express functional FasL on their membranes or, possibly, release soluble FasL.^{9,12,15} It has already been established that such a process plays an important role in several malignant diseases such as colon cancer, melanoma, and hepatocellular carcinoma.^{9,12,15}

In the healthy nervous system astrocytes do not express FasL, but astrocyte-derived glioma cells do express this ligand.¹⁴ Brain tumors in general and gliomas in particular express FasL. Glioblastomas multiforme have the highest rate (81%) of FasL expression among astrocytic tumors.⁶

The efficient killing of Fas-bearing cells by astrocytoma lines in vitro and by tumor cells ex vivo has been demonstrated¹⁴ and supports the theory that FasL expression could enable tumors to mount a Fas counterattack against antitumor immune effector cells. Nevertheless, direct detection of apoptotic T lymphocytes expressing Fas in FasL-positive GBMs has not been previously reported. Our data demonstrate that apoptosis of activated T lymphocytes occurs in malignant gliomas. Dying T cells express Fas, Bcl-2, and Bax and are frequently located in proximity to or in direct contiguity with FasL-expressing tumor cells. These findings support the notion that GBMs that express FasL can induce apoptosis in invading immune cells, thus maintaining an immune privilege and evading an attack by the immune system.

Not all lymphocytes that expressed Fas or Bax appeared apoptotic. This is probably because expression of Fas and Bax precedes the morphological changes that occur during apoptosis. Cells that are about to become apoptotic or are still in the early stages of apoptosis, therefore, could exhibit Fas or Bax positivity without displaying the associated morphological signs of apoptosis.

Although we have demonstrated that all necessary mechanisms responsible for Fas-mediated apoptosis of tumor-infiltrating lymphocytes are present in GBMs, additional evidence demonstrating the direct events leading to apoptosis is needed to state with certainty that the Fas system mediates a tumor counterattack on invading T lymphocytes. We plan to address this issue in future work.

The clinical relevance of the detection of apoptotic immune cells in FasL-expressing GBMs is worthy of further discussion. Deficiencies in both global and local immunological function have been demonstrated in patients with malignant gliomas and include impaired antibody and cell-mediated cytotoxicity.¹⁰ Immunosuppression in GBMs could be explained by the high incidence of FasL expression.¹⁴ It is more likely, however, that several mechanisms contribute to malignancy and immunosuppression in these tumors. Indeed, a number of factors other than

Fas have been implicated in the defective immune response that occurs in these tumors, and these include expression of transforming growth factor- β_2 ² and Bcl-2¹⁹ by tumor cells. It is possible that a diverse set of defense tools allows these tumors to maintain their rapid progression. Diversified protective mechanisms variably expressed in different sets of tumor cells could increase the chances of tumor survival. This notion is supported by the fact that the expression of FasL occurs unevenly throughout glial tumors and is detected in clusters, but not in all tumor cells.⁶ Based on this, in our future work, we plan to assess whether apoptosis of immune cells occurs in FasL-negative GBMs and to evaluate the mechanisms of its induction in such instances.

In a curious way, certain antitumor therapeutic interventions may enhance the contribution of the Fas system in immunosuppression. An interesting and paradoxical effect of some cancer drugs is that they upregulate FasL expression in cancer cells. This effect has been demonstrated for a number of chemotherapeutic agents including doxorubicin and bleomycin, which can upregulate FasL expression on hepatoblastoma cells in vitro.¹⁵ Our evidence of Fas-mediated apoptosis of immune cells in GBMs indicates a need for additional evaluation of the antilymphocyte effects of these drugs because it is possible that upregulation of the tumor Fas system by some anticancer agents could further reduce the efficiency of the body's anticancer immune system.

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Abbreviations used in this paper

DAPI	4',6-diamidino-2-phenylindole · 2HCl
FasL	Fas ligand
GBM	glioblastoma multiforme
TUNEL	terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling

Fig. 1.

Fluorescent micrographs demonstrating apoptotic T lymphocytes in GBMs. A: Triple-stained apoptotic T cells in a GBM. Apoptosis is evidenced by active caspase-3 (green fluorescence) and chromatin condensation seen with DAPI staining (blue fluorescence). The CD3- ϵ T-lymphocyte marker is visualized by red fluorescence. *Arrows* point to one of the many apoptotic T lymphocytes, denoted by both CD3- ϵ and caspase 3 staining. B: Apoptotic T cells identified using DAPI and in situ ligation showing expression of the Fas receptor (FasR) in apoptotic lymphocytes (*upper* and *lower* panels represent two separate cells). *Upper*: A T lymphocyte labeled by the T-cell marker CD3- ζ (red fluorescence) and DAPI counterstaining (blue fluorescence) visualizing apoptotic body formation in the same cell (*arrow*). *Lower*: Apoptotic T cell expressing FasR (green on *left*) and triple-color image of the same cell in which the in situ ligation signal appears blue, CD3- ϵ appears red, and FasR appears green. The magenta area represents superimposition of red and blue fluorescence. C: Direct contact between a FasR-positive immune cell undergoing apoptosis and a GBM cell expressing FasL (triple staining). *Inset* in the far right image shows the magnified DAPI-stained nucleus of the same apoptotic T cell for better evaluation of apoptotic chromatin condensation (*arrows*). The cell detected is in direct contact with a FasL-expressing–FasR-negative tumor cell. Green plus red plus blue yields turquoise and red plus blue yields violet. D: Expression of Bcl-2 in an apoptotic T lymphocyte within a GBM (triple staining). The Bcl-2 appears green; the CD3- ϵ , red; and the

DAPI, blue. Superimposition of red and green fluorescence in areas of coexpression appear yellow. The cells are shown to be directly interacting, with the Bcl-2- and CD3-ε-positive lymphocyte on the left (red plus green yields yellow) and the CD3-ε-negative glioma cell on the right. The *inset* shows chromatin condensation on the nuclear membrane of the lymphocyte. E: Expression of Bax in an apoptotic T lymphocyte within a GBM (triple staining). The *arrows* point to two Bax-positive T lymphocytes (Bax appears green; CD3-ε, red; and DAPI, blue) in GBM tissue. Superimposition of red and green fluorescence appears yellow. The *inset* shows a high-power image of one of the cells. Apoptotic-type chromatin condensation is visualized by DAPI. Bar = 10 μm (A–D); 100 μm (E); the *inset* in E was magnified × 5 from the original.