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Caspase-8 Gene Therapy Using the Human Telomerase Reverse Transcriptase Promoter for Malignant Glioma Cells

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ABSTRACT

Telomerase is a distinctive candidate for targeted gene therapy of malignant gliomas, because the vast majority of malignant gliomas express telomerase activity while normal brain tissues do not. Recently, we developed a telomerase-specific expression system of caspase-8 gene using the promoter of the human telomerase reverse transcriptase (hTERT) gene. However, the transcriptional activity of hTERT-181 promoter (a 181-base pair [bp] region upstream of the transcription start site) was relatively lower in malignant glioma cells than in other tumors such as prostate cancer cells. To establish the hTERT/caspase-8 construct as a novel therapy for malignant gliomas, we need to increase the transcriptional activity of the hTERT promoter in malignant glioma cells. In the present study, we demonstrate that the transcriptional activity of hTERT-378 promoter (a 378-bp region) was 2- to 40-fold higher in hTERT-positive malignant glioma cells (A172, GB-1, T98G, U87-MG, U251-MG, and U373-MG) than that of hTERT-181. We further demonstrate that by using the hTERT-378/caspase-8 construct, apoptosis was restricted to malignant glioma cells, and was not seen in astrocytes or fibroblasts lacking hTERT. Moreover, the growth of subcutaneously established U373-MG tumors in mice was significantly inhibited by seven daily intratumoral injections of hTERT-378/caspase-8 construct and its inhibitory effect persisted during 3 additional weeks without additional treatment. These results suggest that the telomerase-specific expression of caspase-8 under hTERT-378 promoter is a novel targeting approach for the treatment of telomerase-positive malignant gliomas.

OVERVIEW SUMMARY

Because malignant gliomas are considered to be predominantly telomerase-positive in the central nervous system, they are the most promising candidates for the telomerase-specific gene therapy using the human telomerase reverse transcriptase (hTERT) promoter that we have recently developed. In the present study, we demonstrate that using the hTERT-378 promoter (a 378-base pair [bp] region upstream of the transcription start site) the transcriptional activity was significantly enhanced in hTERT-positive malignant glioma cells compared to the hTERT-181 that we used previously. We further demonstrate that introduction of caspase-8 gene under the hTERT-378 promoter (hTERT-378/caspase-8) induced apoptosis only in hTERT-positive malignant glioma cells, but not in astrocytes or fibroblasts lacking hTERT. Moreover, a reduction in tumor volume was effected by seven daily injections of the hTERT-378/caspase-8 into subcutaneous tumors and its effect persisted 3 weeks after cessation of therapy. These findings suggest that the hTERT-378/caspase-8 is a promising tool for the treatment of telomerase-positive malignant gliomas.

INTRODUCTION

MALIGNANT GLIOMAS are the most common neoplasm in the central nervous system. Despite the advances in diagnostic procedures, surgical techniques, radiation therapy, and chemotherapy, the prognosis for patients remains poor, with the

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majority of patients succumbing to the disease within 1 year after diagnosis (Schoenberg, 1983; Mahaley *et al*., 1989; Deen *et al*., 1993; Davis *et al*., 1998). Clearly, new therapeutic strategies are necessary.

Malignancy in general has been considered to be the result of decreased sensitivity to apoptotic signals as well as of enhanced cell proliferation (McDonnel *et al*., 1995; Thompson, 1995; Bold *et al*., 1997). Apoptosis can be described as a process in which cells actively participate in their own death (Wyllie *et al*., 1986; Ellis *et al*., 1991), and this apoptotic pathway may be disrupted in tumor cells (Bold *et al*., 1997). Therefore, a genetic restoration of apoptotic pathway or introduction of apoptosis-inducible gene might be an attractive approach for the treatment of tumors including malignant gliomas. Certainly, there is increasing evidence that the transfer of apoptosis-inducible gene such as caspase or the tumor suppressor p53 is therapeutically effective for tumors *in vitro* and *in vivo* (Kondo *et al*., 1995; Gomez-Manzano *et al*., 1996; Yu *et al*., 1996; Kondo *et al*., 1998a; Marcelli *et al*., 1999). However, if the above apoptosis-inducible gene is transduced to normal cells surrounding tumors, they will undergo apoptotic cell death, resulting in undesirable side effects. Therefore, we need to establish a tumor-specific expression system of apoptosis-inducible gene in order to restrict the induction of apoptosis to tumor cells.

Telomerase, a ribonucleoproteinenzyme, is a particularly attractive target for such a system. It is because approximately 90% of tumors including malignant gliomas express telomerase activity, while most of normal somatic cells do not (Counter *et al*., 1992; Kim *et al*., 1994; Broccoli *et al*., 1995; Hiyama *et al*., 1995). Regarding malignant gliomas, telomerase was detected in 10% to 45% of anaplastic astrocytoma and in 75% to 89% of glioblastoma multiforme (Langford *et al*., 1995; Le *et al*., 1998). Therefore, telomerase can be a prominent candidate for targeted therapy. Activation of telomerase is tightly regulated at the transcriptional level of the human telomerase reverse transcriptase (hTERT) (Meyerson *et al*., 1997; Nakamura *et al*., 1997; Weinrich *et al*., 1997). More recently, the promoter region of hTERT has been cloned and characterized (Horikawa *et al*., 1999; Takakura *et al*., 1999). The transcriptional activity of the hTERT gene promoter was significantly higher in telomerase-positive cells than in telomerase-negative cells. Therefore, we hypothesized that the expression of an apoptosis-inducible gene could be restricted to telomerase-positive cells by the use of the hTERT promoter-driven vector system. More recently, we have constructed the caspase-8 gene (one of initiator caspases) expression vector under hTERT-181 promoter (a 181-base pair [bp] region upstream of the transcription start site) (hTERT-181/caspase-8). We demonstrated that treatment with the hTERT-181/caspase-8 induced apoptosis in a wide range of telomerase-positive tumor cells including malignant glioma cells (Koga *et al*., 2000). In contrast, apoptosis was not seen in normal fibroblast cells without telomerase activity. The report indicated that the hTERT-181 promoter was a tumor-selective promoter with potential application in targeted gene therapy for telomerase-positive tumors (Takakura *et al*., 1999). However, the transcriptional activity of hTERT-181 promoter was relatively lower in malignant glioma cells than in other tumors such as prostate cancer or malignant melanoma cells. To establish the hTERT/caspase-8 construct as a novel therapy for malignant gliomas, we needed to increase the transcriptional activity of hTERT promoter in malignant glioma cells. Among various lengths of DNA fragments upstream of the initiating ATG codon of hTERT promoter, a 181-, 378-, and 1375-bp regions demonstrated significant transcriptional activity in tumor cells (Takakura *et al*., 1999). Because a 181-bp region functions as the core promoter essential for transcriptional activity of hTERT, we previously used the hTERT-181 promoter to construct the hTERT/caspase-8 plasmid (Koga *et al*., 2000). In the present study, we first demonstrated that the use of hTERT-378 promoter significantly enhanced its transcriptional activity in hTERT mRNA-positive malignant glioma cells compared to that of hTERT-181 or hTERT-1375. Next, we demonstrated the antitumor effect of hTERT-378/caspase-8 on malignant glioma cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells

Human malignant glioma (U87-MG, A172, T98G, U373-MG), human breast cancer (MCF-7), human prostate cancer (PC3), and human normal fibroblasts MRC5 were purchased from ATCC (American Tissue Culture Collection, Rockville, MD). Human malignant glioma GB-1 and U251-MG cells were a kind gift from Dr. Tatsuo Morimura (National Utano Hospital, Kyoto, Japan) and Dr. Akiko Nishiyama (University of Connecticut, Storrs, CT), respectively. Human bladder cancer cell line (UM-UC-2) was a kind gift from Dr. H. Barton Grossman (University of Texas M.D. Anderson Cancer Center, Houston, TX). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO BRL), 4 m*M* of glutamine, 100 U/ml of penicillin, and 100 μg/ml streptomycin. Human astrocytes TEN were maintained in RPMI1640 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 4 m*M* of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. TEN astrocytes were characterized by the presence of the astrocytic marker glial fibrillary acidic protein (GFAP) in nearly 100% of cells when evaluated under immunofluorescent microscope as described previously (Liu *et al*., 1997).

RT-PCR analysis for hTERT

The expression of hTERT mRNA was analyzed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) amplification that we have recently described (Wang *et al*., 2000). 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 (glyceraldehyde-3-phosphate dehydrogenase; 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 following primer sets were used as described previously (Nakamura *et al*., 1997): GAPDH, 5′-CTCAGACACCATGGGGAAGGTGA-3′ (forward) and 5′-ATGATC-

TTGAGGCTGTTGTCATA-3′ (reverse); hTERT, 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 [Cambridge, MA] type 667), and photographs were quantitatively scanned using the National Institutes of Health (NIH) image software. The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers.

Transcriptional activity of hTERT promoter

The transcriptional activity of hTERT in normal or tumor cells was determined by the luciferase reporter plasmids as described previously (Takakura *et al*., 1999, Koga *et al*., 2000). Cells were plated at a density of 1.0×10^5 cells/ml 1 day prior to transfection of the luciferase reporter plasmids. Cells were washed and the media was replaced with OPTI-MEM (GIBCO BRL) and transfected by using LipofectAmine (GIBCO BRL) as previously described (Kondo *et al*., 1995). Forty-eight hours 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 for samples and positive control, the standard curve of light units versus relative enzyme concentration were obtained by making serial dilutions of luciferase (QuantiLum® Recombinant Luciferase, Promega CORP-BTC) in 1Xlysis buffer with 1 mg/ml bovine serum albumin (BSA). The following plasmids were used: the SV40 enhancer/promoter (pGL3-control) for a positive control, the hTERT-181, hTERT-378, or hTERT-1375 promoter plasmids (pGL3-181, pGL3-378, or pGL3-1375), and the negative control without promoter (pGL3-basic) as described previously (Takakura *et al*., 1999).

Construction of the hTERT-378 promoter construct carrying caspase-8 gene

To construct the caspase-8 expression vector under hTERT-378 promoter or SV40 enhancer/promoter, the hTERT promoter-luciferase (pGL3-378) and SV40 enhancer/promoter-luciferase (pGL3-control) constructs were used as described previously (Takakura *et al*., 1999). First, luciferase region was removed from the pGL3-378 or pGL3-control using *Hind*III and *Xba*I. The caspase-8 (FLICE) coding region was excised from the pcDNA3-FLICE-HA vector (kindly supplied by Dr. Vishva M., Dixit, University of Michigan, Ann Arbor, MI) (Muzio *et al*., 1996) with *Hind*III and *Xba*I. Then, the cDNA fragment of caspase-8 was inserted into the pGL3-378 or pGL-control construct in place of luciferase, and designated as the hTERT-378/caspase-8 or SV40/caspase-8 expression vector, respectively.

Transient transfection assay

To determine whether the hTERT-378/caspase-8 construct induces apoptosis only in telomerase-positive cells, transient transfection assays using Lipofectamine-mediated gene transfer (GIBCO) were performed as described previously (Kondo *et al*., 1995, 1998a; Koga *et al*., 2000). The plasmid expressing green fluorescence protein, pEGFP-C1 (Clontech, Palo Alto, CA), was used as a reporter gene. The day before transfection, cells were seeded at 5×10^4 cells/ml in Lab-Tek chamber slides. Caspase-8 expression vector under the hTERT-378 promoter (hTERT-378/caspase-8, 1 μg) or SV40 promoter (SV40/caspase-8, 1 μg) together with pEGFP-C1 plasmid (0.3 μg) were transfected into cells, and incubated for 48 hr. The hTERT-378/luciferase construct (pGL3-378) 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 phosphate-buffered saline (PBS), and stained with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (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-positive cells, respectively. An apoptotic index was determined as a percentage of apoptotic cells among 100 GFP-positive cells.

In vivo *effect of caspase-8 expression under the hTERT-378 promoter*

Human malignant glioma U373-MG cells (1.0×10^6 cells in 0.05 ml of serum-free DMEM and 0.05 ml Matrigel [Collaborative Research, Waltham, MA]) were inoculated subcutaneously into the right flank of 8- to 12-week-old male Balb/c nude mice (6 mice for each treatment group) as described previously (Kondo *et al*., 1998a,b). Tumor growth was monitored using calipers every other day. Tumor volume (V) was calculated as $(L \times W^2)/2$, where L = length (mm) and W = width (mm). To simulate the clinical situation, we treated after the establishment of the tumor. When the tumors reached a mean tumor volume of 40 to 60 mm^3, the treatment was initiated. The hTERT-378 promoter plasmid with caspase-8 cDNA (hTERT-378/caspase-8, 10 μg) and cationic lipid (DEMRI, 2 μg, GIBCO BRL) dissolved in 20 μl of sterile PBS were directly injected into the tumor every 24 hr for 7 days. The hTERT-378/luciferase construct (pGL3-378) was used as a control. Mice were sacrificed by cervical dislocation the day after the final treatment, and the tumors were removed, frozen rapidly, and 8.0-μm cryosections were made and used for histologic studies. The sections from treated tumors were used for TUNEL analysis and/or caspase-8 immunohistological staining using anti-caspase-8 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described previously (Kondo *et al*., 1998a). As additional experiments, we treated subcutaneous U373-MG tumors with hTERT-378/caspase-8 for a week as above and further examined if the tumor grew back during 3 additional weeks without additional treatment. During all of the experiments, which were approved by the Institutional Animal Care and Use Committee, the animals were housed and handled in accordance with the National Institutes of Health guidelines.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistical comparison was made using Stat View software and the Mann-Whitney test (nonparametric method). The criterion for statistical significance was taken as $p < 0.05$.

RESULTS

Expression of hTERT mRNA in malignant glioma cells

We previously reported that using the telomeric repeat amplification protocol (TRAP) assay, all six human malignant glioma cells used in the present study were telomerase-positive, while TEN astrocytes and MRC5 fibroblasts were telomerase-negative (Kondo *et al.*, 1998b; Komata *et al.*, 2000; Mukai *et al.*, 2000). To confirm that telomerase-positive tumor cells are hTERT-positive, whereas telomerase-negative normal cells are hTERT-negative, the expression of hTERT mRNA was analyzed by semiquantitative RT-PCR assay as previously described (Wang *et al.*, 2000). As shown in Figure 1, all malignant glioma cells were hTERT mRNA-positive. GB-1, U251-MG, and U373-MG cells were highly positive (the relative band intensity was 1.2, 1.2, and 1.5, respectively) and A172, T98G, and U87-MG cells were moderately positive (the relative band intensity was 0.3, 0.7, and 0.7, respectively). In contrast, hTERT mRNA was not detected in TEN astrocytes or MRC5 fibroblasts.

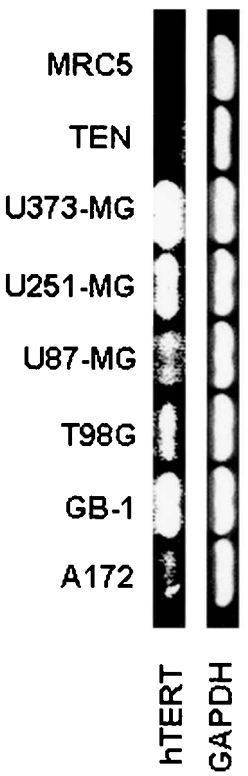

FIG. 1. Expression of human telomerase reverse transcriptase (hTERT) gene mRNA in tumor or normal cells. As telomerase-positive tumor cells, malignant glioma cells (A172, GB-1, T98G, U87-MG, U251-MG, and U373-MG) were tested. As control, telomerase-negative cells (astrocytes TEN and fibroblasts MRC5) were used. After the isolation of RNA from each cell line, reverse transcriptase-polymerase chain reaction (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.

Transcriptional activity of hTERT-181, hTERT-378, or hTERT-1375 promoter in malignant glioma cells

First, using four different tumor cell lines (malignant glioma, U373-MG; breast cancer, MCF-7; bladder cancer, UM-UC-2; and prostate cancer, PC3), we compared the transcriptional activity of hTERT-181, hTERT-378, and hTERT-1375 promoters. To make the comparison easy, the mean transcriptional activity of hTERT-181 promoter for each cell line was considered 100%. As shown in Figure 2A, the level of transcriptional activity varied among tumor types and length of DNA fragments in hTERT promoter. In U373-MG cells, the transcriptional activity of hTERT-378 was significantly higher than that of hTERT-181 or hTERT-1375 ($p < 0.001$). There was no significant difference between hTERT-181 and hTERT-1375 constructs. In MCF-7 cells, hTERT-1375 showed relatively higher transcriptional activity. In UM-UC-2 and PC3 cells, the transcriptional activity of hTERT-1375 promoter was significantly lower than that of hTERT-181 or hTERT-378 ($p < 0.01$). This result suggested that hTERT-378 promoter might be more useful for gene transduction in malignant glioma cells than hTERT-181 or hTERT-1375. To investigate whether hTERT-378 promoter has a similar tendency in other malignant glioma cells, A172, GB-1, T98G, U-87-MG, and U251-MG cells were tested. The transcriptional activity of positive control with SV40 enhancer/promoter in each cell line was considered 100%. As shown in Figure 2B, the transcriptional activity of hTERT-378 promoter was 2- to 40-fold higher than that of hTERT-181. The relative luciferase activities of six of malignant glioma cell lines by hTERT-378 promoter varied from 20% to 84% of the positive control SV40 promoter construct. Interestingly, U87-MG cells showed the highest transcriptional activity of the hTERT-378 promoter although the expression of hTERT mRNA was moderate. In contrast, the relative luciferase activity remained at a baseline level in TEN astrocytes or MRC5 fibroblasts. These results indicated that the hTERT-378 promoter could function as a strong and efficient promoter in malignant glioma cells.

Overexpression of caspase-8 induces apoptotic cell death in malignant glioma cells

The above results suggest that by using the hTERT-378 promoter, we could restrict a gene expression to telomerase- or hTERT mRNA-positive cells. Therefore, we constructed the caspase-8 expression vector under hTERT-378 promoter (hTERT-378/caspase-8). In addition, we prepared the hTERT-378/luciferase (pGL3-378) and SV40/caspase-8 constructs to compare their effects. To determine whether the hTERT-378/caspase-8 construct induces apoptosis only in hTERT mRNA-positive tumor cells, but not in hTERT mRNA-negative normal cells, malignant glioma cells, astrocytes, or fibroblasts were transfected with the pGL3-378, hTERT-378/caspase-8, or SV40/caspase-8 construct together with the GFP gene expression vector (pEGFP-C1). Two days after the transfection, the induction of apoptosis was determined by TUNEL staining. As shown in Figure 3A-a and 3A–d, and Figure 3B-a and 3B-d, malignant glioma U373-MG cells and MRC5 fibroblasts transfected with the pGL3-378 and pEGFP-C1 constructs kept normal morphology of adherent cells, and showed TUNEL-negative (GFP-positive cells were considered transfected cells). Af-

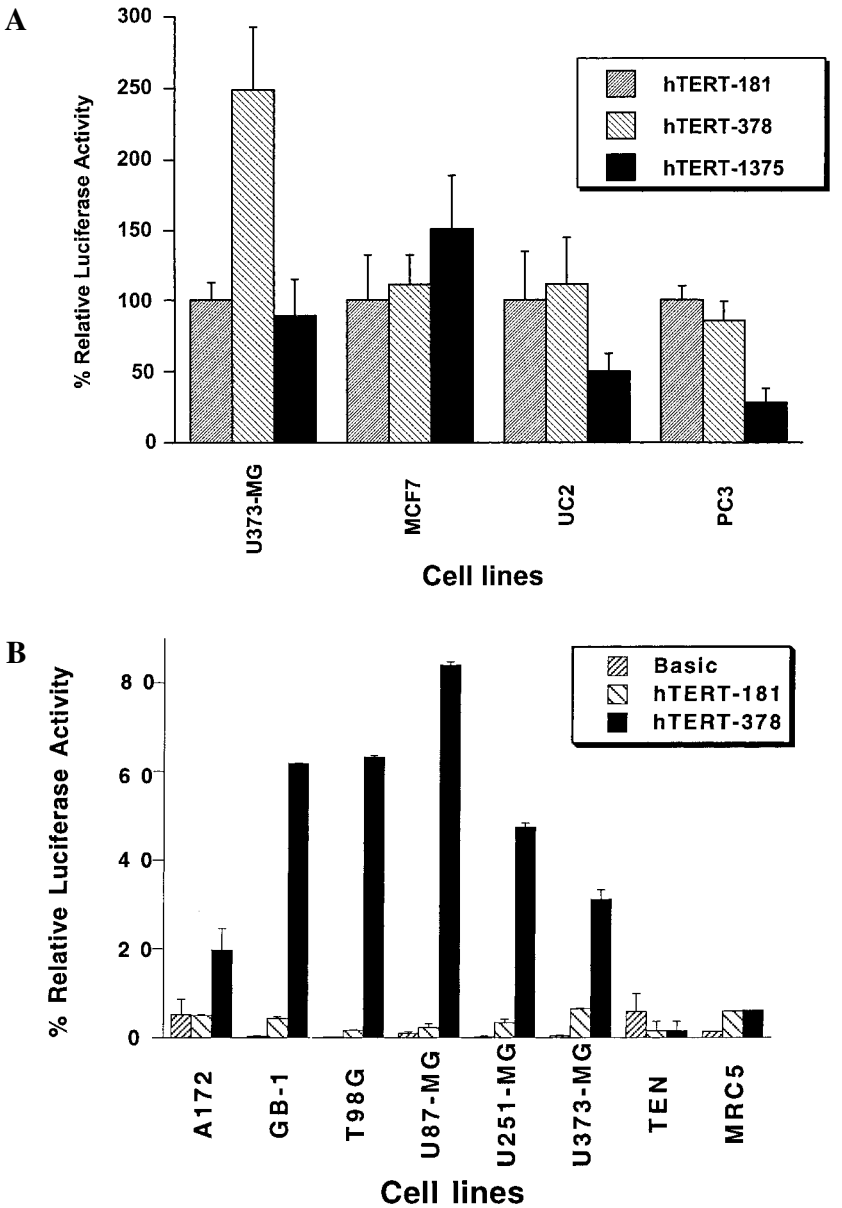

FIG. 2. Transcriptional activity of human telomerase reverse transcriptase (hTERT)-181, hTERT-378, or hTERT-1375 promoter in tumor or normal cells. **A:** Luciferase activity of reporter plasmid with hTERT-181, hTERt-378, or hTERT-1375 promoter was examined in human malignant glioma (U373-MG), breast cancer (MCF-7), bladder cancer (UM-UC-2), and prostate cancer (PC3). Luciferase activity in each plasmid was plotted as a percentage of the hTERT-181 promoter construct. The data shown are the mean ± standard deviation (SD) from three independent experiments. **B:** Luciferase activity of reporter plasmid with hTERT-181 or hTERT-378 promoter was examined in human malignant glioma cells (A172, GB-1, T98G, U87-MG, U251-MG, and U373-MG), astrocytes TEN and fibroblasts MRC5. Luciferase activity in each plasmid was plotted as a percentage of the positive control plasmid (pGL3-control) driven by SV40 enhancer/promoter. pGL3-basic without enhancer/promoter was used as a negative control. The data shown are the mean ± SD from three independent experiments.

ter the transfection with the hTERT-378/caspase-8 construct in the presence of pEGFP-C1 vector, U373-MG cells showed positive TUNEL staining as well as morphologic changes that are typical of adherent cells undergoing apoptosis, round or condensed shape (Fig. 3A-b and 3A-e). In contrast, MRC5 cells transfected with the hTERT-378/caspase-8 and pEGFP-C1 did not undergo apoptosis (Fig. 3B-b and 3B–e). When SV40/caspase-8 and pEGFP-C1 constructs were transfected, both U373-MG and MRC5 cells underwent apoptosis (Fig. 3A-c and 3A–f, and 3B-c and 3B–f). The quantitative representative of these data is shown in Figure 3C. Apoptosis was induced in 30%–50% of malignant glioma cells by the hTERT-378/caspase-8. As expected, apoptosis was detected in 4% of TEN astrocytes and 10% of MRC5 fibroblasts 2 days after the transfer of hTERT-378/caspase-8. These percentages were not significantly different from those by the transfer of pGL3-378

basic plasmid. Furthermore, to investigate whether the use of hTERT-378 promoter is indeed translated into more killing for the corresponding caspase-8 construct than that of hTERT-181, we quantitatively tested the apoptotic activity of hTERT-181 or hTERT-378/caspase-8 construct. As shown in Figure 3D, apoptosis was detected in 34% of U251-MG cells and 48% of U373-MG cells after transfection with hTERT-378/caspase-8 construct, while 18% of U251-MG cells and 21% of U373-MG cells transfected with hTERT-181/caspase-8 was apoptosis. This result indicates that the apoptotic activity of caspase-8 was significantly enhanced in malignant glioma cells by using hTERT-378 promoter compared to hTERT-181 ($p < 0.01$).

Effect of the hTERT-378/caspase-8 treatment on malignant glioma cells in vivo

To determine the *in vivo* antitumor effect of the hTERT-378/caspase-8 construct, U373-MG cells were inoculated subcutaneously in nude mice. After the establishment of subcutaneous tumors, the hTERT-378 promoter vector with luciferase

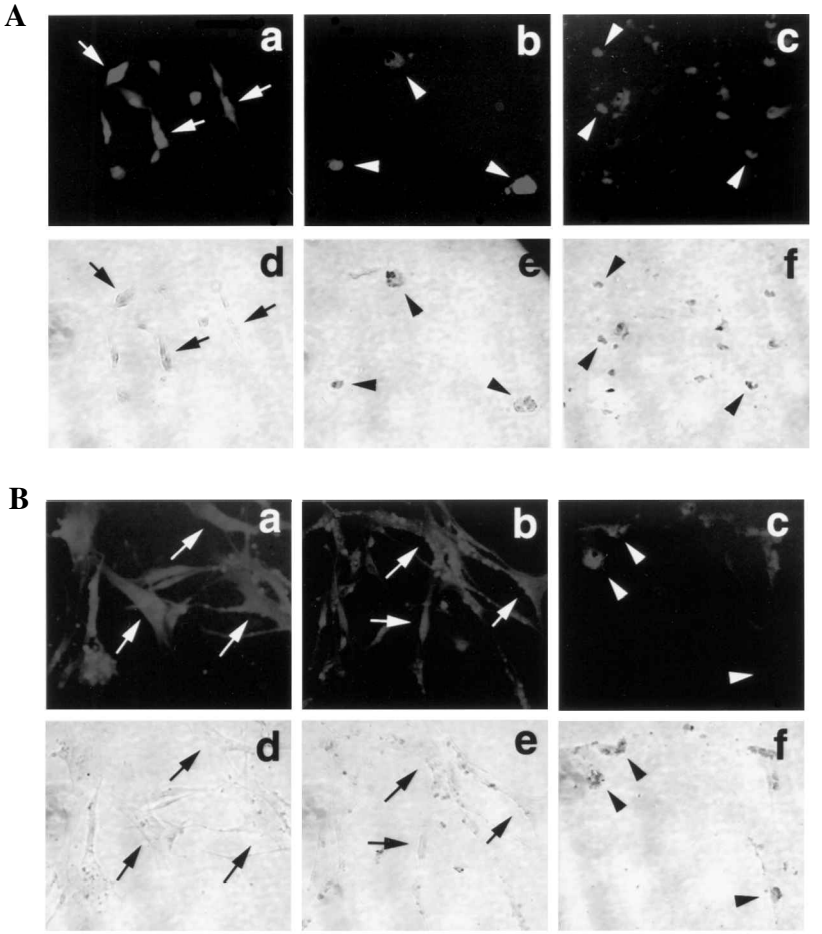

FIG. 3. Induction of apoptosis in tumor or normal cells by introduction of caspase-8. **A** and **B:** U373-MG malignant glioma cells or MRC5 fibroblasts were transiently transfected with pEGFP-C1 (0.3 μg) plasmid and either the control vector (human telomerase reverse transcriptase [hTERT]-378/luciferase), the caspase-8 expression vector with SV40 enhancer/promoter (SV40/caspase-8) or hTERT-378 promoter (hTERT-378/caspase-8), 48 hr later, fixed, and stained using the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling (TUNEL) technique, respectively. A: U373-MG cells. B: MRC5 cells. Arrowheads and arrows indicate representative apoptotic or normal cells (green fluorescence protein [GFP]-positive), respectively (200×). **a** and **d:** Transfected with pEGFP-C1 and hTERT-378/luciferase. **b** and **e:** Transfected with pEGFP-C1 and hTERT-378/caspase-8. **c** and **f:** Transfected with pEGFP-C1 and SV40/caspase-8. Fluorescence microscope (a, b, and c); bright-field microscope (d, e, and f). **C:** Caspase-8 expression vector (SV40/caspase-8 or hTERT-378/caspase-8) and pEGFP-C1 plasmid were transiently transfected into human malignant glioma cell lines, TEN astrocytes, or MRC5 fibroblast cells, respectively. The hTERT-378/luciferase construct was used as a negative control. Cells were fixed 48 hr after transfection and stained using TUNEL technique. The data shown are the mean percentage ± SD of apoptotic cells among total 100 GFP-positive cells counted. The data were collected from at least three independent experiments. **D:** Effect of hTERT-181 or hTERT-378 promoter on apoptosis induction. Caspase-8 expression vector (hTERT-181 or hTERt-378/caspase-8) and pEGFP-C1 plasmid were transiently transfected into U251-MG or U373-MG cells. Cells were fixed 48 hr after transfection and stained using TUNEL technique. The data shown are the mean percentage ± SD of apoptotic cells among total 100 GFP positive cells counted. The data were collected from at least three independent experiments.

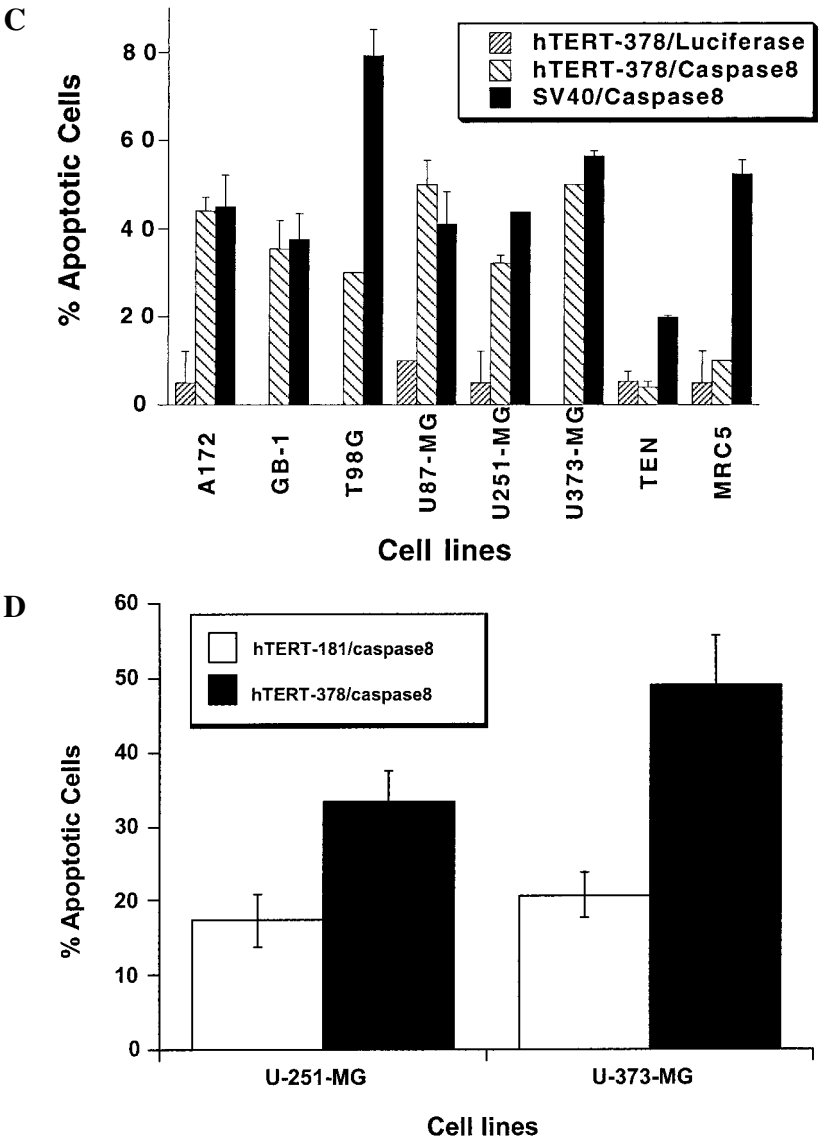

FIG. 3. *Continued.*

gene (pGL3-378) or with caspase-8 cDNA (hTERT-378/caspase-8) (10 μg each) in the presence of DMRIE, a cationic lipid (2 μg) was then injected directly into tumors every 24 hr for 7 days. As shown in Figure 4A, treatment with the hTERT-378/caspase-8 construct significantly suppressed the growth of U373-MG subcutaneous tumors when compared to the control pGL3-378 treatment ($p < 0.001$). In the animals treated with the hTERT-378/caspase-8 construct, the mean tumor volume after 7 days treatment was reduced by 44% from the initial tumor size. In contrast, the mean tumor volume was increased by 41% in control mice treated with the pGL3-378 construct. In tumors treated with the hTERT-378/caspase-8, the density of tumor cells was decreased and some TUNEL-positive cells were detected (Fig. 4B-b). In contrast, few TUNEL-positive cells were observed in tumors treated with the pGL3-378 construct (Fig. 4B-a). To investigate whether the TUNEL-positive (apoptotic) cells are the result of caspase-8 expression by hTERT-378/caspase-8 construct, we performed the double staining of TUNEL and caspase-8 immunofluorescence images. As shown in Figure 4C, a vast majority of TUNEL-positive cells in tumors treated with hTERT-378/caspase-8 construct were caspase-8–positive (c and d). In contrast, few TUNEL- and caspase-8–positive cells were detected in control tumors (Fig. 4C-a and 4C-b). These results indicate that apoptosis induction was because of caspase-8 expression itself. A bystander effect might be slight, if any. As additional experiments, we investigated whether growth delay persists or whether there is a rapid resumption of tumor growth after cessation of hTERT-378/caspase-8 therapy for 1 week. We treated subcutaneous U373-MG tumors with hTERT-378/caspase for 1 week as above and further examined if the tumor grew back during 3 additional weeks without additional treatment. As shown in Figure 4D, the mean tumor volume of U373-MG on day 29 was 43% of the initial tumor size ($p < 0.001$), suggesting that treatment with hTERT-

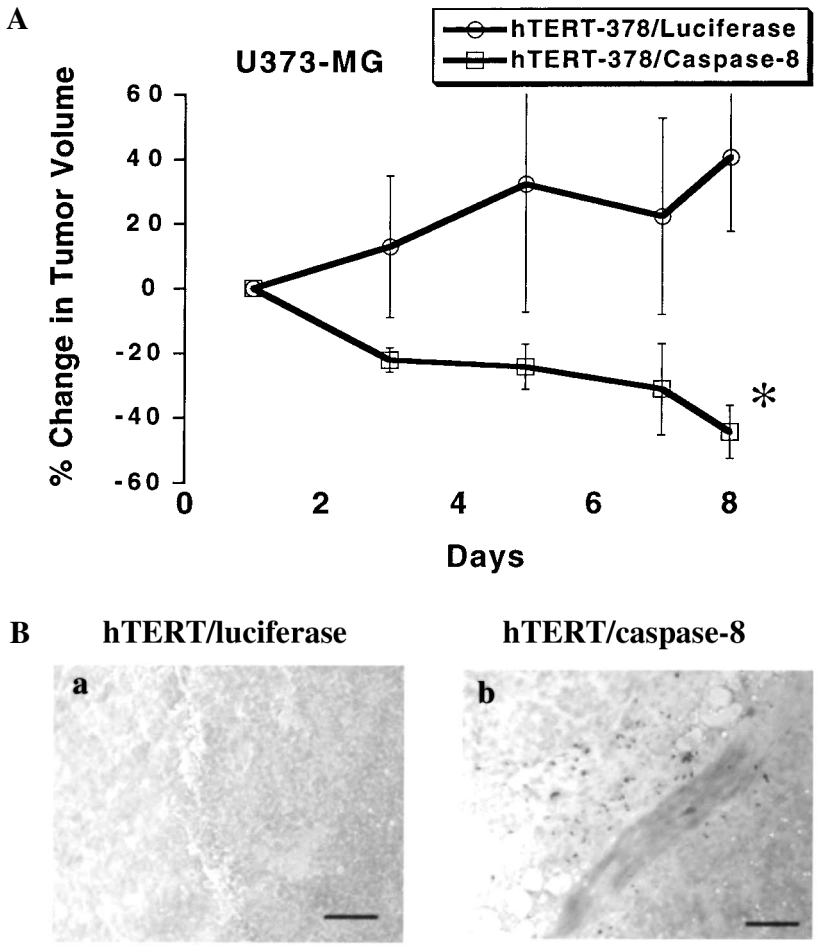

FIG. 4. Effect of caspase-8 gene transfer under the human telomerase reverse transcriptase (hTERT)-378 promoter on subcutaneous U373-MG tumors in nude mice. U373-MG tumors were established subcutaneously in nude mice. When tumors reached a mean volume of 40 to 60 mm^3, the hTERT-378/caspase-8 or luciferase construct (10 μg) mixed with 2 μg of cationic lipid (DMRIE) was directly injected into the tumor every 24 hr for 7 days. Six mice were used in each treatment group. **A:** Tumor volume was determined with calipers every other day. Values represent the means ± standard deviation (SD) of the percentage of change in tumor volume. *$p < 0.001$ compared to controls (on day 8). **B:** *In situ* end-labeling of DNA (terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling [TUNEL] assay) in treated subcutaneous tumors. After treatment for 7 days, the mice were sacrificed and the subcutaneous mass was removed, frozen, and sectioned on a cryostat. The tumor specimens from U373-MG cells were counterstained by methyl green following the TUNEL staining. **a:** hTERT-378/luciferase. **b:** hTERT-378/caspase-8. Bars, 100 μm. **C:** Colocalization of TUNEL- and caspase-8–positive cells. To determine whether the TUNEL-positive cells are caused by hTERT/caspase-8 expression, TUNEL assay (**a** and **c**) and immunofluorescent staining with anti-caspase 8 antibody (**b** and **d**) were performed at the same time. a and b: Treated with the hTERT-378/luciferase construct. c and d: Treated with the hTERT-378/caspase-8 construct. Arrows indicate representative TUNEL- and caspase-8–positive cells. Bars, 38 μm. **D:** When tumors reached a mean volume of 40 to 60 mm^3, the hTERT-378/caspase-8 construct (10 μg) mixed with 2 μg of cationic lipid (DMRIE) was directly injected into the tumor every 24 hr for 7 days; 6 mice were used. Tumor volume was determined with calipers during treatment with hTERT-378/caspase-8 for 1 week and during 3 additional weeks without additional treatment. Values represent the means ± SD of the percentage of change in tumor volume. *$p < 0.001$ compared to the tumor size prior to treatment (on day 1).

378/caspase-8 yielded a more durable antitumor effect than a transient response followed by rapid regrowth after the end of treatment.

DISCUSSION

In this study, we demonstrate the high potentiality of caspase-8 gene transfer under hTERT-378 promoter as a novel telomerase-specific gene therapy against telomerase-positive malignant glioma cells. With regard to targeted gene therapy for malignant gliomas, there are two possibilities. One approach is a selective cell entry system using tumor-specific molecules such as receptors, and the other is a transcriptional regulation using tumor-specific promoters. Regarding the first strategy, there have been several reports describing targeted therapy against malignant gliomas using markers such as glial fibrillary acidic protein (GFAP) (McKie *et al.*, 1998) or epidermal growth factor receptor (EGFR) (Miller *et al.*, 1998). However, only some subpopulations of malignant gliomas present GFAP or

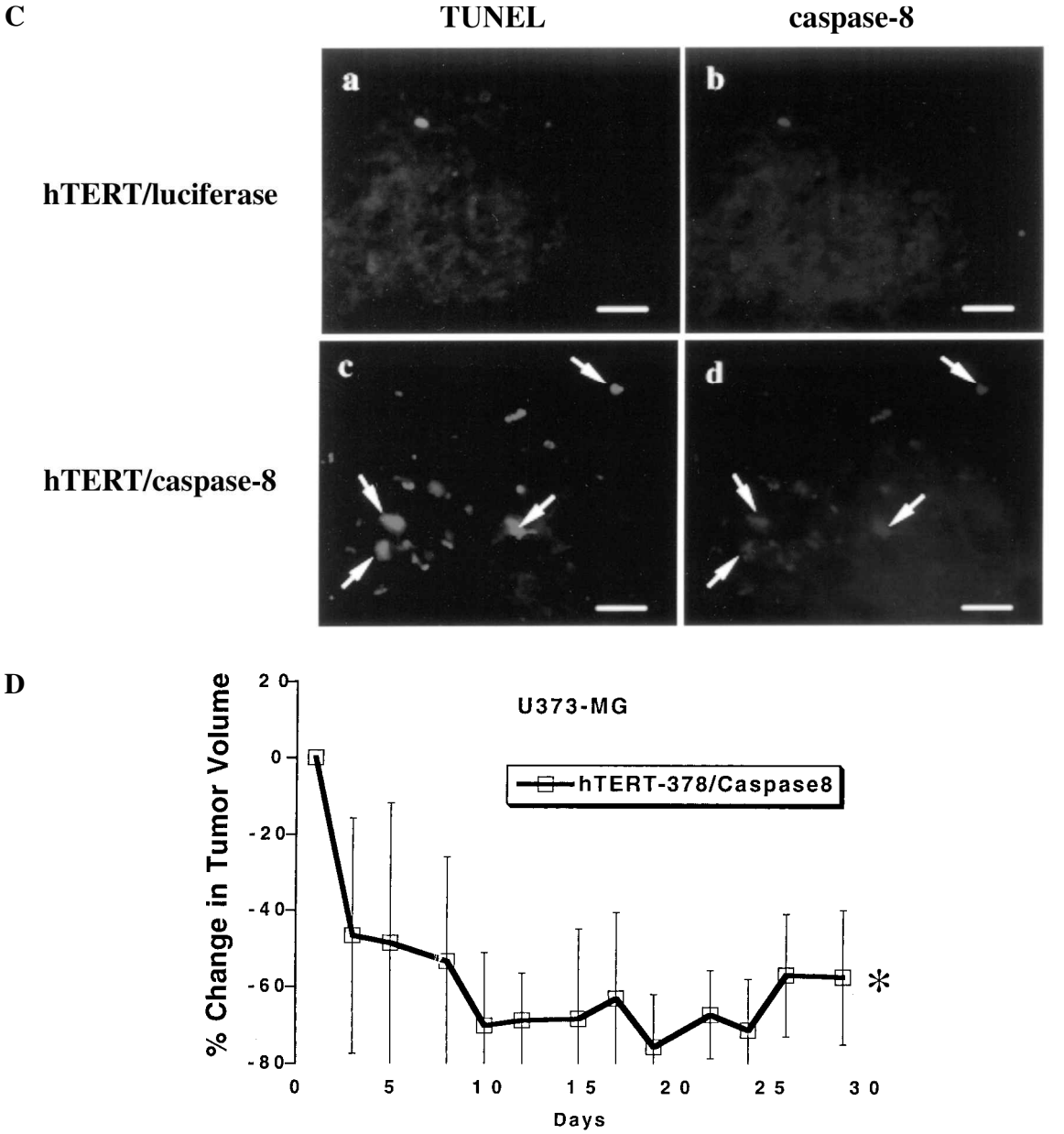

FIG. 4. *Continued.*

EGFR. Regarding the second approach, several gene expression systems via tissue- or cell-specific promoters in tumors other than malignant gliomas are well demonstrated, for example, the prostate-specific antigen (PSA)-promoter in prostate cancer (Pang *et al.*, 1995) or the carcinoembryonic antigen (CEA)-promoter in lung cancer (Osaki *et al.*, 1994). However, there is no specific promoter available for malignant gliomas so far.

Telomerase is highly active in approximately 90% of tumors, but is inactive in most normal somatic cells (Counter *et al.*, 1992; Kim *et al.*, 1994; Broccoli *et al.*, 1995; Hiyama *et al.*, 1995). Therefore, telomerase is expected to be an attractive candidate for targeted gene therapy. Because telomerase activity is tightly regulated at the transcriptional level of hTERT (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Weinrich *et al.*, 1997), we hypothesized that the hTERT promoter could be used for tumor-specific expression of transgenes. To test our hypothesis, we previously constructed the caspase-8 gene expression system under the hTERT-181 promoter (hTERT-181/caspase-8) (Koga *et al.*, 2000). As predicted, apoptosis was restricted to a wide range of telomerase-positive tumor cells by the transfer of hTERT-181/caspase-8 construct, while normal fibroblasts without telomerase did not undergo apoptosis. However, the transcriptional activity of hTERT-181 promoter was relatively lower in malignant glioma cells or breast cancer cells than in prostate cancer, bladder cancer, or malignant melanoma cells, although the mechanisms responsible are still not understood. For example, U373-MG malignant glioma cells showed 6% or 32% of the activity of prostate cancer DU145 or PC3 cells, respectively (Koga *et al.*, 2000). Therefore, to develop the

hTERT/caspase-8 system as a new treatment modality for malignant gliomas, we searched for a promoter that has an increased transcriptional activity. As described in the present study, the application of hTERT-378 promoter significantly enhanced the transcriptional activity and efficiently induced apoptosis in malignant glioma cells when compared to that of hTERT-181 or hTERT-1375. In contrast, apoptosis did not undergo in astrocytes or fibroblasts lacking telomerase activity or hTERT mRNA expression. Because several studies demonstrate that telomerase activity or hTERT mRNA expression is not detected in human normal brain tissue (Le *et al.*, 1998; Huang *et al.*, 1999; Harada *et al.*, 2000), we expected that the hTERT-378/caspase-8 could be available for the treatment of malignant gliomas. Recently, it has been demonstrated that the induction of the Bax (Gu *et al.*, 2000) or thymidine kinase gene (Majumdar *et al.*, 2001) via the hTERT promoter elicited tumor-specific apoptosis *in vitro*, suppressed tumor growth in nude mice, and prevented the toxicity against normal organs. These findings, including the present study, strongly suggest that hTERT promoter is clearly a strong and tumor-specific promoter with high potential in targeted cancer gene therapy.

In summary, the telomerase-specific expression of caspase-8 gene by hTERT-378 promoter was achieved without affecting the telomerase-negativecells. This distinctively telomerase-specific gene expression system is especially useful in treating telomerase-positive tumors surrounded by normal tissues such as malignant gliomas in the brain.

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