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Co-expression of Fas and Fas ligand in malignant glial tumors and cell lines

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Abstract Fas ligand (FasL) is involved in tumor evasion from the immune system. We analyzed 22 human gliomas for expression of FasL and its receptor, Fas. Positive FasL and Fas immunoreactivity was detected in 13 out of 22 tumors by Western blotting and in 15 out of 22 tumors by immunohistochemistry. Immunohistochemistry also showed that Fas and FasL expression was confined to tumor cells. Co-expression of these molecules was confirmed by Western blotting and immunohistochemistry in 4 of 7 glioma cell lines. Co-expression of FasL and Fas within tumor cells suggests that their contribution in vivo to the process of immune system evasion and tumor cell apoptosis is complex and probably involves additional factors.

Introduction

Fas (APO-1/ CD95) is an important mediator of T cell cytotoxicity [7]. Fas is a type I transmembrane protein belonging to the tumor necrosis factor receptor/ nerve growth factor receptor family that is expressed in a variety of cell types, such as activated T and B cells, neutrophils and hepatocytes (reviewed in [8]). Fas binds to its ligand (designated as FasL, CD95L or APO-1 ligand), thereby undergoing trimerization and subsequent activation of the interleukin 1β-converting enzyme (ICE) protease cascade responsible for cellular apoptosis [8].

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FasL expression is restricted to a limited number of cell types, including Sertoli cells, epithelial cells of the anterior chamber of the eye, and neurons [1, 2, 4]. This pattern of expression has been postulated as the basis for the "immune priviliged" status of such sites since binding of FasL to T cells expressing Fas would lead to T cell apoptosis. Recently, expression of FasL has also been detected in melanoma and hepatoblastoma cells [5, 10]. In fact, injection of mouse melanoma cells expressing FasL led to the rapid formation of tumors, while tumor formation was retarded after injection of the same cells in Fas-deficient mice [5]. These findings led to the hypothesis that neoplastic FasL expression may contribute to tumor immune evasion.

Evasion from immune cells may be one of the mechanisms that permit the survival of malignant glial tumors in the brain. Interestingly, it has been reported that malignant brain tumors express Fas and that this expression is preferentially found in perinecrotic cells [11]. We reasoned that FasL production by malignant glial tumors may be a possible mechanism of immune evasion and that this expression would not correlate with expression of Fas within the same tumor. To address this possibility, we evaluated malignant gliomas and glioma cell lines for expression of Fas and FasL by Western blotting and immunohistochemistry.

Materials and methods

Cell lines, tissues, antibodies

Established human glioma cell lines (U87, MGH238, U138, U343, and T98) were originally purchased from the American Tissue Culture Collection. Human astrocytic HYQC cells represent a primary culture from a surgically resected pilocytic astrocytoma. Rat 9L gliosarcoma cells were originally obtained from the University of California Brain Tumor Center. Cells were maintained in culture in Dulbecco's modified essential medium (Cellgro, Mediatech, Va.) supplemented with 10% fetal calf serum, 100 U penicillin, and 0.1 mg streptomycin/ ml medium (Sigma). Tumor tissue from 22 malignant gliomas was collected at the time of surgery and stored by freezing in liquid nitrogen. Tumors were evaluated histologically and graded according to WHO criteria. Samples

consisted of 14 glioblastomas, 3 grade III anaplastic astrocytomas, 3 grade II astrocytomas, 1 pleomorphic xanthoastrocytoma and 1 grade II oligoastrocytoma.

The mouse monoclonal IgG1 anti-FasL (Transduction Laboratories, Lexington, Ky.) is directed against human FasL peptide fragment 116–277. The monoclonal mouse IgG1 anti-Fas was obtained from Kamiya Biomedical Laboratories (Seattle, Wash.). The secondary antibody for the immunohistochemical and Western blot reactions consisted of antimouse IgG (Amersham, Arlington Heights, Ill.).

Western blot analysis

Cells were harvested from confluent cultures and collected by low-speed centrifugation. Pellets were then resuspended in 500 µl phosphate-buffered saline (PBS) containing 1% Triton X-100 (Sigma). Further disruption of cell membranes was achieved by passing this lysate through a 27-gauge needle. The lysate was the stored at –80° C until use. Tumor tissue was homogenized in PBS using a Dounce pestel for 5 min followed by centrifugation at 3000 g for 5 min at 4° C. The supernatant from the tumor lysate was then stored at –80° C until use. The protein content of the cell lysates and of the tissue homogenates was estimated using the Bradford's protein assay (Bio-Rad Laboratories, Hercules, Calif.). Electrophoretic separation of proteins in the cell and tumor lysate was performed in a denaturing sodium dodecyl sulfate (SDS)/ 10% polyacrylamide gel. Rainbow molecular weight markers (Sigma) were employed to estimate the size of detected proteins. Equivalent loading and transfer of protein was ascertained by employing Ponceau S staining (Sigma) after nitrocellulose membrane transfer in a Bio-Rad apparatus. Membranes were then incubated in 5% nonfat milk (Bio-Rad) in TRIS-buffered saline containing 0.1% Tween 20 (TBST), to block nonspecific staining. FasL antibody (1 : 2000 dilution) and Fas antibody (1 : 400 dilution) were then allowed to react with the proteins on the membrane for 1 h. After washing three times with TBST, secondary antibody reactions were carried out with horseradish peroxidase-conjugated antimouse IgG (Amersham) for 45 min, followed by washing. Visualization of the reaction product was performed using the Enhanced chemiluminescence (ECL) system (Amersham).

Immunohistochemistry

Frozen sections (7 µm) from the tumors were mounted on slides, fixed with acetone at –20° C for 10 min, and then incubated in PBS at 37° C for 30 min. After blocking with goat serum (Vector, Burligame, Calif.) for 1 h, sections were incubated with the primary antibody (anti-FasL at a dilution of 1 : 100 and anti-Fas at a dilution of 1 : 50) overnight at 4° C. Subsequent reactions were performed using the ABC Vectastain kit (Vector), using diaminobenzidine as the substrate. Slides were counterstained with hematoxylin.

Results

FasL expression

Representative Western blots of 19 tumors are shown in Fig. 1 A. In total, 13 of 22 tumors were positive for FasL expression, as evidenced by an immunoreactive band at approximately 40,000 Da. The remaining tumors showed no evidence of immunoreactive FasL even after prolonged exposure. The pattern of immunoreactive FasL expression did not correlate with histological grade since 7/14 glioblastomas, 4/6 grade 2 and 3 astrocytoma, the pleomorphic xanthoastrocytoma and the single mixed tumor were positive for FasL expression. Figure 2 A further reveals that FasL expression was seen within tumor cells;

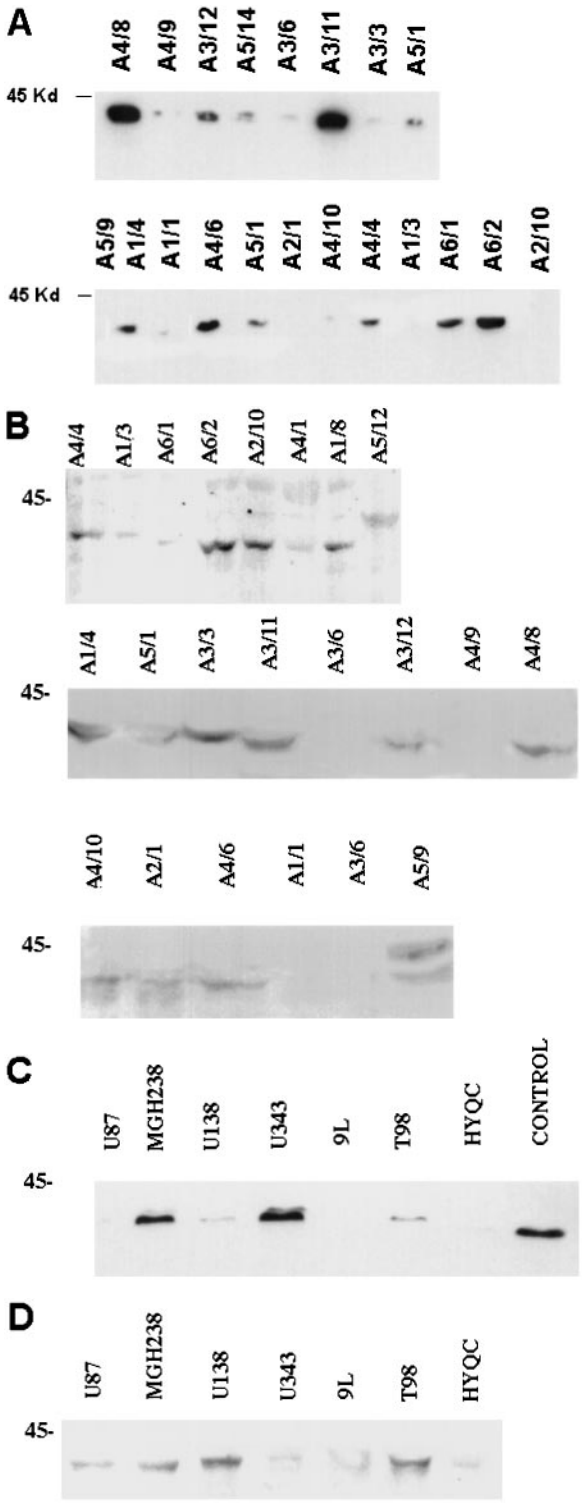

Fig. 1 A–D Western blot analysis of FasL and Fas expression in representative human gliomas and glioma cell lines. **A** Cellular lysates from 19 of the 22 human glioma lysates are shown. Tumors A4–1, A1–8, and A5–12 are not included in the figure but were negative. An immunoreactive band corresponding to FasL is shown at 40,000 Da. **B** The same human glioma lysates were analyzed for Fas expression. A faint band can be seen migrating at approximately 40,000 Da, corresponding to Fas immunoreactivity within the tumors. The blot from tumor A5–14 is not shown but it was positive. **C** Lysates from 6 human glial cell lines and 1 rat gliosarcoma cell line (9L) were analyzed using the FasL antibody. **D** The same analysis was performed using the Fas antibody

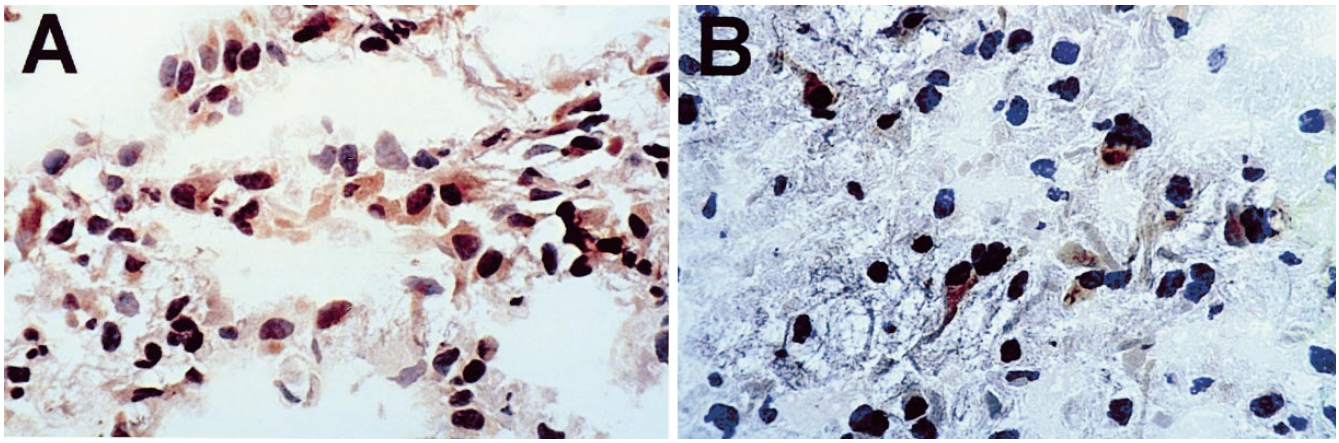

Fig. 2 A, B Immunocytochemical analysis of FasL and Fas expression in human gliomas. **A** Immunoreactive FasL was detected within tumor cells. **B** In an adjacent section, immunoreactive Fas could also be detected within tumor cells

Table 1 Fas and FasL expression in gliomas (*IHC* immunohistochemistry, *G3* grade 3 (WHO), *G2* grade 2 (WHO), *P. xanthoastrocyt.* plemorphic xanthoastrocytoma)

No.	Tumor	Fas		FasL	
		Western blot	IHC	Western blot	IHC
A1-1	Glioblastoma	–	+	+	+
A1-3	Glioblastoma	+	+	–	+
A1-4	Glioblastoma	+	+	+	+
A1-8	Glioblastoma	+	+	–	+
A2-1	Glioblastoma	+	–	–	–
A2-10	Glioblastoma	+	+	–	–
A3-3	Glioblastoma	+	+	+	+
A3-6	Glioblastoma	–	–	+	+
A4-1	Glioblastoma	–	–	–	–
A4-8	Glioblastoma	+	+	+	+
A4-9	Glioblastoma	–	+	–	–
A5-1	Glioblastoma	+	+	+	+
A5-9	Glioblastoma	+	+	–	+
A6-2	Glioblastoma	+	+	+	+
A5-12	Astrocytoma G3	–	–	–	–
A3-11	Astrocytoma G3	+	+	+	+
A6-1	Astrocytoma G3	+	+	+	+
A3-12	Astrocytoma G2	+	+	+	+
A4-6	Astrocytoma G2	+	+	+	+
A4-10	Astrocytoma G2	+	+	–	–
A5-14	P. Xanthoastrocyt.	+	+	+	+
A4-4	Oligoastrocytoma	+	+	+	+

no staining was observed in non-neoplastic cells within the tumor.

Co-expression of Fas and FasL in astrocytic tumors and tumor cell lines

Immunohistochemistry (Fig. 2 B) also showed expression of Fas within tumor cells from the same tumors. Immunohistochemical analysis of the 22 tumors revealed that there was positive immunocytochemistry for both Fas and FasL in 15/22 tumors, while 3/22 tumors were positive only for Fas, 1/22 was positive for FasL, and 3/22 were negative for both.

To co-expression of both Fas and FasL was confirmed by Western blot analysis of the 22 tumor samples (Fig. 1 B). Of the 22 tumors 17 expressed Fas; 11 co-expressed Fas and FasL, while only 6 expressed Fas and only 2 expressed FasL. The Western blot results agreed with the immunocytochemical findings except for tumors A1-1, A2-1, and A4-9 (in which there was discordance for Fas detection between the Western blot and the immunohistochemical analysis) and tumors A1-3, A1-8 and A5-9 (in which there was discordance for FasL detection between the Western and the immunohistochemical analysis) (Table 1).

Fas and FasL were also co-expressed in glioma tumor cell lines. Four of seven cell lines co-expressed both Fas and FasL (Fig. 1 C, D), while human U87 glioma and HYQC (a human pilocytic astrocytoma cell line) cells expressed only Fas. Rat 9L gliosarcoma cells did not show immunoreactivity for either.

Discussion

Expression of FasL is a potential mechanism for tumor evasion of the immune response. Malignant gliomas, however, have been reported to express Fas and, thus, co-expression of FasL would not be expected [11]. In this study, we tested the hypothesis that Fas and FasL expression should occur in different gliomas. Surprisingly, the tumor cells in the majority of the studied gliomas and glioma cell lines co-expressed both molecules, even in the same regions of the tumor. Lymphocytes were not prominent in the analyzed tissues, but did not appear to express either Fas or FasL. However, we cannot exclude expression of these molecules in other regions of the tumor. The issue of Fas and FasL expression in tumor-infiltrating lymphocytes in optimally fixed and embedded specimens should be addressed in future studies using appropriate antibodies.

Co-expression of these molecules in gliomas seems counter-intuitive. It remains unclear whether tumors that

co-express these molecules show higher rates of apoptosis than those that lack co-expression. Kang et al. [6] recently demonstrated FasL expression in pancreatic islet cells and showed that such cells underwent accelerated neutrophilic rejection that was T and B cell independent. Furthermore, up-regulation of Fas expression in FasL-expressing islet cells fails to produce apoptosis. Such results suggest that the interaction between Fas and FasL in cells in vivo is complex and that additional factors may modulate Fas/FasL associations in vivo.

It is also possible that Fas is not fully functional in gliomas. For instance, the levels of Fas detected by Western blotting in our studies appeared lower than those of FasL. If appropriate stoichiometric interactions are important for a full Fas/FasL response, Fas expression in a glioma cell would be less important than the interaction between FasL in the same tumor cell and Fas within activated immune cells infiltrating the tumor. Further studies assessing the ability of freshly explanted Fas-positive and FasL-positive tumor cells to produce apoptosis of T cells purified from the same patient may resolve this question.

Our Western blot results extend the immunohistochemical and reverse transcriptase-polymerase chain reaction studies recently published by Gratas et al. [3]. These authors detected both Fas and FasL co-expression in 12/12 tested gliomas and 10/10 glioblastoma cell lines. They also observed topographic differences in the pattern of Fas and FasL expression within tumors, which were not apparent in our studied samples. It is possible that observed differences may be related to sampling. Saas et al. [9] were able to detect FasL expression in 5/6 cultured astrocytoma cells, in 6/7 malignant and 4/4 benign glioma tumors. Together, these studies show that co-expression of both molecules occurs in the majority of glioma tumors and cells. Furthermore, in our study we show that cases exist in which only Fas or FasL alone (or neither) are expressed.

The pleomorphic xanthoastrocytoma studied in this report had both Fas and FasL immunopositivity. These tumors are frequently infiltrated by lymphocytes, yet this case did not reveal evidence of lymphocytic infiltration. It is interesting to speculate that FasL expression in this tumor may actually have been associated with the failure to find invasion by immune cells, although additional samples would have to be studied before firm conclusions can be drawn.

In conclusion, it seems likely that complex intracellular and intercellular processes are modulating the interaction between Fas and FasL in vivo and that further investigative efforts are required to dissect the contribution of these molecules to the process of tumor progression, immune system evasion, and apoptosis in vivo.

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