Fas/APO-1 Gene Transfer for Human Malignant Glioma

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ABSTRACT

Human malignant glioma cells are susceptible to apoptosis induced by antibodies to Fas/APO-1, a cytokine receptor protein of the nerve growth factor/tumor necrosis factor superfamily. Here we show that a critical level of cell surface expression of Fas/APO-1 is a prerequisite for induction of glioma cell apoptosis via Fas/APO-1. Although Fas/APO-1 mRNA was expressed in three Fas/APO-1 antibody-resistant glioma cell lines, these cells expressed either little Fas/APO-1 protein (LN-319 and LN-405) or an abnormal Fas/APO-1 protein that was not translocated to the cell membrane and therefore functionally inactive (LN-308). Although all glioma cell lines expressed mRNA for Fas/APO-1-ΔTM, a soluble form of Fas/APO-1 lacking the transmembrane domain, none of the cell lines released detectable amounts of soluble Fas/APO-1, a potential endogenous antagonist of Fas/APO-1-mediated glioma cell apoptosis. Stable transfection of three resistant glioma cell lines with a human Fas/APO-1 cDNA expression vector dramatically enhanced cell surface expression of Fas/APO-1 and induced susceptibility to Fas/APO-1 antibody-mediated apoptosis. These data indicate that malignant glioma cells, unlike other tumor cells, uniformly harbor the intracellular cascade required for Fas/APO-1-mediated apoptosis. Low level of Fas/APO-1 expression results from inefficient transcription and translation of the Fas/APO-1 gene or the synthesis of mutant Fas/APO-1 proteins. γ-Interferon, tumor necrosis factor-α, and interleukin 1β augmented Fas/APO-1-mediated apoptosis of Fas/APO-1-transfected glioma cells by acting on the subcellular suicidal cascade triggered by Fas/APO-1 activation. Dexamethasone attenuated Fas/APO-1 antibody-induced apoptosis, not only of constitutively Fas/APO-1-positive glioma cells, but also of Fas/APO-1-transfected glioma cells. The antiapoptotic effect of dexamethasone could be overcome by preexposure of the glioma cells to γ-interferon or by coexpression to Fas/APO-1 antibodies and cycloheximide. Thus, Fas/APO-1 gene transfer and combined immunotherapy using Fas/APO-1 antibodies and cytokines may overcome Fas/APO-1 antibody resistance of Fas/APO-1-negative human malignant glioma cells, which may represent subpopulations within single gliomas or form a separate subgroup of human malignant gliomas.

INTRODUCTION

Human malignant gliomas are highly aggressive neoplasms that are largely resistant to current therapeutic approaches including cellular and antibody-guided immunotherapy (1–4). Glioma cells synthesize a potent immunosuppressive molecule originally termed glioma cell-derived, T-cell suppressor factor that, upon purification and cloning, was identified as a novel member of the TGF-β superfamily and is now known as TGF-β2 (5–8). Immunosuppressive factors like TGF-β may contribute to the absence of a tumor-specific T-cell response in malignant glioma patients via induction of T-cell apoptosis (9). Cytotoxic T cells kill their target cells by perforin-dependent lysis or by Fas/APO-1-dependent apoptosis (10–14). Fas/APO-1 is a transmembrane cytokine receptor protein of the superfamily of receptors for nerve growth factor and TNF-α, which signals apoptosis (15, 16). The natural Fas/APO-1 ligand is a cytotoxic cytokine with significant homology to TNF-α and lymphotxin/TNF-β (17). The role of Fas/APO-1 in the immune network includes a negative feedback control that results in the elimination of activated T cells. The aberrant expression of Fas/APO-1 by various neoplastic cells has attracted interest in Fas/APO-1 as a target for antibody-guided immunological cancer therapy (18–20). Given the synthesis by malignant glioma cells of potent immunosuppressive molecules like TGF-β that paralyze T-cell-mediated tumor immunity, targeting directly those molecules which mediate T-cell-induced cytotoxicity, may be a useful strategy to overcome TGF-β-induced inhibition of cytotoxic T-cell responses.

We have recently reported that malignant glioma cell lines express Fas/APO-1 and are susceptible to Fas/APO-1 antibody-mediated apoptosis (21). Since Fas/APO-1 is also expressed by ex vivo isolated malignant glioma cells, targeting Fas/APO-1 appears to be a promising novel immunotherapeutic strategy to eliminate malignant glioma cells in vivo. The degree of sensitivity of glioma cells to Fas/APO-1 antibody-mediated apoptosis is determined by several factors: (a) expression of a functional Fas/APO-1 molecule on the cell surface is absolutely required for Fas/APO-1-dependent killing. Lpr and lpr/β mice develop autoimmune disorders because they do not express Fas/APO-1 or express mutant Fas/APO-1 incapable of intracellular signaling (14, 22); (b) partial resistance of Fas/APO-1-positive cells to Fas/APO-1-mediated apoptosis can be mediated by short-lived cytoprotective proteins. This type of resistance can be overcome by coexposure of target cells to Fas/APO-1 antibodies and inhibitors of RNA or protein synthesis such as ActD or CHX. These agents dramatically augment Fas/APO-1 antibody-induced cytotoxicity in all Fas/APO-1-positive glioma cell lines that we have examined (21); (c), preliminary evidence has suggested that the antiapoptotic protooncogene, bcl-2, confers resistance to Fas/APO-1 antibody-mediated apoptosis (23). We have recently shown that a murine bcl-2 gene transfer greatly enhances survival of glioma cells exposed to Fas/APO-1 antibodies in the presence and in the absence of ActD and CHX (24); (d), Fas/APO-1-dependent apoptosis may be inhibited by the cellular release of Fas/APO-1-ΔTM, a soluble form of Fas/APO-1 encoded by an alternatively spliced mRNA lacking the transmembrane domain coding sequence. This molecule could act as an extracellular scavenger for therapeutically administered Fas/APO-1 antibodies or Fas/APO-1 ligand (25). Eventually, some nonglial cell lines express Fas/APO-1 but fail to undergo apoptosis when exposed to Fas/APO-1 antibodies, even in the presence of ActD or CHX (26, 27). These cells may harbor an intrinsic defect in the intracellular signaling cascade activated after Fas/APO-1 ligation or may synthesize cytoprotective proteins in the presence of inhibitors of RNA and protein synthesis.

Here we report that glioma cell resistance to Fas/APO-1 antibodies may be due to low level expression or inefficient translation of Fas/APO-1 mRNA, synthesis of mutant Fas/APO-1, or synthesis of cytoprotective proteins. Resistance of human malignant glioma cells to Fas/APO-1 antibodies can be overcome by Fas/APO-1 cDNA gene transfer and combined immunochemotherapy using Fas/APO-1.
antibodies, cytokines like IFN-γ or TNF-α, and chemotherapeutic drugs that target mRNA and protein synthesis.

MATERIALS AND METHODS

Materials. The human malignant glioma cell lines LN-18, LN-215, LN-229, LN-308, LN-319, and LN-405 were obtained from Dr. N. de Triboulet (Lausanne, Switzerland). T98G glioma cells were from American Type Culture Collection (Rockville, MD). Cytotoxic Fas IgM antibody CH-11 and FITC-conjugated Fas IgG antibody UB-2 were purchased from Kamiya (Lausanne, Switzerland). T98G glioma cells were from American Type Culture Collection. FITC-conjugated Faa IgG antibody UB-2 were purchased from Kamiya (Thousand Oaks, CA). Mouse monoclonal APO-1 antibody was generously provided by Dr. P. H. Krammer (Heidelberg, Germany), and rabbit antisera to human Fas/APO-1 by Dr. H. Engelmann (Munich, Germany). Human cytokines were obtained from various commercial sources as previously detailed (9, 21, 24).

Cell Culture and Detection of Apoptosis. Human malignant glioma cell lines were cultured as described (21, 24). To generate cell-free conditioned supernatants, confluent glioma cell cultures were maintained for 24 h in regular medium in the presence of 0.5% or 5% FCS. The supernatants were centrifuged for 10 min at 4000 × g and 4°C and stored in aliquots at −20°C. Viability and proliferation were assessed by crystal violet staining and in situ DNA end labeling, have been outlined previously (9, 21, 24).

Flow Cytometry and Immunocytochemistry. The glioma cells were rinsed in cold PBS (8 g/liter NaCl, 0.2 g/liter KCl, 1.44 g/liter Na2HPO4 and 0.24 g/liter KH2PO4), once in trypsin, incubated for 3 min at 37°C, and harvested into complete medium containing 10% FCS by vigorous pipetting. The cells were centrifuged at 1200 rpm for 10 min at 4°C, resuspended (106 cells/150 μl) in flow cytometry buffer (PBS/1% BSA/0.01% sodium azide), and labeled for 30 min at 4°C with FITC-conjugated Fas antibody UB-2 diluted 1:10 in flow cytometry buffer. Murine FITC-IgG1 was used as an isotype control. The SFI was calculated as the ratio of the mean fluorescence values obtained with the specific Fas/APO-1 antibody and the isotype control antibody.

Immunoblot. Detection of Fas/APO-1 by immunoblot was carried out as described (21). To detect soluble Fas/APO-1, proteins released by the glioma cells into the culture medium were precipitated in 5% ice-cold acetone at −20°C for 30 min and pelleted by cold centrifugation (4°C) at 10,000 × g for 10 min. The protein pellets were air-dried and resuspended in loading buffer for SDS-PAGE.

ELISA for Soluble Fas/APO-1. Ninety-six well plates were coated with mouse monoclonal APO-1 antibody (1 μg/ml; Ref. 19) in PBS/0.01% sodium azide for 24 h at 4°C and blocked with ELISA buffer (0.5% BSA/PBS/0.05% Tween 20). The samples were diluted in ELISA buffer for 8 h at room temperature. The plates were rinsed with ELISA buffer extensively. Bound Fas/APO-1 was detected using polyclonal rabbit antisera to human Fas/APO-1 (1:500; 24 h at 4°C) and peroxidase-linked goat anti-rabbit IgG (Sigma, St. Louis, MO; 1:1000). Soluble Fas/APO-1 was used as a standard.

Preparation of Soluble Fas/APO-1-ΔTM. Recombinant human Fas/ APO-1-ΔTM was prepared from baculovirus cultures 72 h after infection with pBlueBac III (Invitrogen). The cultures were treated with 1 mM EDTA and 50 μM aminomethylbenzenesulfonfluoride and centrifuged at 2000 rpm; then the pellets were washed twice with PBS. The wet pellet (3 ml) obtained from 500-ml cultures was resuspended in 40 ml PBS, vortexed, freeze-thawed three times using dry ice/ethanol with intermittent vortexing and gentle bath sonication, and centrifuged at 15,000 rpm for 20 min; then the supernatant was filtered through nylon (0.45 μm). The filtrate was diluted 3-fold in deionized water and loaded on carboxymethylcellulose Sepharose CL-6B (Sigma) connected to a Pharmacia FPLC system (0.5 ml/min) at 4°C. The starting buffer was 10 mM phosphate (pH 7.5). Fas/APO-1-ΔTM eluted in a salt gradient from 150–200 mM NaCl. Fas/APO-1-ΔTM-containing fractions were collected, brought to 0.5 mM ammonium sulfate, and loaded on phenyl Sepharose CL-4B (Sigma 0.5 ml/min) at room temperature. The initial buffer was 0.5 mM ammonium sulfate and 50 mM phosphate; the final buffer was 50 mM phosphate (pH 7). Fas/APO-1-ΔTM passed through the column, while most impurities bound.

Fas/APO-1-ΔTM-containing fractions were loaded on a S-200 gel filtration column (Pharmacia XK 50/100) containing 1.8 l of gel (5 ml/min at 4°C). The running buffer was 10 mM phosphate/1 mM NaCl (pH 7.5). The collected material was dialyzed overnight to low ionic strength, bound to 0.5 ml CM sepharose, and pelleted at 1500 rpm for 10 min. Fas/APO-1-ΔTM was harvested by repeated washings of the Sepharose with small volumes of high salt buffer (10 mM phosphate/1 mM NaCl, pH 7.5). To enhance its biological activity, Fas/APO-1-ΔTM was subjected to mild chemical cross-linking. The mechanism of activity enhancement by cross-linking is not clear.

Northern Blot Hybridization. Total RNA (20 μg) isolated by acid guanidinium thiocyanate-phenol-chloroform extraction was separated on 1% agarose gel and blotted onto nitrocellulose. The blots were hybridized at 42°C in 50% formamide, 5× SSC, 1× Denhardt’s solution, and 1× SDS (pH 7) with 32P-labeled human APO-1 cDNA or chicken β-actin probes. The blots were washed in 0.2× SSC at 65°C and exposed to X-ray film with intensifying screens at −70°C or analyzed with a Phosphorimager (Molecular Dynamics, Basel, Switzerland).

Reverse Transcription PCR. Reverse transcription and amplification were performed as described previously (28). The first set of primers corresponds to nucleotides 170–191 (5′-CAGTTGGAGGTCTCAACAA-3′) and 1336–1316 (5′-TATGTGGTCTCTCCAGGCTA-3′) and amplifies the full-length Fas/APO-1 cDNA (25). A second set of primers spaced around the transmembrane domain of the Fas/APO-1 cDNA corresponds to nucleotides 5528–5562 (5′-GACGGCAGAATACACAGTGCAAGTGA-3′) and 852–856 (5′-CTCTTGTCACATTGTTGGAAT-3′). For restriction enzyme analysis of the PCR cDNA fragments obtained with the latter PCR primers, the single bands were cut from 4% agarose gels, and the DNA was isolated by phenol extraction and ethanol precipitation using tRNA as a carrier. The DNA was amplified using the same primer pair for 20 cycles, purified with Qiagen PCR columns, and subjected to restriction endonuclease digestion with BamHI and DraIII.

Transfection. A 2.55-kb human APO-1 cDNA generously provided by Dr. P. H. Krammer (Heidelberg, Germany) was cut from the plasmid with NcoI. Blunt ends were generated with T4 DNA polymerase. The fragment was subcloned into the Smal site of pBS-κS (Stratagene, La Jolla, CA), and the orientation was determined. A sense clone was digested with Xhel and NotI, and the fragment was subcloned into the Bcmgs Neo expression vector (29). The glioma cells (5 × 103) were transfected with 10 μg plasmid DNA by electroporation using a Bio-Rad Gene Pulser (25 kV). Selection with G418 (500 μg/ml) was started 48 h later and continued throughout the culture period. Single clones were obtained by limiting dilution 6–8 weeks after transfection and characterized for Fas/APO-1 expression by Northern blot hybridization and flow cytometry and for susceptibility to Fas/APO-1-mediated apoptosis in 96-well plate cytoxicity assays as described previously (21).

Statistical Analysis. EC50 for Fas/APO-1 antibody-induced glioma cell killing were determined by linear regression analysis. Effects of simple treatments were compared by Student’s t test. Composite treatments were analyzed by ANOVA at P < 0.03. Data are expressed as mean and SEM and are representative of triplicate experiments performed at least three times with similar results.

RESULTS

Patterns of Fas/APO-1 mRNA and Protein Expression in Human Malignant Glioma Cells. The susceptibility of human malignant glioma cell lines to Fas/APO-1 antibodies depends in part on the expression of Fas/APO-1 on the cell surface. Flow cytometry showed that the glioma cell lines LN-18, LN-215, T98G, and LN-229 are characterized by a high frequency of Fas/APO-1-positive cells and SFI values above 1.8 (Fig. 1A). These cell lines are constitutively sensitive to the cytotoxic effects of Fas/APO-1 antibodies or can be sensitized to Fas/APO-1 antibody-mediated killing by coexpression to Fas/APO-1 antibodies and inhibitors of RNA and protein synthesis inhibitors like ActD or CHX (Ref. 21, Fig. 1C). Cell surface Fas/ APO-1 expression was hardly detectable (SFI < 1.4) in the glioma cell lines LN-319, LN-405, and LN-308. Absence or low level expression of Fas/APO-1 was paralleled by resistance to Fas/APO-1 antibody-mediated apoptosis (Fig. 1, A and C). Similar results were obtained when the flow cytometric analyses were performed in an indirect
Northern blot analysis showed that all glioma cell lines expressed the two expected mRNA species of 2.0 and 2.7 kb corresponding to Fas/APO-1 (15, 16). There was no consistent pattern of preponderance of one of these mRNA species in the glioma cell lines. The expression levels of Fas/APO-1 mRNA were low in all glioma cell lines. The relative levels of Fas/APO-1 mRNA expression calculated as ratios relative to β-actin mRNA expression (Fig. 1B) corresponded to the data for cell surface Fas/APO-1 protein expression obtained by flow cytometry (Fig. 1A) but not to the immunoblot data on total cellular Fas/APO-1 protein (Fig. 1C). The four Fas/APO-1 antibody-sensitive cell lines, LN-18, LN-215, T98G, and LN-229, expressed higher levels of Fas/APO-1 mRNA levels than the three resistant cell lines, LN-319, LN-405, and LN-308. Thus, the Fas/APO-1 gene is effectively expressed in the four sensitive cell lines and gives rise to cell surface Fas/APO-1 protein expression at the cell surface (Fig. 1A), which is sufficient to mediate a death signal upon binding of agonistic antibody (Fig. 1C). LN-319 and LN-405 have little mRNA expression and little cell surface protein expression; however, total cellular Fas/APO-1 protein levels detected by immunoblot approach those of LN-18 and LN-215 cells, suggesting that LN-319 and LN-405 cells synthesize abnormal Fas/APO-1 proteins that are inefficiently translocated to the cell surface or that exhibit an enhanced half-life, or both. This pattern is even more prominent in LN-308 cells that express little mRNA and no cell surface Fas/APO-1 protein, yet exhibit a prominent band of M, 42,000 immunoblot analysis.

Interestingly, LN-308 cells showed a striking 13-fold increase in Fas/APO-1 mRNA expression after IFN-γ treatment (100 units/ml for 24 h), although IFN-γ-treated LN-308 cells exhibit very little up-regulation of cell surface Fas/APO-1 protein expression (21) and only a moderate increase in total Fas/APO-1 protein levels detected by immunoblot (data not shown). In contrast, of the four sensitive cell lines, LN-18, LN-215, T98G, and LN-229, only LN-215 cells responded to IFN-γ with a significant, that is about 2-fold, increase in Fas/APO-1 mRNA levels, suggesting that IFN-γ enhances cell surface expression of Fas/APO-1 in the sensitive cell lines mainly by enhancing translation of preexisting mRNA. A 2-fold increase in Fas/APO-1 mRNA expression after IFN-γ treatment was also seen in resistant LN-405 cells. No change was noted in LN-319 cells. These observations provide further evidence for abnormal processing of Fas/APO-1 mRNA and protein in LN-308, human malignant glioma cells.

Human Malignant Glioma Cells Express Fas/APO-1-ΔTM mRNA but Do Not Release Soluble Fas/APO-1 Protein. The biological activity of cytokines like IL-2 or TNF-α is regulated by shedding of cell surface receptors or release of soluble receptors. Similarly, the presence of soluble Fas/APO-1 in blood, cerebrospinal fluid, or tumor tissue might interfere with the induction of apoptosis by endogenous Fas/APO-1 ligand or therapeutically administered Fas/APO-1 antibodies. Soluble Fas/APO-1 protein may not only be derived from shedding but may be encoded by an alternatively spliced mRNA species referred to as Fas/APO-1-ΔTM, which lacks the transmembrane domain of the parent Fas/APO-1 mRNA (25). The corresponding protein is released into the cell culture medium and interferes with Fas/APO-1 antibody-mediated cell killing in vitro. The possible role of soluble Fas/APO-1 in Fas/APO-1 antibody-mediated apoptosis of the glioma cells was investigated. A full-length 1167-bp cDNA fragment encoding Fas/APO-1 was amplified by PCR from reverse-transcribed mRNA of all glioma cell lines, indicating that none of the glioma cell lines including LN-308 expressed a grossly abnormal Fas/APO-1 transcript (Fig. 2A). The predicted PCR band for Fas/APO-1-ΔTM cDNA has a size of 1104 bp and is 63 bp smaller than a PCR fragment encoding parent Fas/APO-1 cDNA. Although the PCR gel suggested the presence of a weak band migrating slightly faster than the parent 1167-bp Fas/APO-1 fragment in all glioma cell...
release soluble Fas/APO-1 protein. A, amplification of full-length Fas/APO-1 cDNA by antibody-mediated
amplification products of 682 and 780 bp, corroborating the identity of the PCR fragments shown in Fig. 2, A and B, with Fas/APO-1 cDNA (data not shown). The similar ratios of parent versus transmembrane domain-lacking transcripts among the Fas/APO-1 antibody-sensitive cell lines LN-18, LN-215, T98G, and LN-229, and the resistant cell lines LN-319, LN-405, and LN-308, suggest that Fas/APO-1-ΔTM does not play a significant role in determining sensitivity or resistance of these cell lines to Fas/APO-1 antibodies.

Next we examined whether Fas/APO-1-ΔTM or shedded mature Fas/APO-1 protein was released by the glioma cells into the culture medium, using immunoblot analysis, ELISA, and bioassay. Secreted proteins were acetone-precipitated from conditioned glioma cell medium and analyzed by immunoblot. Fas/APO-1 could not be demonstrated in conditioned medium of any of the glioma cell lines included in this study (data not shown). The failure to detect Fas/APO-1 in the medium was not due to the precipitation procedure because culture medium spiked with an aliquot of LN-308 protein lysate prior to precipitation produced a signal of immunoreactivity comparable to a protein lysate that was not diluted in culture medium and acetone precipitated. Further, none of the conditioned media were positive in an ELISA with a detection limit of 25 pg/ml, using recombinant human Fas/APO-1-ΔTM as a standard and glioma cell lysate as positive control (data not shown). Eventually, the conditioned media of Fas/APO-1 antibody-resistant and of Fas/APO-1 antibody-sensitive glioma cell lines failed to abrogate Fas/APO-1 antibody-mediated apoptosis of LN-18 glioma cells (Fig. 2D). However, Fas/APO-1-mediated apoptosis of LN-18 cells was inhibited in a concentration-dependent manner by recombinant human Fas/APO-1-ΔTM. The masked Fas/APO-1, e.g., of LN-308 cells detected by immunoblot, is thus probably not identical with Fas/APO-1-ΔTM, not only because of little Fas/APO-1-ΔTM mRNA expression in these cells (Fig. 2B), but also because this protein would be released into the cell culture medium (25) and would give rise to a doublet band of M, 40,000–42,000 on immunoblots, neither of which was the case (Figs. 1D and 2D).

Fas/APO-1 cDNA Gene Transfer Induces Susceptibility of Fas/APO-1 Antibody-Resistant Glioma Cell Lines to Fas/APO-1 Antibody-mediated Apoptosis. The data summarized above indicate that absence or low level expression of Fas/APO-1 on the cell surface plays an important role in the resistance of some human malignant glioma cell lines to Fas/APO-1 antibody-induced apoptosis. To prove that the expression of a critical level of Fas/APO-1 protein at the cell surface may be sufficient for Fas/APO-1-dependent glioma cell killing, we transfected the Fas/APO-1 antibody-resistant glioma cell lines LN-319, LN-405, and LN-308 with a human Fas/APO-1 cDNA (16) cloned into an expression vector carrying a neomycin resistance gene for selection (Ref. 29; Fig. 3A). These experiments were also designed to clarify whether resistant glioma cells synthesize an abnormal Fas/APO-1 protein or are unable to translocate a normal Fas/APO-1 protein to the cell surface. Several independent glioma cell clones derived from each of the three transfected cell lines were characterized for Fas/APO-1 transgene mRNA and protein expression by Northern blot analysis and flow cytometry and were compared with pooled vector control cells that were transfected with the empty vector.
BCMGS Neo vector lacking the Fas/APO-1 cDNA insert. Fig. 3B shows a prominent shift of the fluorescent signal for cell surface Fas/APO-1 expression in Fas/APO-1-transfected clones compared with Neo vector control cells of the respective cell lines. The SFI values from 1.02 in parent LN-308 cells to 17.3 in LN-319-A3 cells. C, Northern blot analysis of Fas/APO-1 clones LN-308-C2 and LN-319-A3 and Neo vector control cells performed as described in "Materials and Methods." Equal RNA loading was ascertained by β-actin mRNA quantification (data not shown). At this length of exposure of the blots to X-ray film, no endogenous Fas/APO-1 mRNA is detectable (Lane 1, LN-308-C2; Lane 2, LN-308-Neo; Lane 3, LN-319-A3; Lane 4, LN-319-Neo).

Fig. 3. Generation of Fas/APO-1 cDNA transfectant clones of the LN-308, LN-319, and LN-405 human malignant glioma cell lines. A, the BCMGS Fas/APO-1 expression vector contains a neomycin resistance gene (Neo), a cytomegalovirus (CMV) promoter, and a 2.55-kb human Fas/APO-1 cDNA insert. B, flow cytometric analysis of cell surface Fas/APO-1 expression in LN-308 Fas/APO-1 clone C2 and LN-319 clone A3 (right panels) compared with Neo vector control cells (left panels). Glioma cells (10⁶) were stained with FITC-labeled Fas/APO-1 antibody UB-2 or with control antibody as described in "Materials and Methods." Fluorescence intensity is plotted on the X-axis; cell counts on the Y-axis. Filled areas, fluorescence obtained with a control antibody; open areas, fluorescence obtained with Fas/APO-1 antibody. The transfection increased the SFI values from 1.02 in parent LN-308 cells to 7.8 in LN-308-C2, and from 1.36 in parent LN-319 cells to 17.3 in LN-319-A3 cells. C, Northern blot analysis of Fas/APO-1 clones LN-308-C2 and LN-319-A3 and Neo vector control cells performed as described in "Materials and Methods." Equal RNA loading was ascertained by β-actin mRNA quantification (data not shown). At this length of exposure of the blots to X-ray film, no endogenous Fas/APO-1 mRNA is detectable (Lane 1, LN-308-C2; Lane 2, LN-308-Neo; Lane 3, LN-319-A3; Lane 4, LN-319-Neo).

Fig. 4 (left panel) shows Fas/APO-1 antibody-induced cytotoxicity of selected Fas/APO-1 clones and vector control cells. Transfection with the Fas/APO-1 cDNA expression vector of the three glioma cell lines thought to be resistant to Fas/APO-1 antibodies because of little cell surface Fas/APO-1 expression (21) gave rise to glioma cell clones with significant sensitivity to Fas/APO-1 antibody-mediated apoptosis. Coexposure of the transfected clones to Fas/APO-1 antibodies and ActD or CHX induced a prominent shift of the concentration response curve for Fas/APO-1 antibody to the left and enhanced maximal Fas/APO-1 antibody-mediated apoptosis (Fig. 4, left panel). Fas/APO-1 antibody-induced apoptosis of the transfected glioma cell clones was associated with significant nucleosomal size DNA fragmentation in LN-319 and LN-405 cells but not in LN-308 cells (data not shown). This pattern of DNA fragmentation is typical of apoptotic cell death in numerous cell types, including lymphoid cells (9), but is not a prominent feature of Fas/APO-1-mediated apoptosis in most human glioma cell lines (21). DNA breaks detected by in situ DNA end labeling were uniformly induced by Fas/APO-1 antibodies in Fas/APO-1-transfected clones derived from all three glioma cell lines.

A comparison of the expression levels of Fas/APO-1 achieved by transfection and of the induction of susceptibility to Fas/APO-1-mediated apoptosis showed that a critical level of Fas/APO-1 expression was required to mediate the death signal (Fig. 5). Corresponding to the expression levels of the constitutively sensitive cell lines, LN-18, LN-215, T98G, and LN-229 (SFI 1.8—2.9; Fig. 1A), there was no sensitization in transfected clones with SFI values below 1.35. Yet, the transfected clones were sensitized to Fas/APO-1 antibodies at expression levels that did not dramatically exceed those of constitutively sensitive glioma cell lines (Fig. 5). The data summarized in Fig. 5 also show that variations of Fas/APO-1 expression do not account for all variability in the susceptibility to Fas/APO-1-mediated apoptosis of human malignant glioma cells. The level of Fas/APO-1 expression required to kill more than 50% of the glioma cells within 16 h in the presence of CHX differed significantly among the three cell lines and was lowest in LN-405 (SFI 1.3—1.5) and highest in LN-319 (SFI 2—7), suggesting that these cell lines differ in their intracellular signal transduction cascades. Further, a comparison of different clones derived from the same cell line revealed that, once a critical level of Fas/APO-1 expression is achieved, the correlation between Fas/APO-1 expression and susceptibility to Fas/APO-1-mediated apoptosis is lost, as can be seen in the 3-4 clones with the highest Fas/APO-1 expression in each respective cell line (Fig. 5). These data indicate that additional factors determine the susceptibility to Fas/APO-1 antibodies in those glioma cells that express sufficient levels of Fas/APO-1 to transduce a death signal.

Fas/APO-1 Gene Transfer Modulates Glioma Cell Sensitivity to TNF-α. Fas/APO-1 and its natural ligand share significant homologies with the TNF receptor and TNF, respectively (15–17). The two receptors, Fas/APO-1 and the TNF receptor, transduce their respective death signals via a common domain of their cytoplasmic polypeptide chains referred to as the death domain (32, 33). All glioma cell lines included in this study are resistant to the cytotoxic effects of TNF-α, and some even respond with enhanced proliferation to TNF-α. However, TNF-α resistance can be overcome by coexposure to TNF-α and ActD or CHX in those cell lines that are also susceptible to Fas/APO-1 antibodies because of little cell surface Fas/APO-1 expression (21). Fig. 4 (right panel) shows that stable expression of the Fas/APO-1 cDNA enhanced the sensitivity of most transfected glioma cell clones to TNF-α in the presence of ActD or CHX, as exemplified by the LN-319 and LN-405 clones. The modulation of sensitivity to TNF-α in the presence of ActD or CHX after Fas/APO-1 cDNA transfection led us to study the effects of a combined treatment of parent and transfected glioma cells with TNF-α and Fas/APO-1 antibodies. Co-treatment of LN-308 clone C2 and of LN-319 clone A3 cells with Fas/APO-1 antibodies and TNF-α had additive effects in the presence and in the absence of CHX (P < 0.03.
Fas/APO-1 gene transfer induces susceptibility of resistant malignant glioma cells to Fas/APO-1 antibody-mediated apoptosis. Fas/APO-1 clones LN-308-B3, LN-319-A3, and LN-405-APO-2 (∆, □, ○) or pooled vector control cells (Neo; △, □, ○) were exposed to Fas/APO-1 antibody or to TNF-α in the absence (∘, ○) or presence of ActD (0.5 μg/ml; △, □, ○) or CHX (10 μg/ml; ∆, Δ) for 16 h. Survival was quantified by crystal violet staining and calculated as the percentage of survival of untreated cells or cells exposed to ActD or CHX alone. Data are expressed as the mean; bars, SEM (n = 3).

by ANOVA compared to TNF-α or Fas/APO-1 antibody treatment alone. This contrasted with parent LN-308 or LN-319 cells, which were rather resistant to TNF-α and Fas/APO-1 antibodies added separately or together, both in the presence and in the absence of CHX, and also with constitutively Fas/APO-1-positive glioma cell lines like LN-18 or LN-229, which did not show enhanced cytotoxicity when cotreated with Fas/APO-1 antibody and TNF-α compared with Fas/APO-1 antibody treatment alone (data not shown).

Cytokines Modulate Fas/APO-1 Antibody-mediated Apoptosis of Fas/APO-1-transfected Human Malignant Glioma Cells. Preexposure to various cytokines enhances Fas/APO-1 antibody-mediated apoptosis of Fas/APO-1-positive glioma cells like LN-215, T98G, and LN-229 (21). Flow cytometric analysis of the transfectant Fas/APO-1 clones of the LN-308, LN-319, and LN-405 glioma cell lines showed that none of these cytokines, IFN-γ, TNF-α, IL-1β, IL-8, and transforming growth factor-β2, enhanced Fas/APO-1 expression in glioma cell clones transfected with the BCMGS Fas/APO-1 expression vector (Fig. 6). Thus, the stable cytokine-independent expression of Fas/APO-1 allowed us to study the effects of cytokines on the subcellular suicidal cascade activated by Fas/APO-1 antibodies. Selected transfected Fas/APO-1 clones of each cell line were examined for cytokine-induced changes in susceptibility to Fas/APO-1 antibodies (Table 1). Preexposure of Fas/APO-1 transfectants to IFN-γ, TNF-α, and IL-1β augmented Fas/APO-1 antibody-mediated killing compared with antibody treatment alone. These sensitizing effects were consistent for IFN-γ in all cell lines, whereas TNF-α had effects only in LN-308 and LN-319, and IL-1β only in LN-308. For comparison, these cytokines had no effects on Neo control cells, except for a minor sensitization of LN-319 cells. The most efficient killing of Fas/APO-1-transfected glioma cells (80–95%) was achieved by preexposure to IFN-γ (100 units/ml) and TNF-α (10 ng/ml) for 24 h and subsequent exposure to Fas/APO-1 antibody (1 μg/ml) and CHX (10 μg/ml) for 16 h (data not shown). No significant effects on Fas/APO-1-mediated killing were seen after preexposure of transfected Fas/APO-1 clones to IL-8 or TGF-β2, which were previously found to sensitize some untransfected Fas/
APO-1-positive glioma cell lines for Fas/APO-1-mediated apoptosis (21). Several other cytokines, including IL-4, IL-6, IL-10, IL-13, and macrophage colony-stimulating factor, had no effects on Fas/APO-1 antibody-mediated apoptosis of either parent or transfected glioma cells (data not shown). These cytokines were previously found not to enhance Fas/APO-1-mediated apoptosis of constitutively Fas/APO-1-positive human malignant glioma cells (21). Thus, selected cytokines, notably IFN-γ, but also TNF-α and IL-1β, sensitize human malignant glioma cells to Fas/APO-1-mediated apoptosis by direct actions on the subcellular death cascade triggered by activation of the Fas/APO-1 receptor protein. In previous studies, these cytokines were shown to enhance Fas/APO-1 expression in untransfected glioma cells (21). Taken together, our data indicate that cytokines may sensitize malignant glioma cells for Fas/APO-1-dependent apoptosis by two different mechanisms, one involving stimulation of cell surface Fas/APO-1 expression and one involving the intracellular death pathway.

Preexposure to IFN-γ and Coexposure to CHX Overcome Dexamethasone-mediated Protection of Fas/APO-1 Transfected Glioma Cells from Fas/APO-1 Antibody-mediated Apoptosis. Preexposure to dexamethasone, a drug that is administered to virtually all malignant glioma patients for the control of peritumoral edema, abrogates Fas/APO-1 antibody-induced glioma cell killing (21). The antiapoptotic effect of dexamethasone does not involve down-regulation of cell surface Fas/APO-1 expression on the glioma cells (24) and is, therefore, most likely targeting the intracellular cascade triggered by Fas/APO-1 antibodies. This hypothesis was further tested by studying the Fas/APO-1 cDNA-transfected glioma cell clones. As predicted, dexamethasone inhibited Fas/APO-1 antibody-mediated apoptosis of transfected clones derived from LN-319 and LN-405 (Table 2), although not the LN-308 clones (data not shown). The protective effects were similar to those reported previously for untransfected Fas/APO-1-positive glioma cell lines (21). The antiapoptotic effects of dexamethasone could be overcome partially by cotreatment of the glioma cells with Fas/APO-1 antibodies and CHX or by preexposure to IFN-γ (Table 2). When the antiapoptotic effects of dexamethasone, in the absence and in the presence of CHX, and with or without IFN-γ pretreatment, were compared in constitutively Fas/APO-1-positive glioma cell lines like LN-18 or LN-229 and in the Fas/APO-1-transfected glioma cell clones, no consistent differences emerged, i.e., the biological heterogeneity of various glioma cell lines in their responses to dexamethasone, CHX, and IFN-γ is also reflected in transfected glioma cell clones forced to express Fas/APO-1 by gene transfer (data not shown).

**DISCUSSION**

The induction of apoptosis via activation of Fas/APO-1 is a novel experimental approach to the immunotherapy of human malignant glioma (21). The present study shows that: (a) a critical level of cell surface Fas/APO-1 expression is required to mediate apoptosis in human malignant glioma cells (Fig. 1A); (b) the lack of sufficient Fas/APO-1 expression in some glioma cell lines may result from abnormal processing of Fas/APO-1 mRNA or synthesis of abnormal Fas/APO-1 proteins (Fig. 1, A, B, and D); (c) human malignant glioma cells express Fas/APO-1-DTM mRNA but do not release soluble Fas/APO-1 protein (Fig. 2); and (d) forced expression of Fas/APO-1 mediated by gene transfer induces susceptibility of previously resistant glioma cells to Fas/APO-1 antibody-mediated apoptosis (Figs. 4 and 5).

Expression of a critical level of Fas/APO-1 is the first determinant of susceptibility of human malignant glioma cells to Fas/APO-1-mediated apoptosis since all resistant cell lines had SFI values below 1.4, whereas all sensitive cell lines had SFI values exceeding 1.8 (Fig. 1A). Among the sensitive cell lines with a SFI above 1.8, the SFI values did not correlate with Fas/APO-1 antibody-mediated apoptosis. LN-229 had the highest SFI (2.9) but was resistant to Fas/APO-1 antibodies unless pretreated with IFN-γ or TNF-α or cotreated with inhibitors of RNA and protein synthesis (21). The EC50 for Fas/APO-1 antibody-mediated apoptosis of LN-18 was an order of magnitude lower than the EC50 for other glioma cell lines, although the SFI of 1.87 is in the lower range of the sensitive cell lines. Using Fas/APO-1 gene transfer, we obtained several independent glioma cell clones from three different glioma cell lines (LN-308, LN-319, and LN-405), which express little Fas/APO-1 at the cell surface and are resistant to Fas/APO-1-mediated apoptosis. The Fas/APO-1-transfected clones exhibited SFI values of up to 20 (Figs. 3 and 5), exceeding by an order of magnitude the SFI values of constitutively Fas/APO-1-expressing glioma cell lines like LN-18, LN-215, T9G8, or LN-229 (Fig. 1A). There was a critical lower level of Fas/APO-1 expression for each cell line that had to be achieved to induce susceptibility of the transfected clones to Fas/APO-1 antibodies.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Fas/APO-1 LN-308</th>
<th>Neo LN-308</th>
<th>Fas/APO-1 Neo LN-308</th>
<th>Fas/APO-1 LN-319</th>
<th>Neo LN-319</th>
<th>Fas/APO-1 Neo LN-319</th>
<th>Fas/APO-1 LN-405</th>
<th>Neo LN-405</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>34 ± 3</td>
<td>98 ± 2</td>
<td>20 ± 2</td>
<td>101 ± 2</td>
<td>63 ± 3</td>
<td>98 ± 2</td>
<td>63 ± 3</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>TNF-α (10 ng/ml)</td>
<td>23 ± 2</td>
<td>115 ± 3</td>
<td>12 ± 2*</td>
<td>97 ± 4</td>
<td>37 ± 2*</td>
<td>87 ± 4</td>
<td>58 ± 4</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>IFN-γ (100 units/ml)</td>
<td>25 ± 3</td>
<td>104 ± 2</td>
<td>13 ± 1*</td>
<td>79 ± 3*</td>
<td>37 ± 2*</td>
<td>87 ± 4</td>
<td>58 ± 4</td>
<td>91 ± 4</td>
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<td>TGF-B2 (10 ng/ml)</td>
<td>34 ± 2</td>
<td>106 ± 3</td>
<td>23 ± 2</td>
<td>98 ± 4</td>
<td>58 ± 2</td>
<td>94 ± 5</td>
<td>58 ± 4</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>IL-1β (10 ng/ml)</td>
<td>22 ± 2</td>
<td>96 ± 4</td>
<td>21 ± 2</td>
<td>90 ± 3</td>
<td>21 ± 2</td>
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<td>22 ± 2</td>
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<td>62 ± 3</td>
<td>89 ± 4</td>
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<td>89 ± 4</td>
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</table>

*p < 0.03, ANOVA; cytokine pretreatment compared to vehicle pretreatment.
little amounts of Fas/APO-1-ΔTM mRNA, the present study failed to provide evidence for the release of Fas/APO-1-ΔTM or shedded Fas/APO-1 protein by glioma cells (Fig. 2), suggesting that soluble Fas/APO-1 does not play a significant role in the resistance of malignant glioma cells to Fas/APO-1 antibodies. Prominent constitutive sensitivity of malignant glioma cells to Fas/APO-1 antibodies as in LN-18 cells is uncommon (21). The potentiation of Fas/APO-1-mediated apoptosis by ActD and CHX suggests that the induced or constitutive synthesis of short-lived cytoprotective proteins by glioma cells mediates partial resistance to Fas/APO-1 antibodies. The potentiation by ActD and CHX is an important common feature of cytotoxicity mediated by Fas/APO-1 and by the TNF receptor. The proteins responsible for cytoprotection from Fas/APO-1 antibodies and TNF-α in malignant gliomas await identification but may include superoxide dismutase (35, 36). Reduced expression of bcl-2 (37) is probably not a major factor in ActD- or CHX-mediated potentiation of Fas/APO-1-dependent glioma cell killing, because the potentiation of cytotoxicity mediated by ActD or CHX is observed in glioma cell lines that express little bcl-2 (21) and because the effects of ActD and CHX evolve too rapidly when considering the long half-life of the bcl-2 protein (38).

The interrelations between TNF-α-induced cytotoxicity and Fas/APO-1-dependent apoptosis have remained obscure ever since the initial finding that exposure to Fas/APO-1 antibodies co-down-regulated TNF receptor expression (30). Subsequent work failed to identify functional links between both killing pathways and suggested distinct cascades for each mode of cytokine receptor-mediated cell death (26, 39). Transfection studies using various Fas/APO-1 and TNF receptor fusion proteins in a human melanoma cell line lacking endogenous Fas/APO-1 or TNF receptor indicated that differences in the killing kinetics were the main distinguishing feature of cell killing mediated by the cytoplasmic domains of Fas/APO-1 and the p55 and p75 TNF receptors (34). Our previous studies had revealed a correlation between Fas/APO-1-dependent glioma cell apoptosis and the cytotoxic effects of TNF-α in the presence of ActD or CHX in that lack of cell surface Fas/APO-1 expression predicted resistance to TNF-α (21). Here we report that Fas/APO-1 gene transfer into human malignant glioma cell lines not only induces susceptibility to Fas/APO-1 antibodies but also mediates a moderate but significant sensitization of these cells to the cytotoxic effects of TNF-α in the presence of ActD and CHX (Fig. 4). Moreover, unlike the observations in parent glioma cell lines, including constitutively Fas/APO-1-positive cell lines, TNF-α and Fas/APO-1 antibodies had additive cytotoxic effects in the Fas/APO-1-transfected clones in the presence and in the absence of CHX. These observations may relate to the fact that both Fas/APO-1 and the p55 TNF receptor share a common cytoplasmic domain referred to as the death domain, which is required for the induction of cell death (32, 33).

Detailed immunohistological studies are required to assess whether Fas/APO-1-negative glioma cells are consistently found as a subpopulation of malignant gliomas in vivo or whether there is a subgroup among the malignant gliomas that lacks Fas/APO-1 expression altogether. The present study shows that the enhancement of Fas/APO-1 expression on human malignant glioma cells by pharmacological agents or by gene transfer is a promising approach to enhance Fas/APO-1-mediated apoptosis of resistant human malignant glioma cells. Fas/APO-1-transfected glioma cells show a similar pattern of susceptibility to Fas/APO-1-mediated apoptosis as constitutively Fas/APO-1-positive glioma cells, i.e., Fas/APO-1 antibody-induced apoptosis is augmented by coexposure to ActD or CHX or preexposure to cytokines like IFN-γ and TNF-α and is inhibited by dexamethasone. Effects of both cytokines on major histocompatibility complex antigen expression have been well characterized in malignant glioma cells.
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Fas/APO-1 Gene Transfer for Human Malignant Glioma

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