Interaction of fas and fas ligand in a rat 36b10 glioma model

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Object. An experimental model of the fas and fas ligand (fasL) interaction in malignant glioma was developed.

Methods. Using plasmid-based delivery, 36B10 rat glioma cells were modified to express fas (36B10-fas), and a delivery fibroblast cell line was modified to produce fasL, resulting in the FR-fasL cell line. Evaluation of fas expression was performed with flow cytometry and expression of fasL confirmed by Western blot analysis. Once the cell lines were created and partially characterized, fas-induced cytotoxicity was evaluated using an antibody-mediated assay for 36B10-fas that demonstrated significant toxicity at 24 and 48 hours. To evaluate the potential for activating the fas molecule by using cell-mediated delivery, coculture cytotoxicity studies were performed with a target cell line (36B10-fas) and effector cell line (FR-fasL). Using a series of culture ratios, increasing cytotoxicity was noted, suggesting that activation of the transfected fas receptor by fasL expression on the carrier cell was occurring.

Conclusion. Based on their experiments, the authors describe a model for evaluating the interaction of fas and fasL in a cellular model of malignant glioma.

KEY WORDS • glioma • fas • fas ligand • cytotoxicity • gene transfer

FAS is a type I membrane protein that mediates the induction of apoptosis in selected cells when ligated either by an anti-fas antibody or by interaction with fasL.^{2,6,10,23} Fas is a member of the TNF/nerve growth factor receptor superfamily.^{33,34} Fas ligand is a membrane-type cytokine belonging to the TNF family and is expressed in T cells activated by various factors including interleukin 2, phorbol myristate acetate, and anti-CD3.^{24,30,31} The fas/fasL pathway has been shown to play a role in T cell-mediated cytotoxicity.²⁵

The role of apoptosis, or programmed cell death, in the development of CNS neoplasia has received increasing interest as techniques for studying the signaling pathways and genetics of this process have been developed.^{15,16,28,36,39,42,48} The mechanism of apoptotic cell death is complex. It involves receptor-mediated protease activation, cytolytic plasma protein interactions, and ultimately distinct morphological alterations. These alterations can be observed microscopically and include DNA degradation and margination, condensation of chromatin, cell membrane alter-

ations and the formation of apoptotic cascade. Research specifically focused on apoptosis and CNS neoplasia has included interaction of *p53* with the *retinoblastoma* gene and TNF- α , the identification of apoptosis-protective genes in glioma, and upregulation of protease enzymes important to the apoptotic cascade.^{18-21,35} A recent description of the interaction of fas-induced apoptosis with transforming growth factor- β_1 suggests the central role of these interactions in the modulation of the cell cycle.¹ In a series of studies, Weller, et al.,⁴⁴⁻⁴⁷ have examined fas-induced cytotoxicity in a series of human glioma explants. They have shown, under selective conditions, that apoptosis can be induced when ligated by an anti-fas antibody.

Given the increasing evidence that apoptosis may play a role either in the induction or propagation of neoplastic transformation, we undertook to develop a model in which to examine fas/fasL interaction in glioma cells. In this report we describe the development of a plasmid-based delivery system for fas and fasL that allows the interaction of these molecules to be examined in malignant glial tissue. Because antibodies to these molecules are available, they can be assayed in a semiquantitative fashion to determine expression, and assays for cytotoxicity allow the effect of their interaction to be determined.^{3,4,27} Because of the complexity of the apoptotic cascade and the multifac-

Abbreviations used in this paper: cDNA = complementary DNA; CNS = central nervous system; fasL = fas ligand; FR-fasL = fibroblast cell line modified to produce fasL; MTT = methylthiotetrazole; TNF = tumor necrosis factor.

torial events involved in neoplastic transformation, it is unlikely that any single system will allow these cells to be eliminated.⁷ However, additional examination of the fas and fasL system and other interactions in the apoptotic cascade will provide insight into the steps involved in the maintenance and control of cellular proliferation.

MATERIALS AND METHODS

Cell Lines and Primary Cultures

All cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 10 mmol/L HEPES, pH 7.2. The 36B10 rat glioma cell line was used for transfection and cytotoxicity studies as described later.⁴⁰ These cells were assayed for fas expression by using flow cytometry. We used the Yac-1 (mouse lymphoma) and fibroblast (Sprague-Dawley rat fibroblast) cell lines. The Yac-1 cell line was used as a positive control for mouse fas expression.²⁹

Plasmid Construction. In a plasmid containing cDNA for rat fasL in pBlue Script, the 1.6-Kb fragment containing the fasL cDNA was excised using XhoI and religated into the multiple cloning site of pcDNA3.1/zeo, creating pcDNA3.1zeo-fasL. Orientation was confirmed by restriction digest and appropriate clones expanded. The fas (murine)-containing plasmid was used as previously described.⁴³

Cell Transfection. The 36B10 glioma cells were propagated and 5×10^4 cells were selected for transfection. The fas plasmid (30 μ g) was introduced into the target cells by using calcium precipitation, and positive clones were selected for neomycin resistance by using 500 μ g/ml G418 (36B10-fas). Rat fibroblasts were propagated and 2×10^5 were transfected with 2 μ g pcDNA3.1zeo-fasL by using 5 and 10 μ l of Lipofectin; positive clones were selected for zeocin resistance (400 μ g/ml). For a negative control cell line fibroblast was transfected with 2 μ g pcDNA3.1zeo by using Lipofectin.

Flow Cytometry. A total of 10^6 36B10, 36B10-fas, and Yac-1 cells (positive control) were incubated with 5 μ g/ml biotinylated anti-mouse fas monoclonal antibody, washed, and reincubated with streptavidin-conjugated FITC (1:100). All samples were incubated with their appropriate isotype controls and then analyzed using a FACScan flow cytometer on a logarithmic scale at 488 nm.

Western Blot Analysis. The expression of fasL in the line FR-fasL was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Briefly, total protein from serum-free supernatant (0.5 ml) obtained from FR-fasL cultures was precipitated with acetone and then electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel, transferred to nitrocellulose, and immunostained with polyclonal rabbit anti-fasL antibody (2 μ g/ml). The resultant protein band was reacted with biotinylated anti-rabbit (1:500), followed by serum albumin-horseradish peroxidase for detection. Kaleidoscope molecular-weight markers were used to estimate sizes.

Cytotoxicity Assays

Antibody-Mediated Toxicity. Transfected 36B10-fas rat glioma, 36B10 cells, and Yac-1 cells were plated into 96-well tissue culture plates at a cell density of 1×10^4 cells/well in a total volume of 50 μ l and incubated overnight at 37°C. Anti-mouse Fas was added to cells in concentrations ranging from 0.01 to 10 μ g/ml for 24 and 48 hours in the presence of 1 μ g/ml cyclohexamide as previously described.^{14,45} After indicated times, the wells were assayed for cell viability by using an MTT assay as previously described.⁴⁰

Effector/Target Toxicity. Cocultures of target cells (36B10, 36B10fas, and Yac-1) and FR-fasL were assayed for cytotoxicity. Target cells were plated into 96-well round-bottom plates at 5×10^3 cells/well. Into the same wells, effector cells were plated to achieve final effector/target ratios of 0.25, 1.0, 5.0, and 10.0. The cocultures were incubated for 24 hours at 37°C and assayed for cytotoxicity by using an MTT assay as noted in the preceding section. The percentage of cytotoxicity was calculated by obtaining the absorbance value at 550 nm from the MTT assay for each condition by using the following formula: $(1 - [\{\text{Effectors} + \text{Targets}\} - \{\text{Effectors}\}] / \text{Targets}) \times 100$.¹⁷

SOURCES OF SUPPLIES AND EQUIPMENT

We obtained the Dulbecco's modified Eagle's medium, HEPES, and Lipofectin from Gibco BRL (Grand Island, NY). The fetal bovine serum was purchased from Sigma (St. Louis, MO). A. Spence (University of Seattle, Seattle, WA) kindly provided the rat glioma cell line, and S. Nagata (Osaka Bioscience Institute, Osaka, Japan) kindly provided the cDNA-containing plasmid. The Yac-1 and fibroblast cell lines were obtained from American Tissue Type Collection (Rockville, MD). We acquired the pcDNA3.1zeo from Invitrogen (San Diego, CA). The fas (murine)-containing plasmid came from the laboratory of P. Goldstein (Centre d'immunologie de Marseille-Luminy, France). The calcium precipitation, used to introduce the fas plasmid to the target cells, is produced by Stratagene (La Jolla, CA). Pharmigen (San Diego, CA) manufactures the anti-mouse fas monoclonal antibody and the anti-mouse (Jo2).

The streptavidin-conjugated FITC and the FACScan flow cytometer were purchased from Becton-Dickinson (Franklin Lakes, NJ). We obtained the polyclonal rabbit anti-fasL antibody from Santa Cruz Biotech (Santa Cruz, CA); the SA-HPP from Biogenex (San Ramon, CA); and the kaleidoscope molecular-weight markers from BioRad (Rockville, NY).

RESULTS

Cell Preparation and Confirmatory Studies

The plasmid construction and cell transfection were performed using standard techniques. To analyze the 36B10 glioma cell line for fas-expression before and after transfection with a fas-containing plasmid, we used fluorescent-activated flow cytometry. As seen in Fig. 1, prior to transfection, the 36B10 cell line did not appear to

Fig. 1. Graphs showing the results of flow cytometry of 36B10 (left), 36B10-fas (center) and Yac-1 (right) cells. Each cell culture was incubated with anti-fas antibody and sorted using fluorescent-activated cell sorting analysis. The second peak indicates cells that have been labeled with the anti-fas antibody. In the case of 36B10-fas, this indicates successful cell transfection. The Yac-1 cells (known to express fas) serve as a positive control.

express a significant amount of fas. Following transfection, the second peak depicted in Fig. 1 indicates the occurrence of binding with the anti-fas antibody in a significant number of cells; the Yac-1 cell line serves as a positive control.

The expression of fasL was confirmed in the line FR-fasL by using Western blot analysis. Staining with anti-fasL antibody resulted in a band at approximately 40 kD as expected.

Antibody-Induced Cytotoxicity

Incubation of the target cell line with increasing amounts of anti-fas antibody resulted in cytotoxic effects, presumably caused by activation of the fas receptor complex (Fig. 2). At the 24- and 48-hour intervals, significant cytotoxicity was observed even at the lowest anti-fas antibody concentrations tested ($p < 0.01$, two-tailed t test). These studies confirmed that altered sensitivity to fas-induced cytotoxicity resulted from transfection of the 36B10 (fas-negative) cell line by transfection with the fas-containing plasmid.

Coculture Results

To determine whether a cellular delivery source of a fas-activating molecule could elicit a biological effect on the fas-expressing target cell (site-specific delivery), we used a coculture model of target (36B10-fas) and FR-fasL. By using increasing ratios of fasL-expressing cells, we were able to demonstrate increased cytotoxicity in the cocultures (Fig. 3). The increase in cytotoxicity reached statistical significance at an effector/target ratio of 1.0 ($p = 0.0001$, two-tailed t test) and was clearly significant at the higher ratios of 5.0 ($p < 0.0001$) and 10.0 ($p < 0.0001$). The Yac-1 cells served as positive controls and were clearly even more sensitive to fas activation.

DISCUSSION

Interest in the fas and fasL interaction, including the effect on cellular immunology and induction of apoptotic

cell death, has increased since first described.¹² The fas and fasL system has been postulated to play an important role in how tumor cells evade immune surveillance, and it

Fig. 2. Graphs indicating the presence of anti-fas antibody-induced cytotoxicity. The 36B10- (circles) and 36B10-fas-containing cells (squares) were incubated with increasing concentrations of anti-fas antibody to elicit activation of the fas receptor. Significant toxicity was observed at all concentrations tested at both 24 (upper) and 48 hours (lower). The Yac-1 cells (triangles) served as a positive control.

Fig. 3. Bar graph showing results of coculture of 36B10-fas and FR-fasL. Cytotoxicity studies were undertaken using increasing ratios of effector (fasL expressing) to target (36B10-fas) cells. The resultant increase in cytotoxicity is attributed to increased activation of the transfected fas receptors on the 36B10-fas cells. The higher cytotoxicity observed in the Yac-1 cells suggests that increasing expression of fas should result in an increased biological effect.

has been proposed as a mechanism by which apoptosis in neoplastic tissues is induced and controlled.^{9,32,42} Analysis of our data suggests that fas and fasL can be upregulated and that cytotoxicity can be induced in target cells by activation of the fas receptor.

We were able to demonstrate upregulation of both molecules in the transfected cells and were able to induce cytotoxicity in the target cell line with antibody and ligand activation of fas. The findings in this study confirm those reported by Weller, et al.,⁴⁴⁻⁴⁶ and others³⁰, suggesting that fas can be upregulated and potentially used as a target for cell-specific therapy.

This type of approach, in which genetic material is introduced into the cellular genome in an attempt to elicit a specific effect, is an example of "genetic therapeutics," as opposed to the more common term "gene therapy" in which it is implied that, in some fashion, a faulty genome is being repaired or replaced. Transfection with the fas-containing plasmid is not an attempt to correct a known defect in the genome but, rather, takes advantage of a known biological effect when a specific receptor is activated.⁷

Expression of fas in the normal adult CNS appears to be restricted to endothelial cells; however, there is increasing evidence that fas is expressed in glial tumors and that it is present in amounts corresponding roughly with their biological behavior.^{8,11,38} Expression of the fasL, first described in activated T lymphocytes, has been demonstrated in certain epithelial tissues including neurons and the Sertoli's cells of the testes.⁴⁹ It has also been demonstrated in malignant cells including those found in melanoma, breast carcinoma, and glial-derived tumors.^{13,26} Whether the presence of fas and fasL in glial tumors represents simply an indication of increased apoptosis in a tissue undergoing rapid cell growth or whether their presence indicates an intrinsic defect in normal cell metabolism and turnover is unknown. In our laboratory and those of oth-

ers the coexpression of both of these factors on the same type of malignant glial cell has been demonstrated, and the level of expression appears to correspond with the biological behavior of the tumor.^{5,8,11} The biological advantage conferred on the cell, by the expression of both factors simultaneously, is unknown but suggests an important role for apoptosis and immunoregulation in the progression of malignant glioma. There may also be a critical balance of fas and fasL expression that is disrupted in the progression to the neoplastic state, as is believed to occur in other apoptotic-immunomodulating systems.^{22,37,41}

The present line of research was undertaken to develop a model in which the expression and interaction of fas and fasL could be examined in transformed glia. In this study, plasmid-based delivery with constructs containing fas and fasL were used to upregulate expression of these molecules in selected cell lines (36B10-fas and FR-fasL). Successful transformation was confirmed using Western blot analysis for the FR-fasL line. Both transfection and biological activity of the 36B10 fas rat glioma line was confirmed by performing flow cytometry and by an antibody-induced cytotoxicity assay. To begin the assessment of the interaction between fas and fasL in this model, we cocultured the fas-expressing target cells (36B10 fas) with the fasL-expressing cells FR-fasL in a cytotoxicity assay. In terms of the different ratios of cells, our results suggest an effect of the fasL-expressing cells on the fas-containing target cell population. Controlling the level of both fas and fasL expression should allow the following question to be addressed: how important is the amount of expression in determining the resultant biological effect?

This study was designed to create a model to study the interaction of fas and fasL. We have attempted to begin to characterize this interaction; however, many interesting questions remain unanswered at this time. Given time, we believe this model will greatly facilitate the understanding of the interaction of these molecules.

A major challenge in the use of genetic-based therapy remains the technique of delivery to the target tissue. The use of viral-based therapies in the treatment of glioma continues to be actively investigated, and retroviral and adenoviral fas and fasL vectors are available. We used plasmids because for transfection because of their ease of construction and delivery in the in vitro model. Plasmid delivery is possible in vivo by using lipofection or particle-based delivery systems. Selection of the most appropriate method of delivery will depend on further study. Important considerations are the volume and distribution of tissue required to be transfected, the duration of transgene expression required, and the immune response induced by the expression of foreign molecules in the target tissue. We believe the current results warrant additional study in which the interaction of fas and fasL in malignant glioma is evaluated, and we propose that cell-mediated delivery of fasL may be a feasible technique by which to activate fas and initiate the resultant apoptotic cascade.

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Manuscript received February 15, 2000.

Accepted in final form April 3, 2000.

This work was supported in part by the American Association of Neurological Surgeons VanWagenen Fellowship and the Robert and Molly King Endowed Chair in Neuroscience.

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