

Treatment of Experimental Glioma by Administration of Adenoviral Vectors Expressing Fas Ligand

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ABSTRACT

Fas ligand (FasL) is a cytokine, produced by activated T cells and NK cells, that triggers apoptosis of Fas-positive target cells including human glioma cells. As shown here, *in vitro* infection of rat F98 and human LN18 glioma cell lines with recombinant adenovirus (rAd) expressing FasL cDNA under control of the cytomegalovirus promoter (rAd-CMV-FasL) induced striking cytotoxicity in Fas-positive glioma cell lines but not in the Fas-negative F98 glioma subline F98/ZH. The extent of FasL-mediated cytotoxic effects outranged the expectations based on expression of β -galactosidase (β -Gal) by F98 cells infected with a control virus expressing the *lacZ* gene (rAd-CMV-*lacZ*). The detection of FasL bioactivity in supernatants of infected cells provides evidence of a bystander mechanism involving the cytotoxic action of FasL on uninfected cells. In F98 tumor-bearing rats, infection with rAd-CMV-FasL increased the mean survival time by 50% compared with infection with rAd-CMV-*lacZ* or untreated controls. These data suggest that viral vector transduction of the FasL gene could be part of a successful glioma gene therapy.

OVERVIEW SUMMARY

One of the best characterized pathways leading to apoptosis is initiated by the cell surface receptor Fas (APO-1/CD95). Fas engagement by Fas ligand (FasL) is capable of activating cysteine proteases called caspases, which mediate the intracellular pathway leading to apoptosis. Since human glioma cells express Fas and undergo Fas-mediated apoptosis, we have generated replication-deficient adenoviruses encoding FasL under the control of the cytomegalovirus (CMV) promoter (rAd-CMV-FasL). This article introduces a therapeutic approach for the treatment of brain tumors by expressing FasL locally at the tumor site. This strategy overcomes the problem of systemic toxicity, namely, liver failure induced by FasL. Pronounced killing of Fas-positive gliomas *in vitro* and significant prolongation of survival of glioma-bearing rats *in vivo* were observed. These data suggest that rAd-CMV-FasL may be a useful therapeutic tool in glioma treatment.

INTRODUCTION

MALIGNANT GLIOMAS are the third leading cause of death from cancer in persons 15 to 34 years of age. The delicate structure of the central nervous system (CNS) and the lack of immune response to the tumor may both contribute to the poor prognosis for patients. Impaired tumor immune surveillance may at least in part be due to tumor-derived suppressor factors including transforming growth factor β 2, which inhibits generation of MHC class I-restricted cytotoxic T cells (CTLs) (Fontana *et al.*, 1984, 1989; Wrann *et al.*, 1987). Absence of a CTL response may be overcome by direct killing of glioma cells with Fas ligand (FasL), a cytokine released by activated T cells and natural killer (NK) cells (for review see Nagata, 1997). Indeed, human glioma cells express Fas and can be killed by FasL *in vitro* (Weller *et al.*, 1994; Frei *et al.*, 1998). The impressive efficacy of the death pathway mediated by cross-linking of the Fas receptor *in vivo* has been demonstrated by the administration of antibodies against human Fas in *nu/nu* mice carrying a

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human B cell tumor (Trauth *et al.*, 1989), and by injection of a recombinant FasL-encoding adenoviral vector into a subcutaneous colon carcinoma tumor (Arai *et al.*, 1997). The treatment of metastatic tumors outside the CNS in the clinical situation demands a systemically applied therapeutic agent. However, FasL or antibodies to mouse Fas cannot be administered systemically to mice or rats because they induce liver cell apoptosis and liver failure (Rensing-Ehl *et al.*, 1995; Nagata, 1996). In contrast, intraperitoneal injection of FasL induces apoptosis of Yac-1 lymphoma cells implanted into the mouse peritoneal cavity without leading to systemic side effects (Rensing-Ehl *et al.*, 1995). A similar approach has been used by Walczak *et al.* (1999), who injected intraperitoneal a leucine zipper (LZ) form of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in mammary adenocarcinoma cells that were implanted into the mouse peritoneal cavity and obtained significant increases in mean survival times. Thus, strategies aimed to express FasL locally in tumors, e.g., gliomas, may be envisaged. The CNS is essentially devoid of Fas-positive cells, and gliomas grown in colony-forming assays *ex vivo* are sensitive to FasL treatment (Frei *et al.*, 1998). It is therefore provocative to investigate the efficacy of a gene therapy using adenovirus expressing FasL cDNA.

MATERIALS AND METHODS

Cell lines and reagents

The rat glioma cell lines F98 and D74-RG2 were kindly provided by K.A. Hossmann (Cologne, Germany) (Ko *et al.*, 1980). A subline termed F98/ZH was established by screening of various F98 cultures for their sensitivity to FasL. The human malignant glioma cell line LN-18 was kindly provided by N. de Tribolet (Lausanne, Switzerland). The rat glioma cell lines 9L and C6 were kindly provided by W. Risau (Bad Nauheim, Germany). The murine glioma cell lines GL-261 and MT539MG were kindly provided by P.R. Walker (Geneva, Switzerland). The murine neuroblastoma cell line Neuro-2A was obtained from the American Type Culture Collection (Rockville, MD). The cell lines F98, F98/ZH, D74-RG2, C6, 9L, GL-261, Neuro-2A, and LN18 were grown in Dulbecco's modified Eagle's medium (DMEM) containing D-glucose (4500 mg/liter; GIBCO-BRL, Life Technologies, Basel, Switzerland), supplemented with 10% fetal calf serum (FCS) (Winiger AG, Wohlen, Switzerland), 2 mM *N*-acetyl-L-alanyl-L-glutamine (Seromed, Biochrom KG, and Fakola AG [Basel, Switzerland]), and gentamicin (20 µg/ml; GIBCO-BRL). The murine glioma cell line MT539MG was grown in DMEM containing D-glucose (1000 mg/liter; GIBCO-BRL) supplemented with 10% FCS (Winiger AG), 2 mM *N*-acetyl-L-alanyl-L-glutamine (Seromed), and gentamicin (20 µg/ml; GIBCO-BRL). The kidney embryonic cell line 293 was obtained from Microbix Biosystems (Toronto, Ontario, Canada) and cultured in minimal essential medium supplemented with 10% horse serum (HS), penicillin (100 U/ml), streptomycin sulfate (100 mg/ml), and 2 mM glutamine (all from GIBCO-BRL). The rat glioma cell line CNS-1 was obtained from W.F. Hickey (Lebanon, NH) and cultured in RPMI 1640 medium supplemented with 10% FCS (Winiger AG), gen-

tamicin (20 µg/ml; GIBCO-BRL), and 2 mM *N*-acetyl-L-alanyl-L-glutamine (Seromed). Recombinant human soluble Fas fused to the Fc portion of human IgG₁ (FasFc) and enhancer protein were purchased from Alexis (Läufelfingen, Switzerland). FasFc is known to bind to FasL and thus block its effect. Rabbit polyclonal IgG antibodies specific for rat and mouse Fas (M-20) were purchased from Santa Cruz Biotechnology (Dr. Glaser AG, Basel, Switzerland). Rabbit IgG isotype controls were purchased from BioPur AG (Bubendorf, Switzerland). Fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-rabbit IgG and anti-proliferating cell nuclear antigen (PCNA, clone PC10) monoclonal antibodies were purchased from Sigma (Buchs, Switzerland).

Constructs of adenoviral vectors

The construction and characterization of rAd-CMV-lacZ is described in detail by Morelli *et al.* (1999), using methods described in Lowenstein *et al.* (1996). Briefly, the 880-bp murine FasL cDNA fragment was subcloned from a BCMGS Neo expression plasmid into the *Bam*HI site of the pAL119 shuttle vector. The pAL119 vector plasmid pSM17, the major immediate-early human cytomegalovirus promoter (MIEhCMV), and the simian virus 40 (SV40) poly(A) signal are all described in Shering *et al.* (1997). The murine FasL cDNA, and the SV40 poly(A), were inserted into the *Xho*I sites of the pAL119 shuttle vector. Recombinant adenoviruses (rAds) were generated by cotransfection of 293 cells, using the calcium phosphate precipitation method, with each recombinant pAL119 shuttle vector together with pJM17. The plasmid pJM17 comprises a circular and unpackageable form of the adenovirus type 5 genome, with deletions in E1 and E3 (Microbix). Before and after three rounds of end-point dilution, the viral DNA was checked by *Hind*III digestion followed by Southern blot hybridization (Morelli *et al.*, 1999). rAds containing appropriate transgenic constructs were scaled up and purified by two cesium chloride gradients. Viral titer was measured by standard end-point dilution assay using 293 cells. As control, we used an rAd encoding the *Escherichia coli* β-galactosidase (β-Gal) gene under the control of the MIEhCMV promoter (rAd-CMV-lacZ), previously described as Rad35 (Shering *et al.*, 1997).

Immunohistochemistry

Animals were euthanized by an overdose of pentobarbital (Abbott Laboratories, North Chicago, IL). Brain and various organs were dissected and fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ · 7H₂O, 1.4 mM KH₂PO₄, pH 7.3) (all Fluka Chemie AG, Buchs, Switzerland) for 3 days or directly frozen in isopentane (2-methyl-butane) (Fluka Chemie AG) on dry ice. Paraffin-embedded tissue sections (1 µm) were stained with hematoxylin and eosin and, for the detection of tumor cells, with anti-proliferating cell nuclear antigen (PCNA). The tissue sections were heated in boiling citrate buffer for 3 min and thereafter sequentially incubated with the mouse monoclonal α-PCNA antibody (2 µg/ml) or the appropriate control MAb (mouse IgG_{2a}; Sigma), peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark; 1:400, 30 min, 20°C), and metal-enhanced diaminobenzidine substrate (Sigma) for 15 min at 20°C.

Flow cytometric analysis of Fas expression

F98, F98/ZH, C6, D74-RG2, GL-261, or MT539MG glioma cells (10^6) were plated on 35-mm petri dishes (Falcon Plastics, Oxnard, CA) in 4 ml of culture medium. After 48 hr the cells were washed twice with PBS, scraped off, and incubated for 30 min on ice in 100 μ l of blocking buffer containing PBS (pH 7.4), 10% goat serum (Sigma), and 1% bovine serum albumin (BSA) (Fluka Chemie AG). Subsequently the cells were incubated for 30 min on ice in 100 μ l of blocking buffer containing PBS (pH 7.4), 1% BSA (Fluka Chemie AG), and the rabbit anti-rat/mouse Fas antibody (M20) or isotype control antibody (both 0.4 μ g/ 10^6 cells), followed by incubation with a 1:100 diluted FITC-conjugated goat antibody to whole rabbit IgG. The cells were washed twice with flow cytometry buffer containing PBS (pH 7.4), 0.1% BSA, and 0.01% sodium azide (both Fluka Chemie AG). Dead cells were excluded by gating on a combination of forward and side scatter. A total of 10^4 viable cells was analyzed on an Epics profile analyzer. The specific fluorescence index (SFI) was calculated as follows: SFI = gated mean whole range fluorescence of specific antibody/gated mean whole range fluorescence of isotype control antibody.

Local administration of tumor cells into the right frontal cortex

Male Fischer 344 inbred rats were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland), and kept under specific pathogen-free conditions. The rats ranged from 250 to 300 g in weight at the time of tumor cell implantation. F98 cells were stereotactically implanted into the right frontal cortex, using a modification of the method of Kobayashi (Kobayashi *et al.*, 1980). In brief, rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg; Abbott Laboratories) and placed in a stereotaxic frame (Stoelting, Indulab AG, Gams, Switzerland). A sagittal incision was made through the scalp to expose the skull, and a burrhole was made 2 mm anterior and 3 mm lateral to the right of the bregma. On day 0 all animals were inoculated with 5×10^4 F98 tumor cells in 2 μ l of Hanks' balanced salt solutions (HBSS) containing 1% L-glutamine (both GIBCO-BRL), using a 5- μ l 23-gauge microsyringe (SGE GmbH, Weiterstadt, Germany). The tumor cells were injected at a depth of 4 mm ventral from the dura at a rate of 0.5 μ l/min. The needle was left in place for 3 min and then withdrawn over the course of 5 min. The hole in the skull was plugged with bone wax, and the incision was sutured using Prolene thread (Arnold Bott AG, Zurich, Switzerland). On day 1, animals were injected with either 5×10^7 PFU of rAd-CMV-FasL or rAd-CMV-lacZ in 3 μ l of HBSS containing 1% L-glutamine, at the same coordinates. The animals were weighed daily and sacrificed when they became moribund (weight loss $\geq 5\%$ of their maximal weight), or at the time point indicated.

Assay for cytotoxic activity

Target cells were plated in, per well, 0.15 ml of DMEM containing D-glucose (4500 mg/liter; GIBCO-BRL) supplemented with 1% FCS (Winiger AG), 2 mM N-acetyl-L-alanyl-L-glutamine (Seromed), and gentamicin (20 μ g/ml; GIBCO-BRL), at a concentration of 6×10^4 cells per well in 48-multiwell tissue

culture plates (Costar, Integra Biosciences AG, Wallisellen, Switzerland). In selected wells the number of viable cells was counted by trypan blue staining 24 hr after plating to determine the exact multiplicities of infection (MOIs) necessary. Medium was changed, and the cells were incubated with viral vectors at various MOIs plated in, per well, 0.15 ml of DMEM containing D-glucose (4500 mg/liter; GIBCO-BRL) supplemented with 1% FCS (Winiger AG), 2 mM N-acetyl-L-alanyl-L-glutamine (Seromed), and gentamicin (20 μ g/ml; GIBCO-BRL) in the presence or absence of recombinant human soluble FasFc. Cytotoxicity was assessed after 24 and 72 hr of adenoviral infection, using the Alamar blue (Lucerna Chem AG, Lucerne, Switzerland) assay according to the manufacturer instructions. The supernatant of tumor cells was harvested after 48 hr of adenoviral incubation and preincubated with recombinant human soluble FasFc for 30 min at 37°C, to determine the amount of FasL bioactivity contained in it. A volume of 0.05 ml of this supernatant was added to target cells, plated 24 hr earlier in, per well, 0.05 ml of DMEM containing D-glucose (4500 mg/liter; GIBCO-BRL) supplemented with 1% FCS (Winiger AG), 2 mM N-acetyl-L-alanyl-L-glutamine (Seromed), and gentamicin (20 μ g/ml; GIBCO-BRL) at a concentration of 2×10^4 cells per well in 96-multiwell tissue culture F-plates (Falcon Plastics). To evaluate the cytotoxic effect of membrane form FasL on the glioma cell lines, supernatant of Neuro-2A cells transfected with a pBCMGsneo plasmid containing complete murine FasL was harvested. Transfection of the cells and harvesting of the supernatant were done as described by Rensing-Ehl *et al.* (1995). Neuro-2A/FasL supernatant (0.05 ml) was added to target cells, plated 24 hr earlier in, per well, 0.05 ml of DMEM containing D-glucose (4500 mg/liter; GIBCO-BRL) supplemented with 1% FCS (Winiger AG), 2 mM N-acetyl-L-alanyl-L-glutamine (Seromed), and gentamicin (20 μ g/ml; GIBCO-BRL), at a concentration of 2×10^4 cells per well in 96-multiwell tissue culture F-plates (Falcon Plastics). Cytotoxicity was assessed after an additional 24 hr, using the Alamar blue assay. *Ex vivo* tumor cell cultures were obtained as described (Frei *et al.*, 1998). Briefly, tumor-bearing animals were sacrificed and the tumor tissue was removed, placed on a petri dish, and immediately minced mechanically and digested enzymatically for 1 hr using collagenase/dispase (1 mg/ml; Boehringer GmbH, Mannheim, Germany) in 20 ml of HBSS without calcium (GIBCO-BRL). The digestion was performed in a water bath at 37°C while stirring with a magnetic bar. The dissociated cells were filtered through 100- and 70- μ m pore size cell strainers (Falcon Plastics) to remove tissue debris. Thereafter, the cells were centrifuged (5 min, $250 \times g$) and erythrocytes were lysed by hypotonic treatment for 20 sec in 1.8 ml of distilled water. The hypotonic treatment was stopped by the addition of 0.2 ml of $10 \times$ HBSS (GIBCO-BRL) and the cells were washed and resuspended in DMEM containing D-glucose (4500 mg/liter; GIBCO-BRL) supplemented with 10% FCS (Winiger AG), 2 mM N-acetyl-L-alanyl-L-glutamine (Seromed), and gentamicin (20 μ g/ml; GIBCO-BRL). Cytotoxic assays and flow cytometry (fluorescence-activated cell sorting, FACS) analysis was performed as described for the glioma cell lines. Crystal violet staining (data not shown), and morphological appearance was examined in all cytotoxicity assays to reconfirm the results. Cytotoxicity was calculated as follows: Cytotoxicity = $1 - [\text{fluorescence units (FU) of FasL-ex}$

posed tumor cells-FU of cell-free culture medium]/(FU of untreated tumor cells-FU of cell-free culture medium) \times 100 (%).

β -Galactosidase detection

The following protocol was used for detection of β -Gal activity in Ad-CMV-lacZ-infected glioma cell cultures. The cells were infected as described above, and the medium was discarded after 24 or 72 hr of rAd infection, respectively. A volume of 0.2 ml of 0.05% glutaraldehyde in PBS was added to each well for 10 min at room temperature. Afterward the cells were rinsed three times with 0.2 ml of PBS per well (the second wash was left on for 10 min; the first and third rinses were performed quickly). A volume of 0.2 ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, 1 mg/ml; Sigma) in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, and 2 mM $MgCl_2$ (all Fluka Chemie AG) was added to each well. The cells were incubated at 37°C overnight. Thereafter the X-Gal solution was discarded, and the reaction stopped by addition of 0.2 ml of 0.05% glutaraldehyde (Fluka Chemie AG) in PBS.

A commercially available stain (Specialty Media, Lavallette, NJ) was used for detection of β -Gal-containing cells in the tissue sections. Air-dried serial cryostat sections (5 μ m) were fixed in tissue fixative for 45 min on ice, rinsed in tissue rinse solution A, washed in tissue rinse solution A for 30 min at room temperature, rinsed in tissue rinse solution B, and washed in tissue rinse solution B for 5 min at room temperature. Freshly prepared complete β -Gal tissue stain solution was added to the drained tissues and incubated overnight at 37°C in the dark. The sections were covered with Eukitt (Gribi AG, Belp, Switzerland).

Statistical analysis

The paired two-sided Student *t* test was used to compare the survival of animals among the experimental groups.

RESULTS

Fas expression

In the present study we have investigated adenoviruses encoding mouse FasL cDNA for their potential to infect glioma cells and to induce cell death. Initial experiments tested the susceptibility of rodent glioma cell lines that are currently used in experimental therapeutic glioma studies. When adding FasL in membrane form the F98 glioma cells were found to be susceptible to FasL-mediated killing. The addition of membrane form FasL to the glioma cell lines C6, 9L, D74-RG2, CNS-1, MT539MG, and GL-261 caused a cytotoxicity below 20%. Flow cytometry revealed that F98 glioma cells expressed Fas at a high level (SFI of 5.86) (Fig. 1). In a subline of F98 cells, F98/ZH, no expression of Fas (SFI of 0.99) was detected. In agreement with the flow cytometry analysis only F98 cells, but not F98/ZH cells, were susceptible to FasL-mediated killing (data not shown). A low level of Fas expression was detected by flow cytometry on the rat glioma cell lines C6 (SFI of 1.47), 9L (SFI of 1.07), and D74-RG2 (SFI of 1.57), as well as on the murine gliomas MT539MG (SFI of 1.7) and GL-261 (SFI of

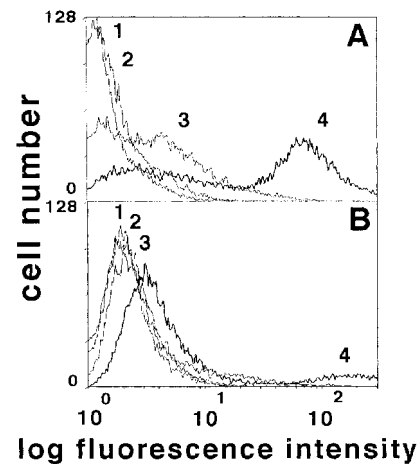


FIG. 1. Expression of Fas by F98 glioma cells but not by its subline F98/ZH. For flow cytometric analysis F98 (A) or F98/ZH (B) glioma cells were stained with a rabbit anti-rat Fas antibody (4) or isotype control (3) followed by an FITC-conjugated secondary goat antibody to whole rabbit IgG. (1) Unstained autofluorescence control and (2) cells stained with the secondary antibody only.

1.34). These expression levels correlate with the low cytotoxicity of membrane form FasL on these cell lines.

Infectivity and cytotoxicity of rAd-CMV-FasL

To assess the potential use of adenovirus-derived replication-deficient vectors expressing FasL, we first examined whether F98 cells expressed a reporter transgene when infected with rAd-CMV-lacZ. Transduction with rAd-CMV-lacZ of F98 and F98/ZH cells 24 hr after plating resulted in substantial expression of β -Gal activity in a time- and titer-dependent manner. Twenty-four hours after rAd transduction, F98 cells infected with 500 or 1000 MOIs showed 12 and 20% β -Gal-positive cells, respectively. Three days after infection, the corresponding values were 11 and 31%. Infection of F98/ZH with rAd-

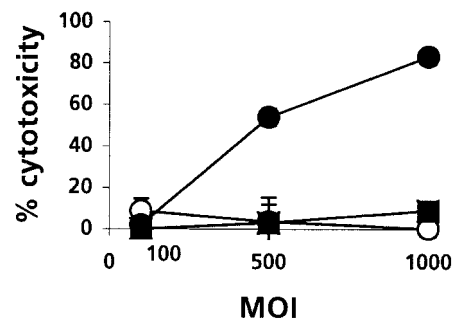


FIG. 2. Infection of F98 glioma cells with rAd-CMV-FasL induces cell death. Glioma cells were infected with various doses of rAd-CMV-FasL or rAd-CMV-lacZ 24 hr after plating. In rAd-CMV-FasL-infected cultures cytotoxicity was assessed 24 hr (○) and 72 hr (●) after infection, or in rAd-CMV-lacZ-infected cells 3 days after transduction (■) using the Alamar blue assay.

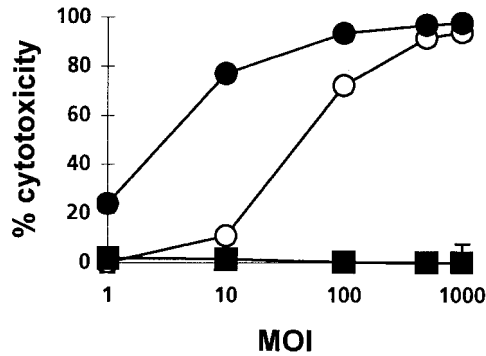


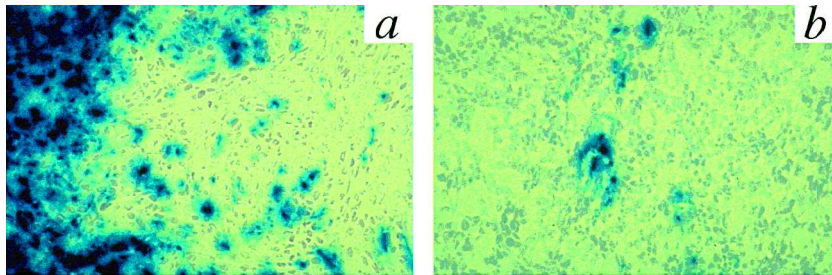
FIG. 3. Sensitivity of human LN18 glioma cells to infection with rAd-CMV-FasL. LN18 cells were infected with various doses of rAd-CMV-FasL or rAd-CMV-lacZ 24 hr after plating. Cytotoxicity was assessed by the Alamar blue assay after 24 hr (○) or 72 hr (●) of infection with rAd-CMV-FasL or 72 hr after infection when using rAd-CMV-lacZ (■).

CMV-lacZ resulted in a high level of expression of β -Gal, e.g., 72 hr of infection with an MOI of 1000 gave rise to 74% β -Gal-positive cells. To explore whether infection of F98 with rAd-CMV-FasL results in FasL-mediated killing, survival of glioma cell cultures was assessed at different time points after infection. No apparent morphological differences among control cells and those infected with adenovirus became evident within the first 24 hr after infection. Thereafter, striking cytotoxicity occurred in rAd-CMV-FasL- but not rAd-CMV-lacZ-treated F98 cells, the extent depending on the amount of rAd-

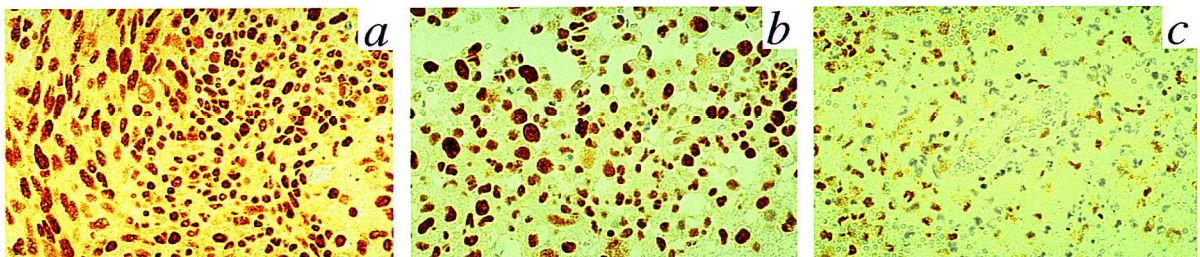
CMV-FasL virus added (Fig. 2). At MOIs of 500 and 1000, cytotoxicity of rAd-CMV-FasL-infected F98 cells was 51.9 ± 4.1 and $81.2 \pm 2.6\%$, respectively. When compared with rAd-CMV-lacZ, no cytotoxic effects were noted in F98/ZH cells infected with rAd-CMV-FasL (data not shown). Cytotoxicity of rAd-CMV-FasL was also tested on LN18 cells, a human glioma cell line described to express Fas and to be sensitive to FasL-mediated killing (Weller *et al.*, 1994). Compared with F98, LN18 cells are much more sensitive. At an MOI of 100, 72% cytotoxicity was observed just 1 days after infection with rAd-CMV-FasL (Fig. 3).

Bystander effect

The extent of cytotoxicity of F98 cells infected with rAd-CMV-FasL was considerably higher than expected from the calculation of the percentage of cells expressing β -Gal on infection with rAd-CMV-lacZ. Therefore we evaluated whether FasL-mediated killing of F98 cells is associated with a bystander effect such that noninfected glioma cells adjacent to rAd-CMV-FasL-infected cells undergo cell death due to interaction of FasL with Fas. Supernatants of cells infected with rAd-CMV-FasL were harvested 2 days after infection and tested on F98 cells, using a 24-hr cytotoxicity assay. Given the latency of cytotoxicity observed in F98 cells infected with rAd-CMV-FasL (Fig. 2), it is unlikely that carrying over of rAd-CMV-FasL will influence cell survival. Indeed, supernatants from F98 cells infected with rAd-CMV-FasL (MOI of 1000) induced 85% cytotoxicity of F98 cells, at a final dilution of 1:2. The cytotoxic effect of the supernatant is mediated by FasL since the addition of FasFc ($2 \mu\text{g/ml}$) caused an 84.4% inhibition of cytotoxicity. These findings demonstrate a bystander ef-



COLOR PLATE 1. F98 tumor cells were stained for β -Gal on day 10 (a) and day 20 (b) after infection with rAd-CMV-lacZ (original magnification: $\times 250$).



COLOR PLATE 2. Necrotic areas in rAd-CMV-FasL-treated F98 tumors: F98 tumor cells were left untreated (a) or infected with rAd-CMV-lacZ (b) or rAd-CMV-FasL (c) and stained with anti-PCNA antibodies on day 4 after treatment (original magnification: $\times 500$).

fect that, besides cell-to-cell contact, depends on freely diffusible FasL that induces Fas-mediated killing of noninfected F98 cells. Analogously prepared and tested supernatants of rAd-CMV-FasL (MOI of 1000)-infected F98/ZH cells induced considerable cytotoxicity (30%) of F98 cells that was neutralized completely by addition of FasFc. Thus the failure of F98/ZH cells to undergo apoptosis after infection with rAd-CMV-FasL is likely due to the absence of expression of Fas but not of FasL.

Survival

On the basis of the encouraging *in vitro* data, we proceeded to evaluate the *in vivo* sensitivity of F98 glioma cells to rAd-CMV-FasL. F98 glioma cells (5×10^4) were injected stereotactically into the right frontal lobes of the brains of syngeneic Fischer 344 rats. One day later, 5×10^7 plaque-forming units (PFU) of either rAd-CMV-lacZ or rAd-CMV-FasL was stereotactically injected at the same coordinates. In the first series of experiments expression of the *lacZ* gene was analyzed on day 10 and day 20 after injection of rAd-CMV-lacZ. Variable results were obtained at both time points, ranging from about 50% β -Gal-positive tumor cells to only single tumor cells expressing β -Gal (Color Plate 1). In the second series of experiments mean survival time of rats inoculated with F98 cells was found to be 21.7 ± 2.6 days. Rats inoculated with F98 cells and treated with rAd-CMV-FasL survived much longer: 32.9 ± 6.5 days ($p = 0.005$). The growth of tumors was not influenced by the injection of rAd-CMV-lacZ, the mean survival time of the animals being 21.4 ± 2.3 days (Fig. 4). In further experiments at day 4 after treatment, rAd-CMV-FasL-treated tumors showed PCNA-stained tumor cells and necrotic areas, which were much less pronounced in rAd-CMV-LacZ-treated tumors and absent in untreated controls (Color Plate 2).

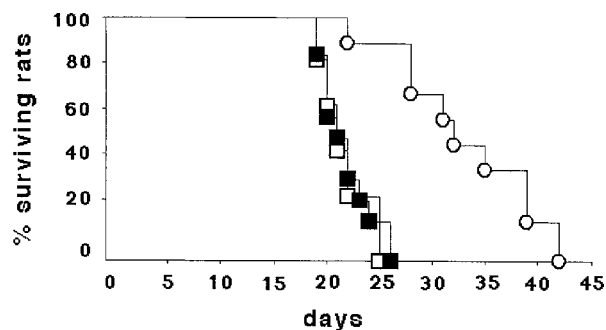


FIG. 4. Animal survival after *in vivo* transduction of F98 gliomas by control or FasL-expressing rAd vector. F98 glioma cells (5×10^4) were implanted into the right frontal lobe. Survival curves for intracerebral F98 gliomas (■, $n = 11$) and for F98 gliomas treated 1 day after tumor cell inoculation with 5×10^7 rAd-CMV-FasL (○, $n = 9$) or rAd-CMV-lacZ (□, $n = 5$) are shown. Survival curves of animals injected with F98 cells alone or with F98 cells and treated with rAd-CMV-lacZ were similar to each other ($p = 0.1416$) and survival of those animals treated with rAd-CMV-FasL was significantly enhanced ($p = 0.0005$).

No acquired drug resistance

Additional experiments were designed to investigate whether tumor cells become resistant to FasL either by downregulation of Fas expression or by recruitment of inhibitors of the intracellular pathway, leading to apoptosis after ligation of Fas receptors. In two rats inoculated with F98 cells and treated with rAd-CMV-FasL, the tumors were excised at the time the animals became moribund (days 39 and 42). The cells were dissociated and analyzed for Fas expression by flow cytometry analysis and for their sensitivity to FasL. The tumor cells in both animals expressed Fas, at a level comparable to the original F98 cell line cultured *in vitro* (data not shown). Furthermore, when adding FasL in membrane form, killing of the *ex vivo* F98 tumor cells from the two rats was 90% and 95%, respectively. Taken collectively, tumor growth in rats treated with FasL is not due to the development of resistance to the cytokine. To assess toxic effects by the therapeutics strategy used, histologic examination were performed on liver, lung, spleen, and muscle and found to be normal.

DISCUSSION

Many human gliomas express Fas and the addition of anti-Fas antibodies or FasL activates the signaling pathway leading to apoptosis of glioma cells (Weller *et al.*, 1994; Frei *et al.*, 1998). We have evaluated the potential of gene therapy of gliomas, using viral vectors encoding murine FasL cDNA. Unfortunately, rat C6 and 9L gliomas, the tumor cell lines most widely used in experimental glioma studies, were resistant to killing by recombinant FasL. This was also true for the rat gliomas CNS-1 and D74-RG2, and the two mouse gliomas GL-261 and MT539MG. Heterogeneity of sensitivity to FasL-mediated cytotoxicity has also been observed when investigating human glioma cell lines (Weller *et al.*, 1994). Three of seven human cell lines analyzed expressed only low levels of Fas and were resistant to anti-Fas antibody-mediated killing. However, all eight *ex vivo*-cultured high-grade gliomas (WHO stages III and IV) grown in colony-forming assays were highly susceptible to FasL ($\geq 85\%$ inhibition of colony formation) (Frei *et al.*, 1998). This may eventually indicate that long-term cultivation of glioma cell lines may select for Fas-negative tumor cell populations and/or that in *in vivo* Fas expression in the tumor is not uniform. In fact, both Fas-positive and -negative tumor cells can be detected in gliomas, the Fas-positive tumor cells being localized mainly in surrounding areas of tumor cell necrosis (Tachibana *et al.*, 1996). Nevertheless, given the low sensitivity of immunostaining for Fas and the striking effect of FasL on *ex vivo* glioma colony formation, it is worthwhile to investigate the efficacy of the Fas/FasL apoptosis pathway in gliomas. Sensitivity to FasL may be enhanced by constitutive expression of FasL by glioma cells, which, however, is not efficient enough on its own to lead to Fas-induced signaling of the caspase 3-initiated apoptosis pathway of glioma cells (Gratas *et al.*, 1997; Saas *et al.*, 1997; Husain *et al.*, 1998). Our decision was to select two Fas-positive glioma lines, the human LN18 and the rat F98 cell lines. Infection of F98 glioma cells with rAd-CMV-FasL resulted in prominent cell killing. Evi-

dence that FasL mediates cytotoxicity in our experiments is based on the observations that (1) the Fas-negative subline F98/ZH was not killed when infected with rAd-CMV-FasL, and (2) F98 cells survive infection with rAd-CMV-lacZ. Since FasL was detected not only in the culture supernatant of rAd-CMV-FasL-infected F98 cells, but also in that of F98/ZH cells, the absence of effects of rAd-CMV-FasL in the latter cells is likely due to impaired expression of Fas but not of FasL.

Animals infected with rAd-CMV-FasL showed a 50% increase in survival when compared with untreated or rAd-CMV-lacZ-treated F98 tumor-bearing animals. Tzeng *et al.* (1990) reported that inoculation of 10^2 F98 cells into the right caudate nucleus led to a survival time of 28.6 ± 3.9 days. The increase in mean survival time to 32 ± 6.5 days in our experiments, after inoculation of 10^5 tumor cells and treatment, indicates that the rAd-CMV-FasL gene therapy was able to kill a high percentage of tumor cells. In general, F98 cells are resistant to various treatment protocols including chemotherapy with ACNU (nimustine), radiotherapy, and adoptive immunotherapy (for review see Barth, 1998). Whereas the variation in the survival time of F98 glioma-bearing rats left untreated or treated with rAd-CMV-lacZ was small (11.98% and 10.7%), in the rAd-CMV-FasL-treated group the respective figure was much higher (19.8%). This may be due to variable expression of the FasL gene in individual tumor-bearing rats. Indeed, by days 10 and 20 after infection with rAd-CMV-lacZ great heterogeneity of expression of β -Gal was observed, ranging from only single β -Gal-positive tumor cells to a maximum of 50% (day 10 after infection). Nevertheless, even low numbers of cells expressing FasL after treatment with rAd-CMV-FasL may interfere with tumor expansion because FasL may interact with Fas on cells not being infected with rAd-CMV-FasL. A bystander mechanism is suggested from our *in vitro* observation that the amount of β -Gal-positive F98 cells was 31% by 3 days after infection, the time point when more than 80% of the cells are killed on infection with rAd-CMV-FasL. Tanaka *et al.* (1995) showed human FasL to be released as a trimer of a cleaved 26-kDa form. The membrane form of FasL (mFasL) was found to be cleaved between Ser-126 and Leu-127 to yield the soluble form of FasL (sFasL), which is, however, more than 1000-fold less active in its apoptosis-inducing capacity (Schneider *et al.*, 1998). Bioactive FasL is detected in supernatants of rAd-CMV-FasL-infected F98 cells. Cytotoxicity induced by the supernatant was significantly inhibited by soluble Fas. Thus, FasL may be released in its entire membrane form. Transfection of the neuroblastoma cell line Neuro2A with murine FasL cDNA was found to lead to release of FasL in its membrane, noncleaved bioactive form (Schneider *et al.*, 1998). Since supernatant of rAd-CMV-FasL-infected glioma cells kills noninfected gliomas efficiently, there is no evidence that sFasL released by rAd-CMV-FasL-infected gliomas counteracts the effect of mFasL. Infection with rAd-CMV-FasL of various types of cells, including the glioma cell lines CNS-1 and C6, leads to release of bioactive FasL (Morelli *et al.*, 1999).

Taken collectively, infection of glioma with rAd-CMV-FasL results in tumor cell killing *in vitro* and *in vivo*. However, significant effects on survival were achieved by using a high dose of rAd-CMV-FasL, which was injected early after F98 tumor

cell inoculation. It is of note that *in vitro* cytotoxic effects on F98 cells required 10 times more rAd-CMV-FasL compared with human LN18 cells. A pessimistic view of the FasL strategy to be used in glioma treatment can be drawn from the failure to achieve long-term survival of rats inoculated with F98 tumors. Strategies aimed at inducing Fas-mediated apoptosis of tumor cells carry the risk of (1) escape of primary Fas-negative tumor cells from FasL killing and (2) induction of treatment resistance by downregulation of Fas expression or overexpression of inhibitors of the caspase pathways. However, in our study rAd-CMV-FasL treatment did not lead to selection of FasL-resistant tumor cells; the failure to induce long-term survival was, rather, the result of insufficient long-term expression of the FasL gene in the tumor. This may be due to the generation of an anti-adenovirus response. Antiviral immunity may be less of a problem because FasL has been shown to impair immune effector pathways locally (Lau *et al.*, 1996). Concerning possible FasL-mediated side effects, overexpression of FasL was neither associated with local morphologic changes such as apoptosis of brain parenchymal cells, nor with systemic alterations such as liver cell failure, a lethal complication when administering FasL intravenously (Rensing-Ehl *et al.*, 1995). It is of note that Fas expression in the CNS is low and FasL does not lead to apoptosis of neurons and oligodendrocytes *in vitro* (Rensing-Ehl *et al.*, 1996; Malipiero *et al.*, 1997). On the other hand, excessive production of sFasL in the CNS could compete with mFasL at the Fas receptor level. FasL has been found to be expressed by brain macrophages Spanaus *et al.*, 1998) and in spinal cord neurons (French and Tschopp, 1996).

ACKNOWLEDGMENTS

We acknowledge the help of Dr. Adriana Larregina in the preparation and provision of recombinant adenoviruses. We thank Seepoei Muljana, Rosmarie Frick, and Therese Michel for excellent technical assistance, and Eva Niederer for flow cytometry analysis. This work was supported by the Swiss National Science Foundation (NFP37: 4037-044702 to A.F.), the Sassella-Stiftung, Zurich, Switzerland (to K.F.), the Swiss Cancer League, Bern, Switzerland (to A.F.), and by grants from the Wellcome Trust, UK (BS1248/2), and Cancer Research Campaign, UK (SP2332/0101), to M.G.C. and P.R.L. A.E.M. was funded by a grant from the British Heart Foundation (PG/97041 to M.G.C. and P.R.L.); P.R.L. is a research fellow of the Lister Institute of Preventive Medicine, UK.

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Received for publication October 2, 1998; accepted after revision April 16, 1999.