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Treatment of Malignant Glioma Cells with the Transfer of Constitutively Active Caspase-6 Using the Human Telomerase Catalytic Subunit (Human Telomerase Reverse Transcriptase) Gene Promoter[1]

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ABSTRACT

Because the apoptotic pathway is often disrupted in tumor cells, its genetic restoration is a very attractive approach for the treatment of tumors. To treat malignant gliomas with this approach, it would be preferred to restrict induction of apoptosis to tumor cells by establishing a tumor-specific expression system. Telomerase is an attractive target because the vast majority of malignant gliomas have telomerase activity whereas normal brain cells do not. Activation of telomerase is tightly regulated at the transcriptional level of the telomerase catalytic subunit [human telomerase reverse transcriptase, (hTERT)]. Therefore, we hypothesized that using a hTERT promoter-driven vector system, an apoptosis-inducible gene may be preferentially restricted to telomerase- or hTERT-positive tumor cells. In this study, we constructed an expression vector consisting of the constitutively active caspase-6 (rev-caspase-6) under the hTERT promoter (hTERT/rev-caspase-6) and then investigated its antitumor effect on malignant glioma cells. The rationale for using the *rev-caspase-6* gene is because it induces apoptosis independent of the initiator caspases. We demonstrated that the hTERT/rev-caspase-6 construct induced apoptosis in hTERT-positive malignant glioma cells, but not in hTERT-negative astrocytes, fibroblasts, and alternative lengthening of telomeres cells. In addition, the growth of s.c. tumors in nude mice was significantly suppressed by the treatment with hTERT/rev-caspase-6 construct. The present results strongly suggest that the telomerase-specific transfer of the *rev-caspase-6* gene under the hTERT promoter is a novel targeting approach for the treatment of malignant gliomas.

INTRODUCTION

Malignant gliomas are the most common tumors in the central nervous system. When treated with conventional therapy such as surgery, γ-irradiation, or chemotherapy, the average life expectancy is usually less than 1 year (1, 2). Clearly, novel therapeutic strategies are necessary.

Generally, malignancy results not only from unregulated cell proliferation but also from decreased sensitivity to physiological programmed cell death (apoptosis) signals (3). Apoptosis is a process in which cells actively participate in their own death (4); however, this pathway is often disrupted in tumor cells (5). Therefore, a genetic restoration of the apoptotic pathway or introduction of proapoptotic molecules is very attractive for the treatment of tumors including malignant gliomas.

Caspases play a major role in the transduction of apoptotic signals and the execution of apoptosis in mammalian cells (6, 7). Currently, the caspase family consists of more than 12 members (8), and implementation of the apoptotic program requires the participation of at least two classes of caspases, the initiators such as caspase-2, -8, -9, or -10 and the executioners including caspase-3, -6, or -7 (8, 9). Rev-caspases-3 and -6[3] have been described recently (10). This was achieved by making contiguous precursor caspases-3 and -6 molecules that have their small subunits preceding their large subunits. Unlike their wild-type counterparts, these recombinant molecules are capable of autocatalytic processing and inducing apoptosis independent of the upstream, initiator caspases. Because caspases-3 and -6 are the most downstream executioners of apoptosis, the constitutively active versions of these caspases could be used at very low concentrations to induce apoptosis in target tumors (10). We have demonstrated recently (11, 12) that the introduction of caspase genes inhibited the growth of malignant glioma cells *in vitro* and *in vivo* through induction of apoptosis, indicating the potential therapeutic usefulness of this approach for malignant gliomas. However, if caspases are transduced to normal brain cells, they would be predicted to also undergo apoptosis, resulting in undesirable brain damage. To restrict induction of apoptosis to tumor cells and increase the safety of this approach, we needed to establish a tumor-specific caspase expression system.

Utilization of a promoter that is predominantly active in tumor cells would be an ideal system to restrict the cytotoxic caspase expression. Telomerase, a ribonucleoprotein enzyme, is a particularly attractive target for specifying tumors, because approximately 90% of tumors have telomerase activity, whereas most normal cells do not express telomerase (13). In malignant gliomas, telomerase activity is detected in 10 to 45% of anaplastic astrocytoma (WHO grade III) and in 75 to 89% of glioblastoma multiforme (grade IV; Refs. 14, 15). In contrast, normal brain tissues do not express telomerase activity (15–17). Therefore, telomerase is expected to represent a very good candidate for the targeted therapy of malignant gliomas. The main components of the human telomerase enzyme are the functional or template RNA component (hTER; Ref. 18) and the telomerase catalytic protein subunit or hTERT (19, 20). Although hTER and hTERT are both necessary for telomerase activity, the expression of hTERT is present specifically in tumor cells whereas hTER is present in both normal

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[3] The abbreviations used are: rev-caspase, constitutively active recombinant caspase; hTERT, human telomerase reverse transcriptase; hTER, human telomerase RNA component; ALT, alternative lengthening of telomeres; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CMV, cytomegalovirus; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; GFP, green fluorescence protein.

and tumor cells (21). Because telomerase expression is predominantly regulated at the transcriptional level of hTERT, there has been recent interest in cloning and characterizing the promoter region of hTERT (22, 23). The transcriptional activity of the hTERT gene promoter is significantly higher in telomerase-positive cells than in telomerase-negative cells (22, 23). Therefore, we hypothesized that the telomerase-specific transfer of caspases under the hTERT promoter could restrict induction of apoptosis to telomerase-expressing malignant glioma cells and prevent normal brain cells lacking telomerase from apoptosis-induced cell death. In the present study, to test our hypothesis, we constructed the rev-caspase-6 expression vector under the hTERT promoter (hTERT/rev-caspase-6) and investigated its antitumor effect on malignant gliomas *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells. Human malignant glioma U87-MG, A172, T98G, and U373-MG cells and human normal fibroblasts MRC5 were purchased from American Tissue Culture Collection (Rockville, MD). Human malignant glioma GB-1 and U251-MG cells were provided by Dr. Tatsuo Morimura (National Utano Hospital, Kyoto, Japan) and Dr. Akiko Nishiyama (University of Connecticut, Storrs, CT), respectively. ALT cell lines (VA13 and SUSM-1) were used as telomerase-independent cell lines. Cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human astrocytes TEN were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. TEN astrocytes were characterized by the presence of the astrocytic marker glial fibrillary acidic protein in nearly 100% of cells when evaluated under immunofluorescent microscope as described previously (24). All of the malignant glioma cells (U87-MG, U251-MG, U373-MG, A172, GB-1, and T98G) were telomerase-positive, whereas TEN, MRC5, VA13, and SUSM-1 cells were telomerase-negative (25–27).

RT-PCR Analysis for hTERT. The expression of hTERT mRNA was analyzed by semiquantitative RT-PCR amplification as described recently (28). The correlation between band intensity and dose of cDNA templates was linear under the conditions described below. RNA of each cell line was isolated using the RNA Isolation Kit (Stratagene, La Jolla, CA). RT-PCR was performed with total RNA (0.1 μg) using the ProSTAR First Strand RT-PCR Kit (Stratagene). The thermal cycles were: 94°C for 1 min, 60°C for 2 min, and 70°C for 2 min for 35 cycles for GAPDH (450 bp), 94°C for 1 min, 58°C for 1 min, 72°C for 1 min for 37 cycles for hTERT (145 bp). The primer sets used were as follows: GAPDH (20), 5′-CTCAGACACCATGGGGAAGGTGA-3′ (forward) and 5′-ATGATCTTGAGGCTGTTGTCATA-3′ (reverse); hTERT (20), 5′-CGGAAGAGTGTCTGGAGCAA-3′ (forward) and 5′-GGATGAAGCGGAGTCTGGA-3′ (reverse). The amplified products were fractionated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide; gels were photographed with Polaroid film (Polaroid type 667), and photographs were quantitatively scanned using the NIH image software. The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers.

Luciferase Assay. The transcriptional activity of the hTERT promoter in malignant glioma cells, astrocytes, fibroblasts, and ALT cells was determined by the luciferase reporter plasmids as described previously (22, 25). Cells were plated at a density of 1.0×10^5 cells/ml 1 day before transfection of the luciferase reporter plasmids (1 μg each). Forty-eight h after transfection, cells were washed twice with PBS and lysed in the lysis buffer provided with the luciferase kit (Promega CORP-BTC, Madison, WI). Transcriptional activity was measured using a Microtiter Plate Luminometer (Dynatech Laboratories, Inc., Chantilly, VA). To ensure that the luciferase assay was within the linear range, the standard curve of light units *versus* relative enzyme concentration was obtained by making serial dilutions of luciferase (QuantiLum Recombinant Luciferase; Promega CORP-BTC) in 1× lysis buffer with 1 mg/ml BSA. The following plasmids were used: the SV40 enhancer/promoter (pGL3-control) for a positive control, the hTERT promoter plasmids (pGL3–378), and the negative control without promoter (pGL3-basic) as described previously (22). The luciferase activity of pGL3-control plasmid in each cell line was considered as 100%.

Construction of the hTERT Promoter Plasmids Carrying Rev-Caspase-6. To construct the rev-caspase-6 expression vector under the hTERT promoter, the hTERT promoter-luciferase (pGL3-378) construct was used as described previously (22). The CMV promoter-expression vector containing the full-length rev-caspase-6 (pRSC-Rev-caspase-6 or CMV/rev-caspase-6) reported previously (10) was used as a template. The 960-bp fragment of rev-caspase-6 was generated by PCR amplification with 5′-TGGCGCCTATGGCTAGCATGA-3′ and 5′-GCTCTAGATTAATCTACTACAT-3′. The sequence of the PCR product was confirmed using ABI PRISM 377 DNA Sequencer system (Applied Biosystems, Foster City, CA). The PCR-amplified product was then ligated into the *Kas*I-*Xba*I site of pGL3-378 instead of luciferase and designated as the hTERT/rev-caspase-6 expression vector.

Transient Transfection Assay. To determine whether the hTERT/rev-caspase-6 construct induces apoptosis only in hTERT-positive cells, transient transfection assays using LipofectAMINE-mediated gene transfer (Life Technologies, Inc.) were performed as described previously (11, 12). The plasmid-expressing GFP, pEGFP-C1 (Clontech, Palo Alto, CA), was used as a reporter gene-plasmid. The day before transfection, cells were seeded at 5×10^4 cells/ml in Lab-Tek chamber slides. The rev-caspase-6 expression vector under the hTERT promoter (hTERT/rev-caspase-6; 1 μg) or the CMV promoter (CMV/rev-caspase-6; 1 μg) together with pEGFP-C1 (0.3 μg) were transfected into cells and incubated for 48 h. The hTERT/luciferase construct was used as a negative control. To detect the induction of apoptosis, cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 5 min, rinsed three times with PBS, and stained with the TUNEL technique (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit; Intergen, Purchase, NY). Cells were visualized by either bright-field or fluorescence microscopy to detect apoptotic cells or GFP-transfected cells, respectively. An apoptotic index was determined as a percentage of apoptotic cells among 100 GFP-positive cells. For detection of exogenous caspase-6, immunohistochemical staining using antihuman-caspase-6 mouse monoclonal antibody (PharMingen, San Diego, CA) was performed instead of TUNEL staining.

***In Vivo* Effect of Rev-Caspase-6 Expression under the hTERT Promoter.** Human malignant glioma U87-MG or U373-MG cells (1.0×10^6 cells in 0.05 ml of serum-free DMEM and 0.05 ml of Matrigel) were inoculated s.c. into the right flank of 8–12-week-old male BALB/c nude mice (six mice for each treatment group), and the tumor growth was monitored using calipers every other day as described previously (25). When the tumors reached a mean tumor volume of 50–70 mm^3, the treatment was initiated to simulate the clinical situation. The hTERT/rev-caspase-6 (10 μg) and cationic lipid (DMRIE; 2 μg; Life Technologies, Inc.) dissolved in 20 μl of sterile PBS were directly injected into the tumor every 24 h for 7 days. The CMV/rev-caspase-6 or hTERT/luciferase construct mixed with DMRIE was used as a positive and negative control, respectively. Mice were sacrificed by cervical dislocation the day after the final treatment. The tumors were removed and frozen rapidly, and 8.0-μm cryosections were made for histological studies. The consecutive sections from treated tumors were used for the TUNEL technique using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit and caspase-6 immunohistological staining using anti-caspase-6 antibody as described previously (12). All of the experiments were approved by the Institutional Animal Care and Use Committee.

Statistical Analysis. The data were expressed as means ± SD. Statistical analysis was performed by Student's *t* test (two-tailed). The criterion for statistical significance was taken as $P < 0.05$.

RESULTS

hTERT mRNA Expression. The expression of hTERT mRNA in malignant glioma cells, fibroblasts, astrocytes, or ALT cells was determined using RT-PCR. As shown in Fig. 1*A*, all of the six malignant glioma cell lines (U87-MG, U251-MG, U373-MG, A172, GB-1, and T98G) were hTERT mRNA-positive, although the expression levels of hTERT mRNA varied considerably depending on the cell line. When the expression level of hTERT mRNA in U373-MG cells was regarded as 1.0, those in U251-MG and GB-1 cells were

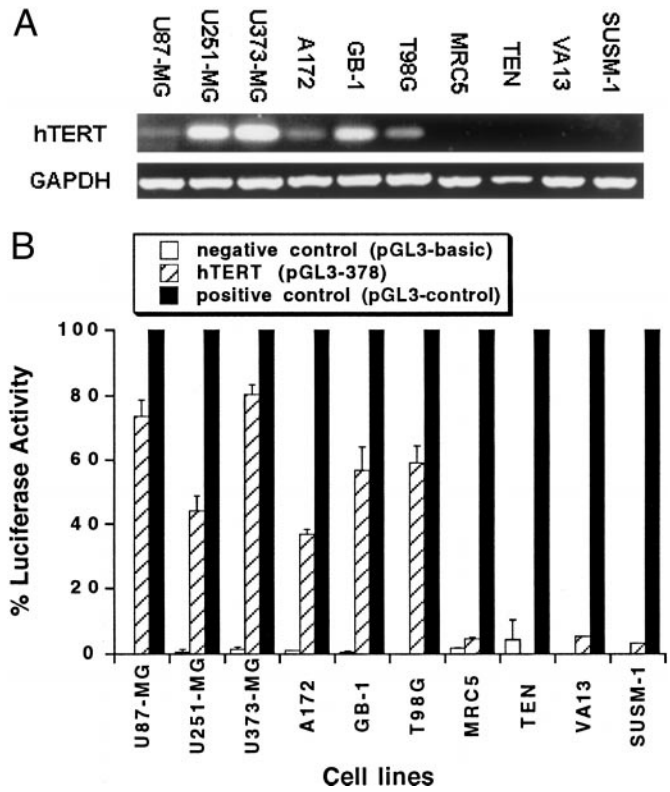

Fig. 1. *A*, expression of hTERT mRNA in malignant glioma cells. As telomerase-positive tumor cells, malignant glioma cells (U87-MG, U251-MG, U373-MG, A172, GB-1, and T98G) were tested. As control, telomerase-negative fibroblasts MRC5, astrocytes TEN, and ALT cells (VA13 and SUSM-1) were used. After the isolation of RNA from each cell line, RT-PCR was performed using primer sets for hTERT mRNA detection. The PCR products were run on 2% agarose gel containing 0.5 μg/ml ethidium bromide. Primer sets for GAPDH were used as a positive control. *B*, transcriptional activity of the hTERT promoter in hTERT-positive or -negative cells. Luciferase activity of reporter plasmids with the hTERT promoter was examined in malignant glioma cells (U87-MG, U251-MG, U373-MG, A172, GB-1, and T98G), fibroblasts (MRC5), astrocytes (TEN), or ALT cells (VA13 and SUSM-1). To standardize the luciferase activity in each cell line, the value was plotted as a percentage of the positive control plasmid (pGL3-control) driven by the SV40 enhancer/promoter in each cell line. pGL3-basic without enhancer/promoter was used as a negative control. The data shown are the mean ± SD from three independent experiments.

1.05 and 0.72, respectively. U87-MG, A172, and T98G expressed moderate levels of hTERT mRNA (0.47, 0.37, and 0.27, respectively). In contrast, hTERT mRNA was not detected in MRC5 fibroblasts, TEN astrocytes, and ALT cells (VA13 and SUSM-1).

Transcriptional Activity of the hTERT Promoter in hTERT-positive or -negative Cells. To confirm that the transcriptional activity of hTERT is observed only in hTERT-positive glioma cells, a transient transfection of luciferase reporter plasmids was performed. The transcriptional activity of a positive control with the SV40 enhancer/promoter in each cell line was considered as 100%. As shown in Fig. 1*B*, the hTERT promoter construct showed significant transcriptional activity in all of the hTERT-positive tumor cell lines, but this did not correlate directly with hTERT mRNA levels (Fig. 1*A*). The relative luciferase activities of six human malignant glioma cell lines by the hTERT promoter were 37 to 80% of positive control (pGL3-control). They were 9 times or greater than the activity of hTERT-negative MRC5, TEN, VA13, and SUSM-1 cells. Interestingly, U87-MG cells with moderate expression of hTERT mRNA among malignant glioma cells had hTERT promoter transcriptional activity as high as U373-MG cells. In contrast, MRC5, TEN, VA13, and SUSM-1 cells showed very low transcriptional activity of the hTERT promoter, and there was no significant difference between the hTERT promoter and pGL3-basic plasmids. These results indicated that the activation of hTERT transcription was significantly up-regulated only in hTERT-positive cells.

***In Vitro* Effect of the hTERT/Rev-Caspase-6 on hTERT-positive or -negative Cells.** To determine whether the hTERT/rev-caspase-6 construct induced apoptosis only in hTERT-positive malignant glioma cells, cells with or without hTERT mRNA were transfected with hTERT/luciferase, hTERT/rev-caspase-6, or CMV/rev-caspase-6 together with the GFP gene (*pEGFP-C1*). Two days after the transfection, the incidence of apoptosis was determined. As shown in Fig. 2*A*, *parts a* and *d*, and Fig. 2*B*, *parts a* and *d*, U87-MG glioma cells and MRC5 fibroblasts transfected with the hTERT/luciferase construct and pEGFP-C1 retained normal morphology of adherent cells and were TUNEL-negative. Next, U87-MG glioma cells that had the hTERT/rev-caspase-6 vector and pEGFP-C1 displayed apoptotic morphology and positive staining for TUNEL (Fig. 2*A*, *parts b* and *e*). In contrast, MRC5 fibroblast cells transfected with the hTERT/rev-caspase-6 and pEGFP-C1 did not undergo apoptosis (Fig. 2*B*, *parts b* and *e*). Both U87-MG and MRC5 cells underwent apoptosis after transfection with the CMV/rev-caspase-6 construct and pEGFP-C1 (Fig. 2*A*, *parts c* and *f*, and Fig. 2*B*, *parts c* and *f*), respectively. The quantitative representation of these data is shown in Fig. 2*C*. Two days after transfection with the hTERT/rev-caspase-6 vector, apoptosis was induced in 21–54% of malignant glioma cells. The incidence of apoptosis by the hTERT promoter system was similar to that by the CMV-promoter, although the transcriptional activity of the hTERT promoter in malignant glioma cells was always lower than that of positive control. Thus, the hTERT promoter is almost as efficient in inducing cell death as the CMV-promoter. This suggests that apoptosis may be induced in tumor cells once the signals for apoptosis reach a certain critical level. In contrast, apoptosis was detected in only 3–5% of MRC5 fibroblasts and TEN astrocytes by the transfer of hTERT/rev-caspase-6. This low level of apoptosis in hTERT-negative MRC5 or TEN cells was not significantly different compared with the hTERT/rev-caspase-6 and the hTERT/luciferase constructs. We then examined the same expression vectors in ALT cells that are immortal but hTERT-negative. As shown in Fig. 2*C*, ALT cells were also resistant to the apoptosis induced by the hTERT/rev-caspase-6. These results indicated that the apoptosis-induction effect of the hTERT/rev-caspase-6 was specific for hTERT-positive cells. To confirm that the apoptosis observed was attributable to the specific activation of caspase-6 activity in the transfected cells, we investigated the expression of caspase-6 after the transfection of hTERT/rev-caspase-6 into U87-MG cells (Fig. 2*D*). Immunohistochemical staining with anti-caspase-6 antibody after 48 h of transfection of either hTERT/rev-caspase-6 (Fig. 2*D*, *part b* and *e*) or CMV/rev-caspase-6 (Fig. 2*D*, *parts c* and *f*) showed high expression of caspase-6 protein in GFP-positive cells as well as morphological changes typical to apoptosis. As shown in Fig. 2*E*, the percentage of apoptotic cells in caspase-6-positive cells treated with hTERT/rev-caspase-6 or CMV/rev-caspase-6 construct was 27% or 45%, respectively. There was a correspondence between apoptotic index and the percentage of caspase-6-positive cells (Fig. 2, *C* and *E*). In contrast, the expression of caspase-6 in hTERT/luciferase-transfected cells (Fig. 2*D*, *parts a* and *d*) remained at basal level, and 2.9% of the caspase-6-positive cells were apoptotic. These results indicated that induction of apoptosis in hTERT-positive tumor cells was correlated with the activated caspase-6 expression.

***In Vivo* Effect of the hTERT/rev-caspase-6 on Malignant Glioma Cells.** To determine the *in vivo* antitumor effect of the hTERT/rev-caspase-6 construct, hTERT-positive malignant glioma cells were inoculated s.c. in nude mice. After the establishment of s.c. tumors, the hTERT/luciferase (negative control), the hTERT/rev-caspase-6, or the CMV/rev-caspase-6 (positive control; 10 μg each) in the presence

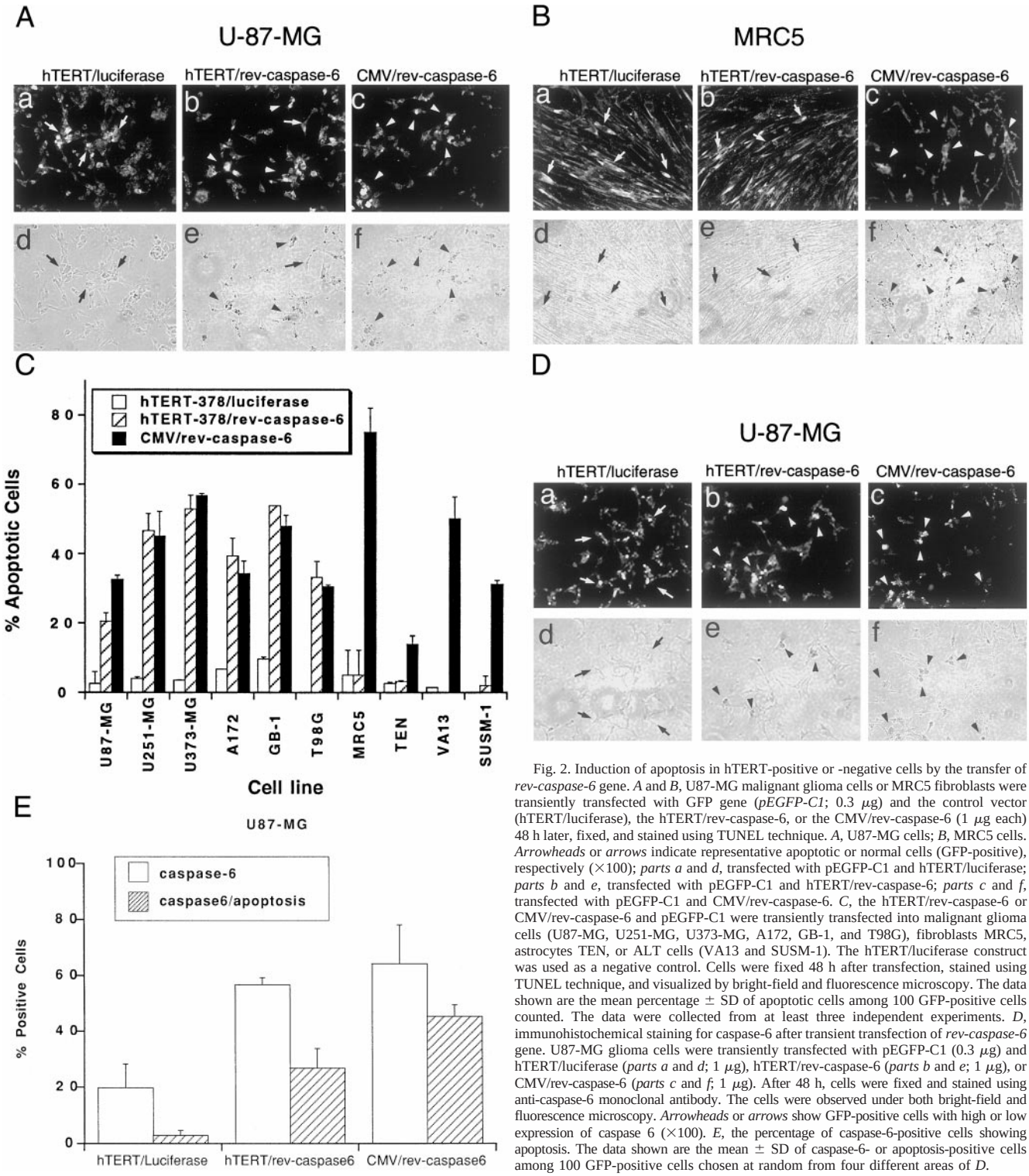

Fig. 2. Induction of apoptosis in hTERT-positive or -negative cells by the transfer of *rev-caspase-6* gene. *A* and *B*, U87-MG malignant glioma cells or MRC5 fibroblasts were transiently transfected with GFP gene (*pEGFP-C1*; 0.3 μg) and the control vector (hTERT/luciferase), the hTERT/rev-caspase-6, or the CMV/rev-caspase-6 (1 μg each) 48 h later, fixed, and stained using TUNEL technique. *A*, U87-MG cells; *B*, MRC5 cells. *Arrowheads* or *arrows* indicate representative apoptotic or normal cells (GFP-positive), respectively (×100); *parts a* and *d*, transfected with pEGFP-C1 and hTERT/luciferase; *parts b* and *e*, transfected with pEGFP-C1 and hTERT/rev-caspase-6; *parts c* and *f*, transfected with pEGFP-C1 and CMV/rev-caspase-6. *C*, the hTERT/rev-caspase-6 or CMV/rev-caspase-6 and pEGFP-C1 were transiently transfected into malignant glioma cells (U87-MG, U251-MG, U373-MG, A172, GB-1, and T98G), fibroblasts MRC5, astrocytes TEN, or ALT cells (VA13 and SUSM-1). The hTERT/luciferase construct was used as a negative control. Cells were fixed 48 h after transfection, stained using TUNEL technique, and visualized by bright-field and fluorescence microscopy. The data shown are the mean percentage ± SD of apoptotic cells among 100 GFP-positive cells counted. The data were collected from at least three independent experiments. *D*, immunohistochemical staining for caspase-6 after transient transfection of *rev-caspase-6* gene. U87-MG glioma cells were transiently transfected with pEGFP-C1 (0.3 μg) and hTERT/luciferase (*parts a* and *d*; 1 μg), hTERT/rev-caspase-6 (*parts b* and *e*; 1 μg), or CMV/rev-caspase-6 (*parts c* and *f*; 1 μg). After 48 h, cells were fixed and stained using anti-caspase-6 monoclonal antibody. The cells were observed under both bright-field and fluorescence microscopy. *Arrowheads* or *arrows* show GFP-positive cells with high or low expression of caspase 6 (×100). *E*, the percentage of caspase-6-positive cells showing apoptosis. The data shown are the mean ± SD of caspase-6- or apoptosis-positive cells among 100 GFP-positive cells chosen at random from four different areas of *D*.

of DMRIE (2 μg) was injected directly into tumors every 24 h for 7 days (days 1 to 7). In this experiment, we used U373-MG cells with high hTERT mRNA expression and U87-MG cells with moderate hTERT mRNA expression. As shown in Fig. 3*A*, treatment with the hTERT/rev-caspase-6 construct significantly suppressed the growth of U373-MG s.c. tumors when compared with the hTERT promoter with the luciferase gene ($P < 0.0005$). As predicted from the *in vitro* experiments, the antitumor effect of hTERT/rev-caspase-6 against U373-MG tumors was not significantly different from that of CMV/rev-caspase-6 ($P = 0.8561$). In the animals treated with the hTERT/rev-caspase-6 construct or CMV/rev-caspase-6, the mean tumor volume on day 8 was reduced by 51% or 52% from the initial tumor size, respectively. In contrast, the mean tumor volume was increased by 39% in control mice treated with the hTERT/luciferase construct. As predicted from the *in vitro* results, the antitumor effect of CMV/rev-caspase-6 on U87-MG tumors was greater than that of hTERT/rev-caspase-6 ($P < 0.005$). However, the treatment with hTERT/rev-caspase-6 also significantly suppressed the tumor growth compared with the hTERT/

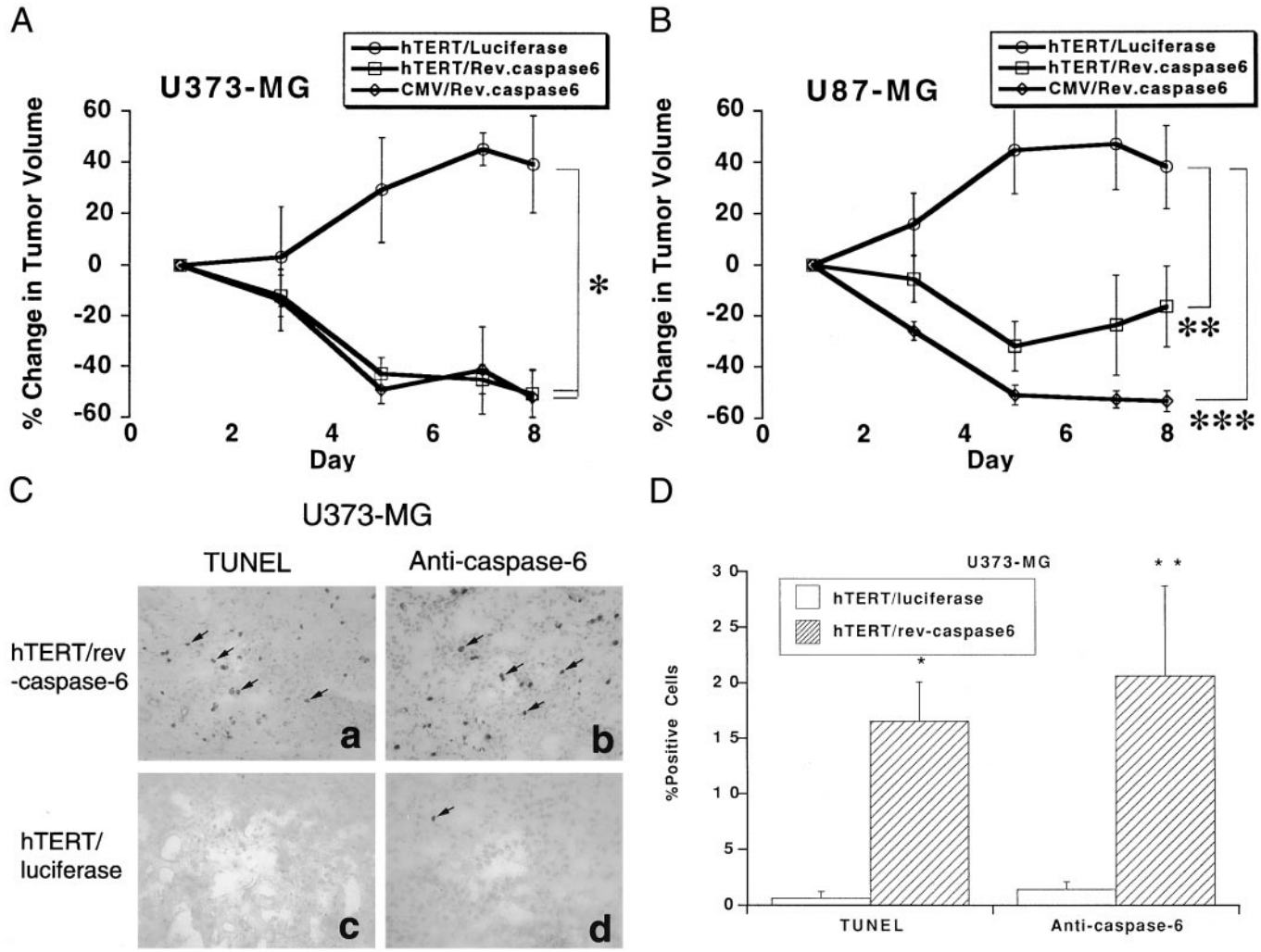

Fig. 3. Effect of *rev-caspase-6* gene transfer under the hTERT promoter on s.c. U373-MG or U87-MG tumors in nude mice. U373-MG or U87-MG tumors were established s.c. in nude mice. When tumors reached a mean volume of 50–70 mm^3, the hTERT/rev-caspase-6, CMV/rev-caspase-6 (positive control), or hTERT/luciferase (negative control) construct (10 μg each) mixed with DMRIE (2 μg) in 20 μl of sterile PBS was directly injected into the tumor every 24 h for 7 days. Six mice were used for each treatment group. *A* and *B*, tumor volume was determined with calipers every other day. Values represent the means ± SD of the percentage of change in tumor volume. *A*, U373-MG tumor; *B*, U87-MG tumor. *, $P < 0.0005$; **, $P < 0.005$; ***, $P < 0.0001$. *C*, *In situ* end-labeling of DNA (TUNEL assay) and caspase-6 expression in U373-MG s.c. tumors. After treatment for 7 days, the mice were sacrificed, and the s.c. masses were removed, frozen, and sectioned on a cryostat. The tumor specimens from U373-MG cells were counterstained by methyl green after the TUNEL staining (*parts a* and *c*; ×150). To detect the expression of caspase-6 protein, immunohistochemical staining was performed (*parts b* and *d*; ×150); *parts a* and *b*, treated with the hTERT/rev-caspase-6 construct; *parts c* and *d*, treated with the hTERT/luciferase construct. The consecutive sections from treated tumors were used for TUNEL and caspase-6 stainings. *Arrows*, representative TUNEL- or caspase-6-positive cells, respectively. *D*, the percentage of TUNEL- or caspase-6-positive cells in U373-MG s.c. tumors. The data shown are the mean ± SD of TUNEL- or caspase-6-positive cells among 100 cells chosen at random from four different areas of *C*. *, $P = 0.0059$; **, $P = 0.0482$.

luciferase treatment ($P < 0.005$). Significant numbers of apoptotic cells were observed in tumors treated with the hTERT/rev-caspase-6 construct (Fig. 3*C*, *part a*), although tumors treated with control vector (hTERT/luciferase) showed almost no apoptotic cells (Fig. 3*C*, *part c*). The percentage of TUNEL-positive cells was 0.6% or 16.5% in hTERT/luciferase- or hTERT/rev-caspase-6-treated tumors ($P = 0.0059$; Fig. 3*D*). The expression of caspase-6 protein was detected throughout the entire tumors treated with the hTERT/rev-caspase-6 construct (Fig. 3*C*, *part b*), whereas few numbers of caspase-6-expressing cells were observed in controls (Fig. 3*C*, *part d*). The percentage of caspase-6-positive cells was 1.4% or 20.6% in hTERT/luciferase- or hTERT/rev-caspase-6-treated tumors ($P = 0.0482$; Fig. 3*D*). These results indicated that the cytotoxic effect was mainly attributable to apoptosis induced by expression of caspase-6 protein. As additional experiments, we treated s.c. U373-MG or U87-MG tumors with hTERT/rev-caspase-6 for 1 week and further examined if the tumors grew back during 3 additional weeks without additional treatment. On day 29, the mean tumor volume of U373-MG tumors treated with hTERT/rev-caspase-6 or CMV/rev-caspase-6 was 34% or 28% of the initial tumor volume. As for U87-MG tumors, the mean tumor volume was 60% or 32% of the initial tumor volume on day 29 after the treatment with hTERT/rev-caspase-6 or CMV/rev-caspase-6, respectively. These results suggested that the effect of hTERT/rev-caspase-6 is more likely to be a robust and durable response rather than a transient response followed by rapid regrowth after the end of treatment.

DISCUSSION

The present study demonstrates that treatment with the hTERT promoter-driven rev-caspase-6 expression vector (hTERT/rev-caspase-6) is an effective and promising tumor-specific gene therapy for telomerase- or hTERT-positive malignant gliomas. There are two gene therapy strategies that are being considered for targeting malignant gliomas. One is a selective cell entry system using tumor-specific molecules or receptors, and the other is transcriptional regulation with

tumor-specific promoter. There have been several reports describing the feasibility of targeting glial fibrillary acidic protein (29) or epidermal growth factor receptor (30), but no distinctive malignant glioma-specific markers or receptors have been found thus far.

Telomerase is thought to be very promising not only as a tumor-specific marker but also as a target for anticancer therapy (31). Studies using antisense oligonucleotides (18, 32, 33), peptide nucleic acids (34), or 2′-*O*-MeRNA oligomers (35) against the hTER or the dominant-negative hTERT (36, 37) demonstrate that inhibition of telomerase effectively leads to cancer cell death. Another novel approach is to use hTERT peptides that bind to human leukocyte antigen-A2.1 to generate a specific CTL response (38, 39). Thus, the development of highly potent and selective telomerase-inhibitors is expected to be clinically useful in cancer therapy. Knowledge of the hTERT promoter system (22, 23) permitted us to determine whether targeting telomerase in cancer cells would be effective. As demonstrated in the present study, the hTERT/rev-caspase-6 vector induced apoptosis in telomerase- or hTERT-positive cells whereas telomerase- or hTERT-negative cells did not undergo apoptosis. In addition, it has been demonstrated recently that the hTERT promoter can be used to restrict expression of caspase-8 (25), the *HSV-tk* gene (40), or proapoptotic *Bax* gene (41) to telomerase-positive tumor cells. These findings strongly support that the hTERT-promoter system could represent a promising approach with potential application in targeted cancer gene therapy irrespective of a kind of therapeutic gene.

There have been reports indicating that some human normal cells, such as lymphocytes (42), endothelial cells, endothelial fibroblasts (43), germ cells (44), and hematopoietic progenitor cells (45) express telomerase activity and/or hTERT at various levels. As for neural tissues, telomerase was detectable only in human embryonic neural precursor cells at low levels (46). Several studies showed no telomerase activity (15–17) or hTERT mRNA expression (17) in human normal brain tissue. Furthermore, in this study we demonstrate that hTERT mRNA is not expressed in cultured human astrocytes. Therefore, malignant gliomas are considered to be predominantly hTERT-positive in the central nervous system, although we further need a regional assessment of hTERT expression in human brain, including ependymal cells, choroid plexus, brain vascular endothelial cells, and microglia cells. However, these results suggest that there may be a minimal risk that normal brain tissues would be adversely affected for treatment of intracranial tumors. If extracranial tumors are treated with the hTERT/rev-caspase-6 vector, additional studies would be needed to determine whether or not telomerase-positive normal cells would be affected.

The experimental design in this present study did not permit us to follow whether surviving tumor cells after the treatment with the hTERT/rev-caspase-6 construct will undergo apoptosis several days later, acquire resistance, and still have telomerase activity or not. Additional studies using an inducible system of hTERT/rev-caspase-6 are expected to address the above questions.

In summary, telomerase- or hTERT-specific expression of *rev-caspase-6* gene by the hTERT promoter was successfully achieved without affecting telomerase- or hTERT-negative cells. This distinctive tumor-specific gene expression system is especially useful in treating the tumors such as malignant gliomas within the brain. Future experiments using animal models with intracranial tumors should determine whether this approach could be applied for the treatment of patients with malignant brain tumors. Because the hTERT/rev-caspase-6 is specific for hTERT expression, not for malignant gliomas alone, we expect that a wide range of telomerase- or hTERT-positive tumors may be successfully treated with this approach.

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