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Research Article

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Fas Ligand Expression by Astrocytoma In Vivo: Maintaining Immune Privilege in the Brain?

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Abstract

Astrocytomas are among the most common brain tumors that are usually fatal in their malignant form. They appear to progress without significant impedance from the immune system, despite the presence of intratumoral T cell infiltration. To date, this has been thought to be the result of T cell immunosuppression induced by astrocytoma-derived cytokines. Here, we propose that cell contact–mediated events also play a role, since we demonstrate the in vivo expression of Fas ligand (FasL/CD95L) by human astrocytoma and the efficient killing of Fas-bearing cells by astrocytoma lines in vitro and by tumor cells ex vivo. Functional FasL is expressed by human, mouse, and rat astrocytoma and hence may be a general feature of this nonlymphoid tumor. In the brain, astrocytoma cells can potentially deliver a death signal to Fas^+ cells which include infiltrating leukocytes and, paradoxically, astrocytoma cells themselves. The expression of FasL by astrocytoma cells may extend the processes that are postulated to occur in normal brain to maintain immune privilege, since we also show FasL expression by neurons. Overall, our findings suggest that FasL-induced apoptosis by astrocytoma cells may play a significant role in both immunosuppression and the regulation of tumor growth within the central nervous system. (*J. Clin. Invest.* 1997. 99:1173–1178.) Key words: central nervous system • CD95 • glioma • immune tolerance • apoptosis

Introduction

The relationship between the central nervous system (CNS)[1] and the immune system has been considered to be rather limited due to the physical isolation provided by the blood–brain barrier and the absence of a well-defined lymphatic drainage (1). However, it is now clear that considerable trafficking of immune cells such as lymphocytes can and does occur (2). Despite this potential for immune interaction, evidently there are other means that generally maintain the immune privilege of the brain, including weak constitutive MHC molecule expression (2, 3), low concentrations of complement components (3), and the release of soluble factors, among which TGF-β is the best described (2).

These multiple processes inhibiting immune activation in the CNS may be preferable to inducing an inflammatory response, which could damage normal brain structures. However, the lack of an adequate immune response may facilitate the development and the growth of brain malignancies. In the particular case of astrocytomas, which constitute > 40% of all brain tumors and are the most lethal, specific mechanisms have been suggested that further inhibit a productive immune dialogue in the CNS. These include the secretion of PGE_2 (4) and high levels of TGF-β_2 in its active form (5). However, it is not clear whether such soluble factors alone can account for the lack of immunoreactivity in astrocytoma. Indeed, the role of TGF-β in vivo is complex, since it is not always inhibitory, but can also act as a T cell costimulatory factor under certain conditions (6). Moreover, TGF-β has been shown to decrease the homing of T cells into the brain (7), and yet tumor infiltrating lymphocytes, albeit in variable number, are commonly found in astrocytoma (8, 9). Thus in this glial tumor, T lymphocytes cross the blood–brain barrier and make intimate contact with tumor cells. However, since the prognosis for astrocytoma patients with such an infiltration is generally not improved (9), this suggests that the T cell–tumor cell relationship is functionally inadequate. There is clearly the potential for cell contact–mediated events to exert an inhibitory effect in these critical interactions, but mechanisms have not yet been defined.

A candidate molecule for inhibiting T cell function by a cell contact–mediated event is Fas ligand (FasL), a 40-kD type II membrane protein belonging to the TNF family (10). Indeed, FasL is responsible for the induction of apoptosis in Fas-bearing cells (10, 11), which is a major mechanism of clonal downsizing in immune homeostasis (11, 12). FasL is also a cytolytic effector molecule used by cytotoxic T lymphocytes (13, 14). Moreover, the FasL–Fas interaction has been suggested to actively mediate immune privilege in mouse testis (15) and in the anterior chamber of the murine eye (16).

In this study, we show that FasL is expressed on astrocytoma cells and that it can functionally induce a death signal in

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1. *Abbreviations used in this paper:* CNS, central nervous system; FasL, Fas ligand.

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Fas-bearing cells. We discuss the possibility that this finding may not only impede tumor–immune system interactions, but could also directly influence human astrocytoma growth.

Methods

Established cell lines. Human astrocytoma cell lines (LN18, LN229, LN308, LN443) have been described previously (17). Human astrocytoma cell line U-118MG (ATTC HTB15) and rat astrocytoma cell line C_6 (ATTC CCL107) were obtained from the American Type Culture Collection (Rockville, MD). A spontaneous murine astrocytoma cell line MT539MG (18) was kindly provided by Dr. G.Y. Gillespie (Birmingham, AL). Murine mastocytoma P815 and its murine Fas cDNA transfectant were generous gifts from Dr. P. Vassalli (Geneva, Switzerland). Cell lines were maintained in DME (Gibco BRL, Life Technologies, Paisley, United Kingdom) supplemented with 10% FCS (Seromed, Biochrom KG, Berlin, Germany), 100 mg/ml streptomycin (Sigma, Buchs, Switzerland), 100 IU/ml penicillin (Sigma), and 10 mM Hepes (GIBCO BRL).

Brain tissue. Biopsies from patients with astrocytoma were collected during surgery, after informed consent. These included four benign (two grade I, two grade II) and eight malignant (two grade III and six grade IV) astrocytomas, histologically graded according to World Health Organization nomenclature (19). Nontumoral human brain tissue was obtained from epilepsy patients. Rat brain samples were from Wistar-Furth rats (Iffa-Credo, L'Arbresle, France). Mouse brain tissue was from normal NMRI mice (Kleintierfarm, Madörin, Basel, Switzerland). Samples were snap-frozen in 2-methylbutane (Merck, Wertheim/Main, Germany) and stored at −80°C.

Primary cultures and ex vivo tumor cells. Cell suspensions derived from tumor biopsies were obtained by enzymatic digestion with medium containing 0.1% collagenase type IA, 0.002% DNase type II, and 0.05% protease type I (Sigma). Tumor cells were isolated by adherence to plastic culture vessels for 36 h in a 5% CO_2 incubator at 37°C, which also allowed reexpression of enzyme-sensitive cell surface molecules. In one patient (Ge87, grade IV) sufficient tumor cells were available for testing immediately as effectors in a cytotoxicity assay. For this patient and two further patients (Ge60, grade II and Ge33, grade IV) primary cultures were derived and tested for functional FasL expression as soon as sufficient cells were available.

Antibodies. Anti-FasL Ab PE62 (affinity-purified rabbit polyclonal Ab) and anti-FasL A11 mAb (rat IgM) were obtained by immunization with peptide 196–220 of FasL (20) which is a conserved amino acid sequence between mouse, rat, and humans (10, 21). A rat IgM antimurine CD8 mAb 3.168 (22) was used as an isotype-matched negative control. Antibodies against human glial fibrillary acidic protein (6F2, mouse IgG1) and an FITC-conjugated $F(ab')_2$ fragment of goat anti–mouse Ig were obtained from Dako (Zug, Switzerland). This latter Ab cross-reacts with rat Ig. Hamster antimurine Fas (CD95) mAb (Jo2) and a peroxidase-conjugated goat anti–rat Ig were obtained from PharMingen (San Diego, CA) and Biosource International (Camarillo, CA), respectively. Neutralizing anti–human FasL mAb NOK-1 (mouse IgG1) (23) was obtained from PharMingen.

Immunoblotting. Membrane protein extraction was performed as described previously (24). Briefly, dry pellets of astrocytoma cell lines (2×10^6 cells) or tumor biopsy material were lysed with Triton X-114 (Fluka, Buchs, Switzerland). Proteins were isolated by centrifugation, then separated by SDS-PAGE under nonreducing conditions, and subsequently transferred to nitrocellulose (25). The loading and transfer of equivalent levels of protein was confirmed by staining with Ponceau S (Sigma). FasL was detected with PE62 Ab followed by peroxidase-conjugated donkey anti–rabbit Ab (Dianova, Hamburg, Germany) and visualized by a chemiluminescence reaction using the ECL system (Amersham, Little Chalfont, United Kingdom). Specificity was confirmed by inhibition with a competing peptide (20, 25).

Flow cytometry. Analysis of astrocytoma cell lines was performed on a FACScan® (Becton Dickinson, Mountain View, CA) using Lysys II software. Cells were detached from culture vessels using PBS containing 1 mM of EDTA (Sigma), stained with A11 mAb or the isotype-matched control mAb and revealed by FITC-conjugated goat anti–mouse Ab. Dead cells were excluded by propidium iodide (Sigma) gating.

Cytotoxicity assays. For ^{51}Cr release assays, P815 or Fas-transfected P815 target cells were labeled with 100 μCi (^{51}Cr) sodium chromate (Amersham) for 1 h at 37°C, washed twice in DME/10% FCS, and then incubated at 37°C for 2 h to minimize spontaneous release. After a final wash, labeled cells were aliquoted at 2×10^3 cells/well with astrocytoma cells at the indicated E/T ratios in round-bottomed microtiter plates. After incubation for 20 h at 37°C in a 5% CO_2 incubator, supernatants were assayed for radioactivity using a γ-counter. Experiments were performed in triplicate (in three to six independent assays), with spontaneous ^{51}Cr release always < 30% (mean value 18%). Percentage specific lysis was calculated as: 100 × (*experimental ^{51}Cr release − spontaneous ^{51}Cr release*)/(*maximum ^{51}Cr release − spontaneous ^{51}Cr release*).

DNA fragmentation was measured by the JAM test, as described previously (26). Briefly, P815 target cells were labeled with 5 μCi/ml of [^{3}H]thymidine (Amersham) for 20 h. Before the assay, labeled cells were washed twice and aliquoted at 5×10^3/ml in V-bottomed microtiter plates with astrocytoma cells at the indicated E/T ratios (replicates of three to six wells). After 5 h of incubation at 37°C in a 5% CO_2 incubator, cells were harvested and counted in a liquid scintillation β-counter. Percentage of specific DNA fragmentation was calculated as: 100 × (*spontaneous − experimental*)/*spontaneous*. Experimental is counts per minute of retained DNA in the presence of effector cells and spontaneous is counts per minute of retained DNA in the absence of effector cells.

Immunohistochemical staining. 5-μm sections from biopsy material were cut from each sample: 10 sections were used for immunoblotting (see above), the rest were mounted on microscope slides and air dried for staining. Sections were fixed in acetone (Fluka), endoge-

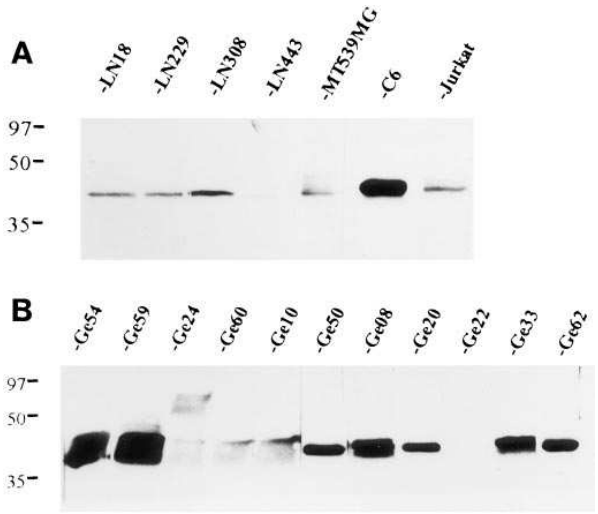

Figure 1. FasL is expressed in vitro and in vivo by astrocytomas. A 40-kD protein revealed by Ab PE62 and corresponding to the membrane-bound form of FasL was detected by immunoblot analysis of (*A*) human astrocytoma cell lines LN18, LN229, LN308, and LN443; murine astrocytoma line MT539MG; rat astrocytoma line C_6; and (*B*) human tumor biopsies from six malignant astrocytomas (two grade III: Ge10, Ge50; four grade IV: Ge8, Ge62, Ge20, Ge33) and four benign astrocytoma (two grade I: Ge54, Ge59; two grade II: Ge24, Ge60). One human malignant astrocytoma (grade IV: Ge22) was found negative for FasL expression. Jurkat cells served as a positive control.

nous peroxidase was quenched with 0.6% H_2O_2 and 0.1% sodium azide (Sigma), and nonspecific sites were blocked with PBS containing 1% BSA (Sigma), 0.1% Tween 20 (Merck), and 3% normal goat serum (Dako). The predetermined optimal concentration of A11/A11-biotinylated mAb or isotype-matched control mAb was added to the sections and revealed by peroxidase-conjugated goat anti–rat Ab or by StreptABComplex/HRP (Dako) for the biotinylated mAb. Then a freshly prepared solution of AEC substrate (3-amino-9-ethylcarbazole, Sigma) and H_2O_2 was added. Hematoxylin was used as counterstain.

Results

FasL expression by human, murine, and rat astrocytoma cell lines was analyzed using immunoblotting (Fig. 1 *A*). The FasL-specific Ab PE62 clearly identified a 40-kD protein (27) in the cell lysate of 5/6 cell lines tested; a faint signal at the same size was also obtained for LN443. This protein corresponds to the membrane-bound form of FasL, rather than the soluble form that migrates at 26-kD (25). Furthermore, flow cytometric analysis of the same cell lines demonstrated the cell surface expression of FasL using the mAb A11 (Fig. 2). This basal level of FasL expression by astrocytoma cells is constitutive, with significant variation between lines.

To test whether the constitutive expression of FasL by astrocytoma cells could induce death of Fas-bearing cells, astrocytoma cell lines were used as cytotoxic effectors in a ^{51}Cr release assay in which they were incubated with Fas-transfected P815 cells as targets. It was possible to use the same target cells for all species (transfected with murine Fas cDNA) since there is functional cross-reactivity between murine Fas and FasL from mouse, rat, and humans (10, 21). Mouse (MT539MG), rat (C_6), and human (LN18, LN229) astrocytoma cell lines all killed Fas^+ targets at modest E/T ratios, but not the parental P815 cells (Fig. 3, *A–D*). Sensitivity of target cells to Fas-mediated lysis was checked in all experiments using the lytic anti-Fas mAb Jo2. Parental P815 cells, which express barely detectable levels of Fas, were resistant to killing induced by Jo2 mAb (mean of specific lysis in seven experiments 3.4±1.3%), whereas this mAb induced 56.2±5.6% specific lysis in Fas-transfected P815 cells in the same experiments. These data show that lysis depends upon high levels of Fas expression by target cells, thus strongly implicating the FasL–Fas interaction as the responsi-

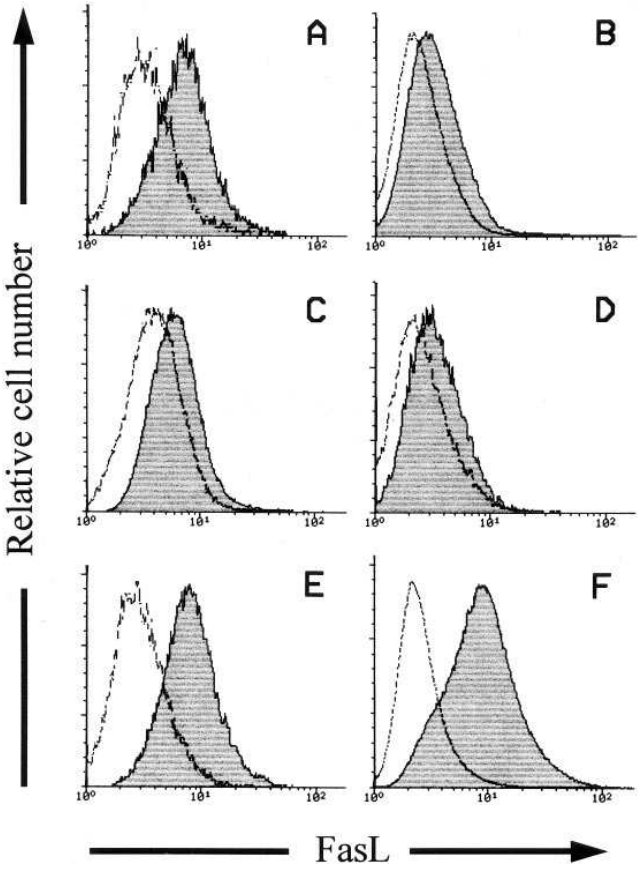

Figure 2. Human, mouse, and rat astrocytoma cell lines express cell surface FasL. Human astrocytoma cell lines LN18 (*A*), LN229 (*B*), LN308 (*C*), LN443 (*D*), murine astrocytoma line MT539MG (*E*) and rat astrocytoma line C_6 (*F*) were stained either with anti-FasL A11 mAb (*filled curve*) or with control mAb 3.168 (*open curve*) followed by FITC-conjugated anti-Ig and analyzed by flow cytometry. Data from a representative experiment (out of a total of three independent experiments) are shown.

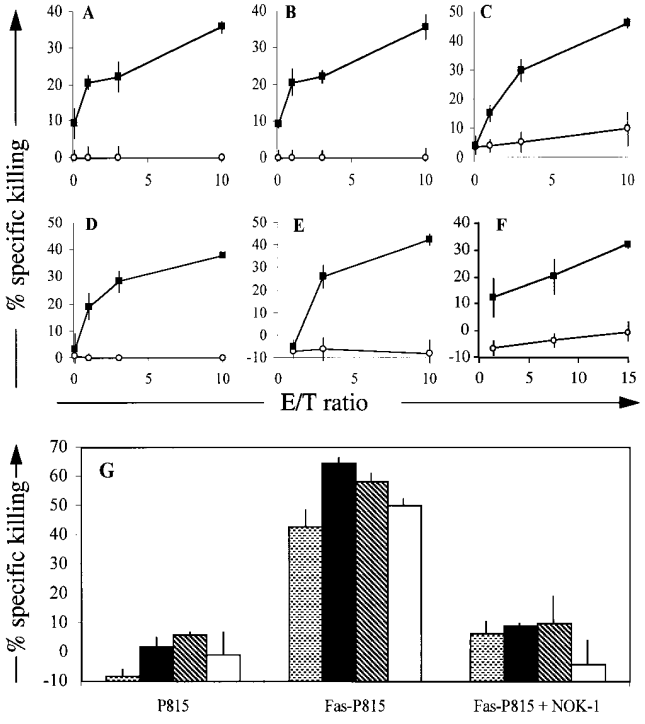

Figure 3. Killing of Fas (CD95)-transfected P815 cells by astrocytoma cells. Cytotoxic activity of established astrocytoma cell lines derived from mouse (*A*, MT539MG), rat (*B*, C_6), and humans (*C*, LN18 and *D* and *E*, LN229) and cytolytic activity of ex vivo tumor cells (*F*, Ge87) were tested against Fas-transfected P815 (*black squares*) or P815 (*open circles*) cells in a ^{51}Cr release assay (*A–D*) or by the JAM test (*E* and *F*). For *G*, DNA fragmentation induced in the indicated target cells by either the established cell line LN229 (*stippled bars*) or by early passage cell lines (Ge33, *solid bars*; Ge60, *hatched bars*; Ge87, *open bars*) was assayed by the JAM test. E/T ratio was 10/1 for LN229, Ge60, and Ge87, and 3/1 for Ge33. The mean±SD of three to six replicate samples is indicated. Specificity was confirmed by inhibition with neutralizing anti–human FasL mAb NOK-1 (added to effectors at 0.75 μg/ml for 30 min before addition of target cells).

ble mechanism. In further cytotoxicity assays in which three other human astrocytoma cell lines were tested, tumor line U-118MG specifically lysed Fas^+ targets, but LN308 and LN443 were consistently nonlytic (assessed in four to six independent experiments, data not shown). We present results of ^{51}Cr release (reflecting membrane damage) measured at 20 h. However, we were able to more directly assess an earlier apoptotic event (DNA fragmentation) using the JAM test (Fig. 3, *E–G*). Indeed, the established human cell line LN229 showed similar levels of specific killing of Fas-transfected P815 cells when DNA fragmentation rather than ^{51}Cr release was measured, this was detectable 5 h after coincubation of effectors and targets (Fig. 3 *D*, ^{51}Cr release; Fig. 3 *E*, JAM test), and as early as 2 h in other tests (data not shown). Furthermore, specificity was confirmed using the neutralizing anti–human FasL mAb NOK-1, which reduced specific killing from 42.6 to 6.5% (Fig. 3 *G*).

To assess whether FasL-mediated function is only a feature of established cell lines cultured long-term in vitro, astrocytoma cells directly obtained after enzymatic dissociation of tumor biopsy (Ge87) were also tested. These cells efficiently and specifically killed Fas^+ targets (Fig. 3 *F*), with DNA fragmentation totally inhibited by NOK-1 (0.1% specific killing at an E/T of 15/1 in the presence of 0.75 μg/ml mAb). In addition, early passage cell lines Ge33, Ge60, and Ge87 (derived from the same biopsy that was tested ex vivo) also exhibited FasL-mediated killing, with specificity clearly defined by inhibition with NOK-1 mAb and absence of activity against parental P815 (Fig. 3 *G*).

To confirm our data on astrocytoma cell lines and cells ex vivo, we also studied expression of FasL on a series of human astrocytoma biopsies by immunoblotting and immunohistology. As in the astrocytoma cell lines, the membrane-bound form of FasL was readily detected in cell lysates from 6/7 malignant and 4/4 benign tumors (Fig. 1 *B*). The FasL protein detected by immunoblotting predominantly derives from astrocytoma cells, since analysis of glial fibrillary acidic protein–stained sections revealed that only homogeneous tumor tissue had been selected (data not shown). Further immunohistochemical analysis of some tumor sections confirmed that astrocytoma cells express FasL in vivo (Fig. 4, *A–E*). Staining was heterogeneous in intensity and distribution, even within the same tumor. Nontumoral control tissue (temporal cortex) showed no evident FasL expression by normal human astrocytes in contrast to the clear expression by neurons (Fig. 4 *F*). Similar staining was observed with normal rodent brain tissue, i.e., no detectable expression of FasL by normal astrocytes but a constitutive FasL expression on neurons (Fig. 4, *G* and *H*; positive staining of mouse Purkinje neurons and rat cortical neurons is shown).

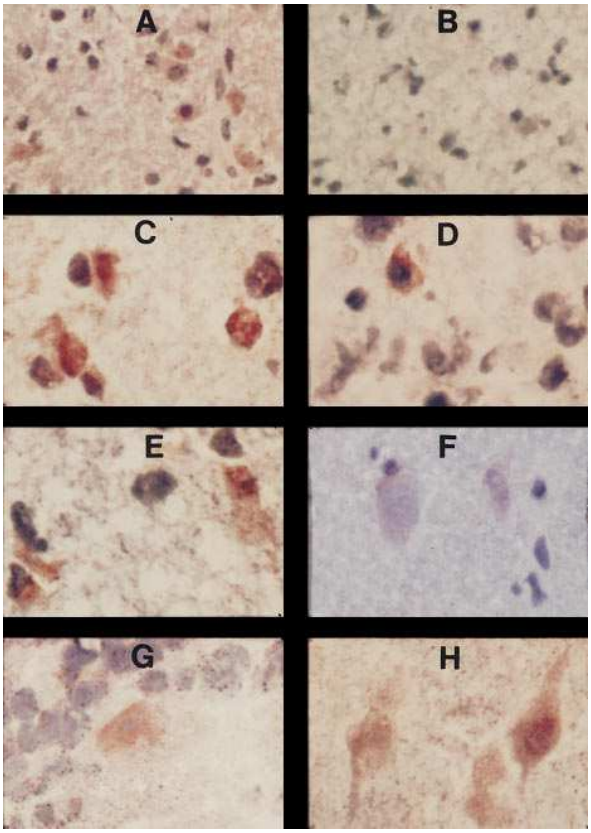

Figure 4. FasL is expressed on astrocytes in tumor tissue but only on neurons in nontumoral brain tissue. Cryostat sections from human grade IV astrocytomas Ge33 (*A–C*) and Ge62 (*D*); human grade II astrocytoma Ge60 (*E*); human nontumoral tissue (temporal cortex) (*F*); normal mouse brain tissue (*G*) and normal rat brain tissue (*H*) were stained with anti-FasL mAb A11 (*A* and *C–G*), A11-biotin (*H*), or control mAb 3.168 (*B* as a representative example), followed by a peroxidase-conjugated Ab (or StreptABComplex/HRP) and revealed by AEC. Sections were counterstained with hematoxylin. Final magnifications: *A* and *B*, ×160; *F*, ×350; and *C–E*, *G*, *H*, ×400.

Discussion

In this study, we have analyzed expression of FasL on astrocytoma and normal CNS tissue. A series of human astrocytoma lines were $FasL^+$ as well as the rat C_6 and mouse MT539MG lines. However, in common with many tumors, the in vitro phenotype of astrocytoma does not always reflect the in vivo situation (28). Therefore, it was essential to confirm these findings in vivo, and indeed, the majority (10/11) of astrocytomas were positive for FasL protein expression. Furthermore, purified astrocytoma cells tested by immunoblot ex vivo were also FasL positive (data not shown). Some cell lines and tumor biopsies were also analyzed by RT-PCR to confirm FasL expression at the mRNA level: all samples tested were positive, whereas a control Schwannoma biopsy was negative (data not shown). The hierarchy of FasL expression levels between different cell lines in immunoblot, flow cytometry, and PCR was not always identical. This may reflect differences between surface expression (by flow cytometry) and total expression of FasL. However, it is clear that even modest levels of FasL surface expression are sufficient for functional activity (10). It was noteworthy that in vivo expression of FasL was restricted to tumoral astrocytes, but in normal tissue, we also observed a strong FasL expression on neurons, as reported previously in mouse (29).

Does the expression of FasL on astrocytoma cells (deriving from a nonlymphoid lineage) influence tumor–immune system interactions? FasL expression is not always linked to lytic potential: the murine thymoma line EL-4 expresses FasL without detectable cytotoxicity against Fas^+ targets (30) and *gld* mice express a mutant form of FasL that is nonfunctional (20). However, not only with the established astrocytoma cell lines used for this study, but also with tumor cells tested directly af-

ter digestion and early passage cell lines, we observed efficient lysis of Fas^+ target cells at modest E/T ratios. Such a level of cytotoxicity is relevant to cell–cell interactions in vivo, where leukocytes infiltrating astrocytoma are generally outnumbered by tumor cells (8, 9). Monocytes (31), neutrophils (31), and activated T cells (12) express Fas and thus may be susceptible to a death signal delivered by the functionally active FasL expressed by astrocytoma cells. These findings may be of importance at various stages of the antitumor immune responses. During the initiating steps, cells such as neutrophils and macrophages may be affected, since they express Fas in their resting state and are sensitive to Fas-mediated cell death (31). If these cells are eliminated, the local accumulation of inflammatory cytokines would be diminished, thus limiting a subsequent specific immune response that is dependent upon induction or upregulation of MHC and adhesion molecules (2). While such processes may limit immune dialogue, we believe that the response is not totally abrogated at this induction stage, as evidenced by the lymphocytic infiltration (8, 9) and substantial T cell oligoclonal expansion observed in astrocytoma (Dietrich, P.Y., V. Schruriger, G. Perrin, P. Saas, A.L. Quiquerez, and P.R. Walker, manuscript in preparation). Why is such a T cell response ineffective against tumor growth? The constitutive nature of FasL expression by astrocytoma cells, rather than being solely inducible as for T cells, is probably important, particularly in the tumor microenvironment in which T cell function is compromised by soluble factors. For example, TGF-β may reduce perforin mRNA levels in $CD8^+$ T cells (32), thus impeding this fast-acting lytic pathway. This could confer a kinetic advantage to the tumor allowing time for a FasL-mediated death signal to induce T cell deletion/inactivation.

FasL expression may be a general mechanism of preserving immune privilege in the brain (29, 33). Even in a pathological state, the brain maintains FasL expression in both tumoral and normal compartments (neurons). In the tumoral tissue, in which no neurons are present, there is still FasL expression, but in this case by the astrocytoma cells. It will be important to determine in future studies if FasL is inducible on normal astrocytes, as is MHC expression (2), and whether malignant transformation mimics such an "activated" state. It is interesting to note that functional FasL was reported recently in a single colon cancer cell line. However, expression in vivo or on normal tissue was not analyzed in this study (34). Furthermore, melanoma cells also express FasL, which was shown to influence the rate of tumor growth in vivo (35).

A further complexity in astrocytoma–lymphocyte interactions is that Fas is expressed on some human astrocytoma cell lines and is also present, although not uniformly, on astrocytoma cells in vivo (28). Thus, even without lymphoid cell contact, Fas–FasL interactions may regulate tumor growth by autocrine suicide or "fratricide." However, in vivo susceptibility to Fas-mediated death may be influenced by the particular conditions found in localized areas of the tumor (cytokines, hypoxia, etc.). Indeed, it has been shown that cytokines such as IFN-γ or TNF-α modify Fas expression and susceptibility to anti-Fas Ab-induced death of astrocytoma lines (28). It is clear that there are differences between Fas and FasL expression not only between tumor cells and lymphoid cells, but also between various lineages of tumor cells such as melanoma (35), colon cancer (34), and now astrocytoma. This raises important questions regarding the regulation of these molecules in these different cell types.

In conclusion, we have demonstrated that functional FasL is widely expressed by a nonlymphoid tumor, astrocytoma. This could profoundly influence the efficacy of the immune response against astrocytoma and the rate of tumor growth. Furthermore, the distribution of FasL expression in both normal CNS and astrocytoma was conserved in rat, mouse, and humans, suggesting that this may be a universal mechanism to limit immune reactivity in a brain tumor as well as in the normal CNS.

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