Chimeric Tumor Suppressor 1, a p53-derived Chimeric Tumor Suppressor Gene, Kills p53 Mutant and p53 Wild-type Glioma Cells in Synergy with Irradiation and CD95 Ligand

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ABSTRACT

Adenoviral chimeric tumor suppressor 1 (CTS1) gene transfer was evaluated as a novel approach of somatic gene therapy for malignant glioma. CTS1 is an artificial p53-based gene designed to resist various pathways of p53 inactivation. Here, we report that an adenovirus encoding CTS1 (Ad-CTS1) induces growth arrest and loss of viability in all glioma cell lines examined, in the absence of specific cell cycle changes. In contrast, an adenovirus encoding wild-type p53 (Ad-p53) does not consistently induce apoptosis in the same cell lines. Electron microscopic analysis of Ad-CTS1-infected glioma cells reveals complex cytoplasmic pathology and delayed apoptotic changes. Ad-CTS1 induces prominent activation of various p53 target genes, including p21 and MDM-2, but has no relevant effects on BCL-2 family protein expression. Although Ad-CTS1 strongly enhances CD95 expression at the cell surface, endogenous CD95/CD95 ligand interactions do not mediate CTS1-induced cell death. This is because Ad-CTS1 promotes rather caspase activation nor mitochondrial cytochrome c release and because the caspase inhibitors, z-val-Ala-DL-Asp-fluoromethylketone (zVAD)-fmk or z-Ile-Glu-Thr-Asp-fluoromethylketone (z-IE TD)-fmk, do not block CTS1-induced cell death. Ad-CTS1 synergizes with radiotherapy and CD95 ligand in killing glioma cells. In summary, Ad-CTS1 induces an unusual type of cell death that appears to be independent of BCL-2 family proteins, cytochrome c release, and caspases. CTS1 gene transfer is a promising strategy of somatic gene therapy for malignant glioma.

INTRODUCTION

Glioblastoma, the most common malignant intrinsic brain tumor, is rather resistant to current approaches of therapy. Thus, median survival after aggressive multimodality treatment consisting of cytoreductive neurosurgery, involved-field radiotherapy, and adjuvant chemotherapy has remained in the range of 12 months for at least two decades. Accordingly, human glioblastoma has been and still is a common target of novel strategies of somatic gene therapy for cancer. For instance, suicide gene therapy involving thymidine kinase gene transfer and ganciclovir treatment has been evaluated in several clinical trials (1, 2). However, the clinical results have been disappointing thus far (3), and recent studies in laboratory animals have raised unexpected concerns about neurotoxicity and long-term safety of this approach (4).

Transfer of the wild-type p53 gene, which is one of the most common targets of mutation in human cancers, is another strategy of somatic gene therapy for cancer. Adenoviruses encoding p53 inhibit the growth of experimental gliomas in rodents (5, 6) and of human glioma cell lines in vitro (7–10), and p53 gene therapy is currently being evaluated in human patients with lung cancer (11). CTS1 is a p53-derived chimeric tumor suppressor gene designed to resist physiological pathways of p53 inactivation, including resistance to inactivation by MDM-2 and oncogenic p53 mutants and decreased sensitivity to E6-induced degradation (12). Compared with wild-type p53, CTS1 exhibits enhanced transcriptional and proapoptotic activity both in vitro and in vivo (12, 13). Here, we generated an adenoviral vector encoding CTS1 (Ad-CTS1) and examined its effects on human malignant glioma cells in vitro.

Materials and Methods

Materials and Cell Lines. Cytotoxic drugs were obtained from the following sources: (a) teniposide, Sandoz (Basel, Switzerland); (b) vincristine, Bristol (Munich, Germany); and (c) lomustine, Medac (Hamburg, Germany). zVAD-fmk, DEVD-ame, and zIETD-fmk were obtained from Bachem (Heidelberg, Germany). 4′,6-Diamidino-2-phenylindole (DAPI) and all other reagents were obtained from Sigma Chemical Co. (Deisenhofen, Germany). CD95L-containing supernatant was harvested from CD95L-transfected N2a neuroblastoma cells (14). U87MG, LN-18, LN-229, and LN-308 human malignant glioma cells and SKN-LE and SKN-BE human neuroblastoma cells were kindly provided by N. de Tribolet (Neurosurgical Service, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). HTC116 colon cancer cells and Ad-p53 were a kind gift of B. Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center, Baltimore, MD). SV-FHAS SV40-transformed human astrocytes were provided by A. Muruganandan (National Research Council, Ottawa, Canada). MCF-7 breast carcinoma (HTB22), MRC5 fibroblasts (CCL-171), and DAOY medulloblastosoma cells (HTB186) were obtained from the American Type Culture Collection (Manassas, VA). Rat hippocampal neuronal cultures were prepared as described previously (15). All cell lines except HCT116, which grow in McCoy’s 5A medium, and rat primary neurons, which grow in conditioned N2-medium, were maintained in DMEM (Life Technologies, Inc., Eggenstein, Germany) containing 10% FCS, 2 mM glutamine, and 100 IU/ml penicillin/100 μg/ml streptomycin (16).

Construction of Adenoviral Vectors and Infection Procedure. CTS1 is a synthetic p53 analogue, that is, a chimeric tumor suppressor gene in which the NH2-terminal domain of p53 (amino acids 1–75) has been substituted by the transactivator domain of the viral protein VP16 (amino acids 420–490). An optimized dimerization leucine zipper substitutes the p53 oligomerization domain (amino acids 325–355). The COOH-terminal autoregulatory inhibitory domain of p53 is deleted in CTS1 (12). Replication-deficient recombinant adenoviruses that had the E1a region replaced by the expression cassette (Ad-CTS1) were constructed by cotransfection of shuttle vector pMH4 containing CTS1 cDNA and genomic adenoviral DNA (17). The shuttle vector pMH4-CTS1 was constructed by inserting CTS1 cDNA. The expression of CTS1 cDNA is controlled by the human cytomegalovirus immediate early promoter and enhancer, β-Galactosidase (Ad-LacZ) or EGFP-expressing adenoviruses (Ad-EGFP) were constructed the same way. After cotransfection of adenoviral genomic DNA and shuttle vectors into the adenovirus-transformed MRC-5 cells, the recombinant adenoviruses were purified by ultracentrifugation.

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3 The abbreviations used are: CTS1, chimeric tumor suppressor 1; CD95L, CD95 ligand; EGFP, enhanced green fluorescent protein; M0I, multiplicity of infection; SFI, specific fluorescence index; zVAD-fmk, z-Val-Ala-Asp-fluoromethylketone; zIETD-fmk, z-Ile-Glu-Thr-Asp-fluoromethylketone; DEVD-ame, Acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin; DAPI, 4′,6-Diamidino-2-phenylindole.

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human embryonic kidney cell line 293 (ATCC CRL-1573) by calcium phosphate-mediated gene transfer, plaques were screened for the presence of transgene expression by reverse transcription-PCR or Northern blot analysis. Viral plaques containing transgene sequences and devoid of adenoviral E1a sequence were grown in 293 cells and purified by cesium chloride gradient (17). Infection with recombinant viruses was accomplished by exposing cells to different concentrations of adenovirus in PBS containing 10% glycerol for 15 min followed by addition of serum-containing medium for 1–5 days. The efficacy of adenoviral gene delivery and expression was ascertained by EGFP fluorescence or immunoblot analysis using a p53 COOH-terminal antibody for p53 and a VP16 antibody for CTS1.

**Immunoblot Analysis.** The general procedure has been described previously (16, 18). Lysates for the detection of cytochrome c were prepared as described by Hicke (19). The following antibodies were used: (a) anti-VP16 (SC7545; Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA); (b) anti-p21 (C19; Santa Cruz Biotechnology SC397); (c) anti-p53 [Ab-6 (Oncogene Research Products, Cambridge, MA) and Bp53-12 (Santa Cruz Biotechnology SC263)]; (d) anti-MDM-2 (SMP14; Santa Cruz Biotechnology SC965); (e) anti-BAX (G23; Santa Cruz Biotechnology SC832); (f) anti-BAX (N20; Santa Cruz Biotechnology SC498); (g) anti-BCL-2 (clone 124; Dakopatts, Glostrup, Denmark); (h) anti-BCL-X (S18; Santa Cruz Biotechnology SC694); (i) anti-actin (1-19; Santa Cruz Biotechnology SC1616); (j) anti-cytochrome c (65981A; PharMingen-Becton Dickinson, Heidelberg, Germany); (k) anti-caspase 3 (C31720; Transduction Laboratories, Lexington, KY); (l) anti-caspase 8 (kindly provided by P. H. Krammer; DKFZ, Department of Immunogenetics, Heidelberg, Germany); and (m) anti-caspase 9 (kindly provided by Y. Lazebnik; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

**Proliferation and Viability Assays.** For most assays, the cells were seeded into 24- or 96-well plates and allowed to attach for 24 h. Growth and viability were assessed at different times or at variable MOIs by crystal violet staining or trypan blue exclusion. For acute drug cytotoxicity assays, the cells were seeded at 10^4 cells/well in microtiter plates, allowed to attach, and infected with adenovirus at 50–300 MOI. The cells were harvested from the supernatant by centrifugation and added to the wells. After the indicated time, the cells were irradiated at 3 Gy at 24 h after virus infection and allowed to grow for 10–15 doubling periods. Viable colonies were stained by crystal violet and counted.

**Cell Cycle Analysis.** The cells were treated as indicated. Detached cells were harvested from the supernatant by centrifugation and added to the nonattached cells harvested by trypsinization. The cells were washed with PBS, fixed in 70% ice-cold ethanol, centrifuged, and washed with PBS, and 10^6 cells/well in microtiter plates, allowed to attach, and infected with adenovirus at different MOIs. At 24 h after infection, the cells were exposed to CD95L or cancer chemotherapy drugs for 72 h in serum-free medium. For radiosensitivity assays, 10^5 cells were seeded on 35-mm dishes, allowed to attach, and infected with adenovirus at 50–300 MOI. The cells were irradiated at 3 Gy at 24 h after virus infection and allowed to grow for 10–15 doubling periods. Viable colonies were stained by crystal violet and counted.

**Electron Microscopy.** Glioma cells treated as indicated were fixed in 2.5% glutaraldehyde (Pausel-Lorei, Frankfurt, Germany) in Hank’s modified salt solution, postfixed in 1% OsO4 in 0.1 M cacodylate buffer, scraped off, and dehydrated in a series of ethanol. The 70% ethanol step was saturated with uranyl acetate for contrast enhancement. Dehydration was completed in propylene oxide, and the specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultrathin sections were produced on a FCR Reichert Ultracut ultramicrotome (Leica, Bensheim, Germany), mounted on pioloform-coated copper grids, and contrasted with lead citrate. Specimens were analyzed and documented with an EM 10A electron microscope (Zeiss, Oberkochen, Germany).

**DEVD-ame Cleaving Caspase Activity Assay.** The cells (10^6) were seeded on microtiter plates, allowed to attach, and infected with adenovirus. At 24–48 h after infection, the cells were lysed in 25 mM Tris-HCl (pH 8.0), 60 mM NaCl, 2.5 mM EDTA, and 0.25% NP40 for 10 min, and DEVD-ame was added at 12.5 μM. Caspase activity was assessed by fluorescence using a Millipore FLUORO 360 nm excitation and 480 nm emission wavelengths as described previously (20).

**Statistical Analysis.** In general, all data are from experiments performed three times with similar results. To assess synergy, the fractional product method of Webb (21) was used. Here, the (additive) effect of two independently acting agents is defined as the product of the unaffected survival fractions after treatment with either agent alone: fu(1,2) = fu(1) × fu(2). This formula allows us to calculate the predicted (additive) effect of cotreatment, based on the assumption that two agents do not interact or cooperate in inducing their effects. If the unaffected fraction, that is, the relative percentage of surviving cells compared with untreated control cells, is below the calculated product fu(1,2) after cotreatment with two drugs, then synergy is assumed.

## RESULTS

**Modulation of Glioma Cell Proliferation and Viability by Ad-p53 and Ad-CTS1.** Four human glioma cell lines with different p53 status were selected for this study (16, 22). U87MG is p53 wild type. LN-229 is heterozygous for p53 and exhibits transcriptional activity in a reporter assay (23) and p53 accumulation in response to genotoxic stress (18). LN-18 is mutant for p53. LN-308 cells express no p53 protein (deletion mutant). To assess the susceptibility of the glioma cells to adenoviral infection, the degree of infection at different MOIs of adenovirus was measured by green fluorescent protein fluorescence using Ad-EGFP. The degree of infection was assessed 48 h after infection by staining nuclei with 4',6-diamidino-2-phenylindole and monitoring EGFP protein levels by fluorescence microscopy. Approximately 30% of the cells were infected at 30 MOI. All cells were EGFP-positive at 100 MOI. Using Ad-EGFP, cellular morphology was unaltered at MOIs as high as 3000. Similar results were obtained in all four glioma cell lines (data not shown). The growth of all cell lines was inhibited by Ad-CTS1 in a time- and concentration-dependent manner (Fig. 1, A and B). Compared with Ad-p53, there were ranges of MOI where Ad-CTS more effectively inhibited proliferation in all cell lines (P < 0.05, t test). The differences between Ad-p53 and Ad-CTS1 were more prominent in U87MG and LN-18 cells than in LN-229 and LN-308 cells. Light microscopic monitoring indicated that Ad-CTS1 did not simply arrest proliferation of the cell lines but induced cell death. To confirm actual induction of cell death, we analyzed the glioma cells for trypan blue dye exclusion in parallel. There was a strong time- and concentration-dependent increase in trypan blue-positive LN-229 cells in cultures exposed to Ad-CTS1 compared with cultures exposed to Ad-LacZ (Fig. 1C). As expected, within the respective range of MOI, Ad-CTS1 treatment resulted in a higher percentage of trypan blue-positive cells than Ad-p53 in all cell lines (data not shown). To further characterize the mode of Ad-CTS1-induced cell death, we monitored changes in cell cycle distribution in response to CTS1 or p53 gene transfer in all four cell lines. Interestingly, although both G1/G0 and G2/M arrest have been associated with wild-type p53 activation, no such change was observed in either of the four cell lines infected with Ad-CTS1. In contrast, cell cycle analysis revealed a progressive increase in sub-G1 cells, corresponding to dying or dead cells, but no specific cell cycle change at any time point.
after Ad-CTS1 infection. Representative data are shown in Fig. 1D and Table 1. Note that the inhibition of growth detected in Fig. 1A precedes the detection of cell death in Fig. 1D.

Electron Microscopic Characterization of CTS1-induced Cell Death. U87MG and LN-18 cells were infected with 30, 100, or 300 MOI for 24 or 72 h and then analyzed for morphological changes by electron microscopy. Here, we show and describe the findings obtained in U87MG cells. Similar results were obtained in LN-18 cells (data not shown). Untreated U87MG cells (Fig. 2A) are characterized by a high content of electron-light mitochondria that are frequently associated with cisterns of the rough endoplasmic reticulum. In addition, many free ribosomes are located in the cytoplasm. The nuclei are...
GLIOMA CELL KILLING BY CTS1, A SYNTHETIC P53 HOMOLOGUE

Table 1  Modulation of cell cycle distribution by Ad-p53 and Ad-CTS1

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* The data correspond to the curves depicted in Fig. 1D.

oval shaped, often showing a remarkably large nucleolus. The heterochromatin is located mainly beneath the inner nuclear membrane. The cells are elongated, and their surface is moderately increased by slender cytoplasmic evaginations. After transfection with Ad-EGFP (Fig. 2B), classical apoptosis or other forms of cell death were not observed. Ad-CTS1 induced a striking variability of changes in structure and viability in the glioma cells, ranging from virtually unaltered cells to necrotic debris. Many cells rounded, lost their flat-shaped character and cell-cell contacts. The nucleus accumulated condensed chromatin beneath the nuclear envelope, signaling early apoptotic events (Fig. 2C). Moreover, the cells contained different organelles, including dense lamellar inclusions, lipid droplets, or polymorphous cytosomes. Rarely were 50-nm vesicles observed, which resembled the structures described as a characteristic feature of cells in early apoptosis (stage 1; Ref. 24). Other cells were characterized by surface blebbing and by an increased accumulation of polymorphous organelles. Sometimes, an invagination of the nuclear envelope was observed (Fig. 2D). A stage 2 apoptotic cell (24) is shown in Fig. 2E: the hypercondensation of the chromatin has advanced, and the cytoplasm filled with masses of vesicular and vacuolar organelles seems to signify incipient disruption of cellular integrity. Cells in the final stage of apoptosis also were found (Fig. 2F); membranes, including the nuclear envelope, were interrupted; or the perinuclear space was swollen, and the chromatin was extremely condensed beneath the nuclear membranes.

Modulation of p53 Target Gene Expression by Ad-CTS1. To assess which classical p53 target genes were induced by CTS1 and which of these changes might be responsible for the CTS1-induced phenotype shown in Figs. 1 and 2, the glioma cell lines were infected with Ad-CTS1 or with Ad-EGFP as a control and assessed for changes in target protein expression. CTS1 transgene expression was verified by VP16 labeling (Fig. 3A). Irrespective of the p53 status of the cell lines, endogenous p53 levels decreased in response to Ad-CTS1 gene transfer, except in LN-308 cells, which express no endogenous p53 protein (Fig. 3B). This loss of endogenous p53 levels was probably the result of enhanced MDM-2 expression induced by CTS1 because MDM-2 targets wild-type p53 in LN-229 and U87MG cells as well as the mutant form of p53 in LN-18 for degradation (Fig. 3C). Mutant p53 in LN-18 cells may be targeted for degradation in these cells because the mutation p53C238S does not affect the MDM-2 binding site of p53 (22, 25). Furthermore, the levels of the growth arrest-associated cyclin-dependent kinase inhibitor, p21, were uniformly and strongly increased by Ad-CTS1 in all cell lines (Fig. 3D). To assess the modulation of BCL-2 family protein expression by CTS1, we examined changes in the expression levels of two proapoptotic BCL-2 family proteins known to be positively regulated by p53, BAX and BAK (26, 27), and two antiapoptotic proteins, BCL-2 and BCL-XL. Representative experiments are shown in Fig. 3, E–H. There was no significant change in the levels of pro- or antiapoptotic BCL-2 family proteins in response to infection with Ad-CTS1. Equal protein loading was verified by immunoblot analysis for actin (Fig. 3J). The failure of Ad-CTS1 to induce BAX expression in the glioma cell lines corresponded to the failure of Ad-p53 to induce BAX expression (Fig. 3L), whereas p21 was also strongly induced by Ad-p53 in all cell lines (Fig. 3K).

The Role of the CD95/CD95L System, Caspases, and Mitochondrial Cytochrome c Release in CTS1-induced Death of Glioma Cells. CTS1-mediated changes in CD95 and CD95L expression were monitored by flow cytometry. CTS1 led to a strong increase in CD95 expression in all cell lines. In contrast, CD95L expression was unaffected by CTS1 gene transfer. Representative flow cytometry profiles are shown in Fig. 4A. The SFI values for CD95 with Ad-LacZ and Ad-CTS1 were 1.76 and 4.1 in U87MG cells, 1.14 and 2.2 in LN-229 cells, 0.99 and 5.2 in LN-18 cells, and 0.99 and 1.91 in LN-308 cells. CD95-mediated apoptosis of human glioma cells involves activation of caspases 3, 8, and 9 and mitochondrial cytochrome c release and is blocked by pseudosubstrate caspase inhibitors (19, 28). To assess whether the enhanced expression of CD95 and hence endogenous death ligand/receptor interactions were involved in CTS1-induced cell death, we screened for these events in Ad-CTS1-infected glioma cells, using exogenous CD95L as a positive control. In contrast to CD95L, Ad-CTS1 induced neither caspase 3 nor caspase 8 nor caspase 9 processing in either cell line (Fig. 4B), nor did it promote mitochondria...
for actin (in CTS1-infected LN-308 cells). Equal loading was ascertained by immunoblot analysis of p53 that has been deleted in CTS1. This explains why CTS1 is not labeled in BCL-XL (48 h and analyzed for BAX and p21 levels as described for Ed). Caspases was detected in a DEVD-amc cleavage assay after infection of p53, BCL-2, BAX, BAK, MDM-2, p21, or CGG transactivator domain (12).

Because in the CTS1 protein, the transactivator region of p53 is replaced by the VP16 transactivator domain of VP16 (12). A, the glioma cell lines were also examined for the expression levels of endogenous p53 (B), MDM-2 (C), p21 (D), BAX (E), BAK (F), BCL-2 (G), or BCL-X (H) proteins. Note that we chose a p53 antibody recognizing the COOH-terminus of p53 that has been deleted in CTS1. This explains why CTS1 is not labeled in B (e.g., in CTS1-infected LN-308 cells). Equal loading was ascertained by immunoblot analysis for actin (J). K and L, the glioma cell lines were infected with Ad-EGFP or Ad-p53 for 48 h and analyzed for BAX and p21 levels as described for E and D.

drial cytochrome c release in LN-18 and LN-229 cells (Fig. 4C), nor did zVAD-fmk or zLETD-fmk block CTS1-mediated cell death (Fig. 4, D and E), except for a minor effect of zVAD-fmk at a high MOI of Ad-CTS1 in U87MG and LN-229 cells. Finally, no activation of caspasas was detected in a DEVD-amc cleavage assay after infection of LN-18 cells with Ad-CTS1 or Ad-EGFP at 16 h postinfection, when only low transgene expression was detected, or at 36 h, when transgene expression was high (data not shown), and the cells were killed (Fig. 4F). As a positive control, the cells were treated with CD95L.

**CTS1 Gene Therapy in Human Glioma Cells: Synergy with Irradiation, Cancer Chemotherapy, and Cytotoxic Cytokines.** Next we examined whether CTS1 inhibited glioma cell proliferation and viability in synergy with other proapoptotic agents. The cells were infected with Ad-EGFP or Ad-CTS1 at various MOIs and then irradiated or treated with cancer chemotherapy drugs or CD95L. There was strong synergistic inhibition of glioma cell growth by irradiation (3 Gy) and Ad-CTS1 in all cell lines (Fig. 5A). For the combination with cytotoxic drugs, we selected three drugs with different modes of action, lomustine, teniposide, and vincristine (Fig. 5, B–D). No interaction between Ad-CTS1 and lomustine became apparent (Fig. 5B). Cotreatment with Ad-CTS1 and teniposide (VM26) resulted in synergistic growth inhibition in U87MG and LN-229 cells, but not in LN-18 or LN-308 cells. In fact, there was antagonism of growth inhibition of Ad-CTS1 and teniposide in LN-18 cells (Fig. 5C). Cotreatment with Ad-CTS1 and vincristine was moderately synergistic in U87MG cells but antagonistic in all other cell lines (Fig. 5D). Given the striking induction of CD95 expression in response to CTS1 gene transfer (Fig. 5A), we also examined whether Ad-CTS1 sensitized glioma cells to CD95L-induced apoptosis. This was indeed the case. All cell lines except LN-18 cells were strongly sensitized to CD95L-induced apoptosis by CTS1 gene transfer (Fig. 5E). For comparison of Ad-CTS1 and Ad-p53, we performed similar combination experiments corresponding to those shown in Fig. 5 with Ad-p53. We used Ad-p53 at (with regard to Ad-CTS1) concentrations and combined these with the concentrations of drugs or doses of irradiation used in combination with Ad-CTS1 in Fig. 5. These experiments showed major differences between Ad-p53 and Ad-CTS1: for Ad-p53, most combinations were negative in that there was neither synergy nor antagonism, with the exception of the combination of Ad-p53 and vincristine in LN-229 cells, which, like that with Ad-CTS1, yielded antagonistic effects, and the combination of Ad-p53 and CCNU in LN-308 cells, which yielded antagonistic effects not observed previously with Ad-CTS1. Moderate synergy was observed when Ad-CTS1 was combined with irradiation in U87MG and LN-308 cells, but not in LN-18 or LN-229 cells (data not shown).

**Ad-CTS1 Inhibits the Growth of Nonglial Cells in a Cell Type-specific Manner.** Because Ad-CTS1 was uniformly active against the human glioma cell lines examined here, we also assessed its effects on various other cell lines. First, we assessed whether nonglial cells could be infected by Ad-CTS1 (Fig. 6A), using VP16 immunoreactivity as described for Fig. 3A. Transgene expression was high in DAOY, SV-FHAS, HCT116, and MRC5 cells but low in SKN-LE, SKN-BE, and MCF-7 cells. Next, we examined the effects of Ad-CTS1 on the growth of these cells in a time- and concentration (MOI)-dependent manner (Fig. 6, B and C). The two human neuroblastoma cell lines, SKN-LE and SKN-BE, were refractory to Ad-CTS1, whereas the human medulloblastoma cell line, DAOY, was highly sensitive. The differential response to Ad-CTS1 among these three cell lines was probably the result of lower CTS1 transgene expression in the neuroblastoma cells, given that the rate of infection as assessed by EGFP fluorescence was comparable (data not shown). SV-FHAS, an immortalized nonneoplastic astrocytic cell line, was highly sensitive to CTS1, suggesting that the CTS1 effect is not specific for the neoplastic phenotype of glioma cells. MFC-7 breast cancer and MRC5 fibroblast cells were rather resistant to CTS1, whereas HCT116 colon carcinoma cells were rather sensitive to CTS1. Eventually, we treated primary hippocampal neurons, as an in vitro
Fig. 4. CTS1-induced cell death does not involve death ligand-receptor interactions, caspase activation, or mitochondrial cytochrome c release. A, glioma cells were infected with Ad-LacZ or Ad-CTS1 at 300 MOI. Cell surface expression of CD95L or CD95 was assessed by flow cytometry 48 h later (gray profiles, isotype controls; open profiles, specific antibody). The SFI is provided in the top right corner of each panel. B, U87MG, LN-229, LN-18, and LN-308 cells were infected with Ad-EGFP or Ad-CTS1 at 300 MOI for 48 h and examined for activated cleavage products of caspases 8, 3, or 9. Caspase activation is visualized by the detection of p28 and p18/caspase 8, p17/caspase 3, and p35/caspase 9 in
indicator of possible neurotoxicity, with Ad-EGFP or Ad-CTS1. Ad-CTS1 was devoid of toxicity at concentrations of up to 1000 MOI. However, there was prominent loss of neurites and neuronal cells as confirmed by light microscopy at 3000 MOI of Ad-CTS1 but not at 3000 MOI of Ad-EGFP (Fig. 7A). In contrast, prolonged exposure to Ad-CTS1 at 300 MOI was not toxic (Fig. 7B).

**DISCUSSION**

CTS1 is a synthetic p53-based gene designed to overcome some of the mechanisms that interfere with the efficacy of p53 gene therapy for human cancers (12). Here, we show that an adenoviral vector encoding CTS1 (Ad-CTS1) induces growth arrest and cell death in all of four human malignant glioma cell lines. Compared with an adenovirus encoding wild-type p53, Ad-CTS1 was more effective (e.g., U87MG and LN-18 cells underwent growth arrest but not cell death in response to Ad-p53 but readily died in response to Ad-CTS1; Fig. 1). CTS1-induced cell death exhibited some changes consistent with an apoptotic mode of cell death (Fig. 2). Moreover, there was complex cytoplasmic and organelle pathology in glioma cells induced to die by Ad-CTS1. CTS1-induced growth arrest and cell death were not associated with a specific pattern of cell cycle arrest (Fig. 1D; Table 1). However, classical p53 response genes such as MDM-2 or p21 were uniformly induced by Ad-CTS1 (Fig. 3).

Fig. 5. Synergy of Ad-CTS1 and irradiation, cancer chemotherapy, and cytotoxic cytokines. The glioma cells were treated with Ad-CTS1 alone (light gray bars), Ad-LacZ plus a second proapoptotic stimulus (dark gray bars), or Ad-CTS1 plus this second proapoptotic stimulus (■). The third bar (○) indicates the predicted effect of cotreatment according to the fractional product method (for details, see “Materials and Methods”). Ad-CTS1 was combined with irradiation in a clonogenic survival assay (A) or with lomustine (CCNU; B), temposide (VM26; C), vincristine (VCR; D) or CD95L (E) in a 72-h cytotoxic cell death assay. Data are expressed as mean percentage of survival and SE [n = 3; *, P < 0.05; **, P < 0.01 (t test for synergy, observed effect compared with predicted effect)].

CD95L-treated LN-18 glioma cells. C, for the detection of cytochrome c release, LN-18 or LN-229 cells were infected with 300 MOI Ad-EGFP or Ad-CTS1. Cytoplasmic cytochrome c was examined by immunoblot analysis 16 h after infection. CD95L was used as a positive control. D, the cells were untreated (○), treated with zVAD-fmk (100 µM; ■) and infected with Ad-LacZ or Ad-CTS1 at different MOIs for 96 h. E, LN-18 cells were untreated (○), treated with zIETD-fmk (100 µM; ■) and infected with adenovirus, or treated with CD95L for 72 h. Survival in D and E was assessed by crystal violet staining [n = 3; SE < 10%; *, P < 0.05 (t test, effect of caspase inhibitors versus control conditions)]. F, LN-18 cells were infected with Ad-EGFP or Ad-CTS1 or treated with CD95L. DEVD-amc cleavage was measured 16 h after adenoviral infection or 6 h after the addition of CD95L (left panel). Survival was assessed at 48 h by crystal violet staining (right panel).
BCL-2 family protein expression was not consistently altered by Ad-CTS1, and BAX was not induced (Fig. 3). Although we were not able to ascertain that new protein synthesis is required for CTS1-induced apoptosis, genes other than Bax that have been hypothesized to mediate p53-induced apoptosis are also candidate mediators of CTS1-induced apoptosis include p53-apoptosis effector related to PMP-22 (PERP; Ref. 29), p53-inducible gene (PIG; Ref. 30), Noxa (31), and p53-regulated apoptosis-inducing protein 1 (p53AIP; Ref. 32).

CTS1-induced cell death is also an unusual type of apoptotic cell death at a biochemical level because we failed to confirm a role for endogenous death receptor-ligand interactions, caspases, or mitochondrial cytochrome c release in the CTS1-dependent killing cascade (Fig. 4). The CTS1-induced increase in CD95 expression, which corresponds to our previous observations on p53-induced changes in CD95 expression (27), is thus epiphenomenal. These data are consistent with the apparent lack of regulation of BCL-2 family protein expression because these proteins regulate cell death at the level of caspase activation and mitochondrial cytochrome c release. Additional studies are needed to determine whether other BCL-2 family members, other non-caspase proteases, or other mitochondrial factors such as apoptosis-inducing factor mediate CTS1-induced cell death.

Adenoviral p53 gene transfer has been reported to induce apoptosis in p53 mutant glioma cell lines but not in cell lines retaining wild-type p53 activity (7, 8). Our data indicate that the type of p53 alterations may determine the response to Ad-p53 infection in glioma cell lines. We confirm that U87MG cells, which are wild type, resist Ad-p53-induced cell death. In contrast, heterozygous LN-229 cells, which retain p53 activity in a reporter assay, were nevertheless susceptible to Ad-p53-induced cell death. Furthermore, among the p53 mutant cell lines, although LN-308 cells were almost as susceptible to Ad-p53 as to Ad-CTS, LN-18 cells were much more resistant to Ad-p53 than to Ad-CTS1. In any case, Ad-CTS1 induced cell death irrespective of the endogenous p53 status of the cell lines. These data suggest that CTS1 gene therapy may be superior to wild-type p53 gene therapy, notably in view of the fact that less than 50% of all human glioblastomas exhibit p53 mutations (33).

In regard to a possible clinical application of Ad-CTS1, we were also interested in examining whether the anti-glioma activity of Ad-CTS1 could be synergistically enhanced by combination with other strategies (Fig. 5). Most intriguingly, we found that irradiation, the most important treatment modality for malignant glioma, and Ad-CTS1 acted in synergy in all cell lines. Enhancement of radiation lethality in glioma cell lines has also been observed after p53 gene transfer (34, 35), but Ad-CTS showed synergy with irradiation in all cell lines, as opposed to two of four cell lines with Ad-p53, in this study. In contrast, lomustine, representing nitrosoureas, the drugs used most frequently in the adjuvant chemotherapy setting in human malignant glioma patients, showed little interaction with the effects of Ad-CTS1. For two other drugs used in the treatment of gliomas, teniposide and vincristine, the interactions varied among the cell lines, with robust antagonistic effects of vincristine in three of four cell lines. Interestingly, death ligand-induced apoptosis, one of the most promising experimental strategies to treat malignant gliomas (36–38), was synergistically enhanced by Ad-CTS1 in three of four cell lines (Fig. 5). LN-18 cells, which failed to show this synergy, are most sensitive to death ligands (39), indicating that CTS1 may overcome some pathway of resistance to death ligand-induced apoptosis that is constitutively disabled in this cell line.

A screening of additional cell lines for sensitivity to CTS1-induced cell death revealed significant variability (Fig. 6). Although stronger CTS1 transgene expression may explain the high sensitivity of DAOY and SV-FHAS cells compared with the neuroblastoma cell lines (SKN-LE and SKN-BE), MRC5 cells were resistant to cell death despite strong transgene expression. Importantly, primary hippocampal neurons were significantly more resistant to Ad-CTS1 gene transfer than the glioma cells, as assessed by the MOI required to cause toxicity. However, neurons were not fully resistant to Ad-CTS1 cytotoxicity. Thus, primary rat neurons were killed by Ad-CTS at 3000 MOI, whereas no killing was seen at a MOI of 300 (Fig. 7). At
Ad-CTS1 gene therapy in animal models of glioma are required before a possible evaluation of Ad-CTS1 in human malignant glioma patients. It is important to note that, considering the poor therapeutic effect of radiotherapy or chemotherapy in glioblastoma and their narrow therapeutic window (doses in excess of 54 Gy are necessary, but these doses, when applied to normal brain, carry a significant risk of neurotoxicity on long-term survival), the therapeutic window and efficacy of CTS1 may well turn out to be superior to current approaches of glioblastoma therapy.

Fig. 7. Effects of Ad-CTS1 on primary hippocampal neurons. Primary rat hippocampal neurons were seeded at $1.5 \times 10^4$ cells/35-mm dish and infected with variable amounts of Ad-EGFP or Ad-CTS1 for 72 h (A) or at 300 MOI for different lengths of time (B).

300 MOI, however, all glioma cell lines were strongly inhibited on prolonged exposure.

Cultured neurons are postmitotic and very resistant to irradiation within the time frame of culturing, and the neurotoxic effects of irradiation in vivo are more likely to be mediated by damage to vasculature and myelin-forming oligodendroglial cells than by direct toxic effects on neurons. Therefore, the possibility remains that Ad-CTS and irradiation not only synergize against glioma cells but also synergistically induce neurotoxicity in vivo. Thus, careful studies of

REFERENCES

GLIOMA CELL KILLING BY CTS1, A SYNTHETIC P53 HOMOLOGUE


Chimeric Tumor Suppressor 1, a p53-derived Chimeric Tumor Suppressor Gene, Kills p53 Mutant and p53 Wild-type Glioma Cells in Synergy with Irradiation and CD95 Ligand

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