A novel treatment of human malignant gliomas in vitro and in vivo: FADD gene transfer under the control of the human telomerase reverse transcriptase gene promoter

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Abstract. Telomerase activity has a close relationship with malignancies in many cell types and it is tightly regulated at the transcriptional level of human telomerase reverse transcriptase (hTERT). Utilizing the hTERT promoter, the authors developed a gene delivery system of Fas associated protein with death domain (FADD) (hTERT/FADD). FADD is a protein which plays an important role in the apoptotic pathway of Fas. Overexpression of FADD induces apoptosis in the cells regardless of Fas expression on the cell surface. We hypothesized that we might be able to restrict the expression of FADD in malignant glioma cells if we use the gene transfer system under the control of hTERT promoter. This study was designed to investigate whether the hTERT/FADD construct induces apoptosis effectively in malignant glioma cells while keeping normal cells intact. First, using the reverse transcription-polymerase chain reaction (RT-PCR) technique, we confirmed that hTERT mRNA was expressed in human malignant glioma cells (U373-MG, A172 and GB-1), but not in cultured astrocytes (TEN) or fibroblasts (MRC5). After transient transfection with the hTERT/FADD construct, a significant number of FADD-positive cells and apoptotic cells were detected in hTERT-positive malignant glioma cells. In contrast, hTERT-negative astrocytes and fibroblasts remained intact. Furthermore, subcutaneously implanted U373-MG tumors treated with the hTERT/FADD construct reduced in volume significantly compared to the control treatment (p=0.0001). Gene transfer of FADD under the control of the hTERT promoter may be a novel and promising therapy to kill hTERT-positive malignant glioma cells while sparing normal brain cells lacking hTERT.

Introduction

Current treatment modalities for patients with glioma are surgery, radiotherapy, and chemotherapy. Advances in these therapies extended the prognosis for patients with low-grade glioma from 5 years to 10 years over the time period of 1984-1995 (1). However, the prognosis for patients with high-grade glioma, especially with glioblastoma multiform (GBM), remains less than 1 year (2-4). To improve the prognosis for patients with GBM, it is crucial to explore novel therapeutic modalities.

Recently, we have reported that the transduction of the Fas associated protein with death domain (FADD) gene effectively induces apoptosis in human malignant glioma cells (5). The FADD gene is a down-stream death signal for the apoptosis pathway of Fas/APO-1, a transmembrane receptor for tumor necrosis factor α (TNFα) (6-10). There are several reports demonstrating the effectiveness of the treatment with Fas ligand for cancer cells with Fas/APO-1 (11-13). However, Fas ligand does not lead cells to apoptosis unless the target cells express Fas/APO-1, as shown in some malignant glioma cell lines (5). In contrast, the FADD gene transfer induces cell death regardless of the expression of Fas/APO-1 in the target cells, indicating high potential in cancer gene therapy (5). Nevertheless, a major drawback of the FADD gene therapy is that the FADD gene can cause cell death not only in malignant cells but also in normal brain cells.

To overcome this problem, we developed a gene transfer system under the control of the human telomerase reverse transcriptase (hTERT) promoter (14). This system enables us to restrict transfer of the therapeutic gene to those cells which have telomerase activity, because telomerase activity is tightly regulated at the transcriptional level of hTERT (15,16). We hypothesized that FADD gene transfer under the control of the hTERT promoter (hTERT/FADD) might induce apoptosis.
only in telomerase-positive malignant glioma cells while keeping telomerase-negative normal cells intact. The fact that a vast majority of GMBs has telomerase activity while normal human brain tissues lack the enzyme (17-21) makes hTERT/FADD a very attractive and promising strategy to target telomerase-positive malignant gliomas.

In the present study, we developed FADD gene transfer system under the control of the hTERT promoter. Then, we investigated whether the hTERT/FADD construct successfully induces the expression of FADD, resulting in induction of apoptosis in hTERT-positive human malignant glioma cells. It is of great importance to observe if normal cells without hTERT remain intact or not with this strategy.

Materials and methods

Cells. Human malignant glioma U373-MG, A172 cells and human fibroblast MRCS cells were purchased from ATCC (American Tissue Culture Collection, Rockville, MD). Human malignant glioma GB-1 cells were kindly gifted by Dr Tatsuo Morimura (National Utano Hospital, Kyoto, Japan). Cells were cultured in DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL), 4 mM glucose, 100 U/ml penicillin and 100 ìg/ml streptomycin. Human astrocyte TENG cells were kindly gifted by Dr Barbara P. Barna (Metrohealth Medical Center, Cleveland, OH), and maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 4 mM glucose, 100 U/ml penicillin and 100 µg/ml streptomycin.

RT-PCR analysis for hTERT. The expression of hTERT mRNA was analyzed by semi-quantitative RT-PCR amplification as recently described (22). 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 ProStarTM First Strand RT-PCR Kit (Stratagene, La Jolla, CA). 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 (23): GAPDH, 5'-CTCAAGACCATGGAAGGTGA-3' (forward) and 5'-ATGACCTTGGAGGCTTTCATA-3' (reverse); hTERT, 5'-CGGAAGTGTGTTGAGAAA-3' (forward) and 5'-GGATGAAAGGATCTTGGGA-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 amount of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers.

Luciferase assay. Transcriptional activity of hTERT in normal or tumor cells was determined by the luciferase reporter plasmids as described previously (24,25). Cells were plated at a density of 1.0x10^5 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 (26). 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). Luciferase activity was measured using a Microtiter Plate Luminometer (Dynatech Laboratories, Inc., Chantilly, VA). The following plasmids were used: the hTERT promoter plasmid (pGL3-181), the negative control without promoter (pGL3-basic), and the positive control with SV40 enhancer/promoter (pGL3-control) as described previously (25). The luciferase activity of the pGL3-basic plasmid in each cell line was considered as 1.0.

Construction of the hTERT promoter plasmid carrying FADD gene. To construct the FADD expression vectors under hTERT promoter, the hTERT promoter-luciferase (pGL3-181) construct was used as described previously (25). The vector including the full length coding region of FADD (pDNNA3-AU1-FADD) (6) was a generous gift from Dr Vishva M. Dixit (University of Michigan, Ann Arbor, MI) and used as a template. The 712 bp fragment of FADD was generated by PCR amplifying with 5’-GAAAGATCTGTCGACACGTG-3’ and 5’-ACACAGATCTGGATACGCTG-3’. The PCR amplified product was then ligated into BglII-XbaI site of pGL3-181 instead of luciferase, and designated as the hTERT/FADD expression vector.

Transient transfection assay. Transient transfection assays by Lipofectamine-mediated gene transfer (Gibco) were performed as described previously (5,26,27). Co-transfection of B-galactosidase gene plasmid (Promega Corp.-BTC) was performed to detect transfected cells. One day before transfection, cells were seeded at 10^5 cells/ml in Lab-Tek chamber slides. The hTERT/FADD construct (1 µg) together with B-galactosidase plasmid (0.5 µg) were transfected into cells, and incubated for 48 h. The hTERT/luciferase construct was used as a negative control. To detect transfected tumor cells, cells were fixed with 1% formamide and 0.2% glutaraldehyde for 5 min, rinsed with PBS, and incubated with X-Gal buffer (0.4 mg/ml 5-bromo-4-chloro-3-indoxyl B-galactosidase, 4 mM K,F(CN), 4 mM K4Fe(CN)6, 2 mM MgCl2 in 0.1 M sodium phosphate buffer (pH 7.5)) at 37°C for overnight. Cells were visualized by phase-contrast microscopy to detect morphological alterations typical of adherent cells undergoing apoptosis, such as round-shape cells or condensed nuclei as described previously (5,27,28). Apoptotic index was determined as a percentage of apoptotic cells among 100 X-Gal-positive cells.

Effect of FADD expression under the hTERT promoter in vivo. Human glioma U373-MG cells (1.0x10^6 cells in 0.05 ml serum-free DMEM and 0.05 ml Matrigel) were inoculated subeutaneously into the right flank of 8 to 12-week-old male Balb/c nude mice (6 mice for each treatment group) as described previously (29). Tumor growth was monitored using calipers every other day. Tumor volume (V) was calculated as (L x W^2)/2, where L, length (mm) and W, width (mm) (29). To simulate the clinical situation, treatments were started after tumors were established. When the tumors reached a mean volume of 40 to 60 mm^3, the treatment was initiated. The hTERT/FADD construct (10 µg) and a cationic lipid (DEMRI,
Expression of hTERT in tumor or normal cells. To examine the expression of hTERT, semi-quantitative RT-PCR for hTERT mRNA was performed. As shown in Fig. 1, the expression of hTERT was clearly detected in malignant glioma cell lines, U373-MG, A172 and GB-1. In contrast, hTERT mRNA was not clearly detectable in cultured astrocytes TEN and fibroblasts MRC5. Relative expression level of hTERT mRNA against GAPDH mRNA was 3.05, 1.72, 1.81, 0.01 and 0.003 for U373-MG, A172, GB-1, TEN and MRC5 cells, respectively. These results suggest that exogenous expression of a therapeutic gene may be restricted to malignant glioma cells by utilizing the hTERT promoter.

Transcriptional activity of hTERT promoter in tumor or normal cells. A luciferase plasmid under the control of the hTERT promoter (hTERT/luciferase) was constructed and its transcriptional activity was compared with the pGL3 basic plasmid (pGL3/luciferase). Since luciferase activity of a plasmid differs considerably among cell lines, relative activity of the hTERT/luciferase construct against the activity of the pGL3/luciferase construct was calculated for each cell line and compared. Forty-eight hours after a transient transfection, relative luciferase activity of the cells transfected with the hTERT/luciferase construct was 98, 30, 32, 0.5 and 1 for U373-MG, A172, GB-1, TEN and MRC5 cells, respectively (Fig. 2). The three malignant glioma cells had strikingly high luciferase activity with the hTERT/luciferase construct compared to the pGL3/luciferase construct. These results suggest that the construct under the control of the hTERT promoter can deliver a therapeutic gene specifically into hTERT-positive malignant glioma cells. Additionally, a luciferase expression vector with SV40 enhancer/promoter (SV40/luciferase) was transfected to malignant glioma cells to compare the transcriptional activity with the hTERT/luciferase construct. When the transcriptional activity with SV40/luciferase was regarded as 100%, relative luciferase activity using the hTERT promoter was 6.0, 7.9 and 4.3% for U373-MG, A172 and GB-1 cells, respectively. Relatively low transcriptional activity with the hTERT/luciferase raises a question whether the construct expresses the cytotoxic gene enough to kill glioma cells.

Overexpression of FADD induces apoptotic cell death in hTERT-positive tumor cells. A FADD expression vector under the control of the hTERT promoter (FADD/hTERT) was constructed to examine the effect on malignant glioma cells. First, the expression of FADD protein after the transfection was studied. Immunohistochemical staining using FADD antibody was performed on U373-MG cells transfected with the hTERT/FADD or hTERT/luciferase construct. As shown in Fig. 3A, immunoreactivity was detected in some of the cells 48 h after the treatment with the hTERT/FADD construct. Furthermore, FADD-positive tumor cells exhibited apoptotic morphology for adherent cells (condensed or rounded) (Fig. 3A, b). These results suggest that overexpression of FADD induced apoptosis in U373-MG cells. In contrast, scarcely any cells treated with the hTERT/luciferase construct showed positive staining, indicating little endogenous FADD. Second, U373-MG and MRC5 cells were transfected with either the hTERT/luciferase or hTERT/FADD construct. To detect cells taking up the above construct, the b-galactosidase gene expression vector was co-transfected and X-Gal-positive cells were regarded as cells expressing the hTERT/luciferase or hTERT/FADD, respectively. With the hTERT/luciferase vector, almost all the cells with X-Gal-positive staining...
Figure 3. Induction of apoptosis in tumor or normal cells by overexpression of FADD. (A), Immunohistochemical staining of FADD in U373-MG cells transfected with the hTERT/luciferase (a) or the hTERT/FADD (b) construct. The cells were fixed 48 h after the transfection and incubated with FADD antibody. Arrowheads indicate FADD-positive cells. Note that FADD-positive cells show an apoptotic feature, round shape. Bars, 50 μm. (B), U373-MG cells (a and b) or MRC5 cells (c and d) were transiently transfected with β-galactosidase gene and the hTERT/luciferase or hTERT/FADD construct, respectively. Forty-eight hours later, cells were fixed and stained with X-Gal solution. The hTERT/luciferase construct was used as a control. Arrows or arrowheads indicate representative normal or apoptotic cells (X-Gal positive), respectively. (a and c), transfected with β-galactosidase gene and the hTERT/luciferase construct. (b and d), transfected with β-galactosidase gene and the hTERT/FADD construct. Bars, 100 μm. (C), Percentage of apoptotic cells in X-Gal-positive cells. Cells were transiently transfected with β-galactosidase gene and the hTERT/luciferase or hTERT/FADD construct, respectively. The transfected cells were fixed 48 h later, stained with X-Gal solution, and visualized by phase-contrast microscopy. The data shown are the mean percentage ± SD of apoptotic cells among total 100 cells of X-Gal positive cells counted. The data were collected from at least three independent experiments.

Figure 4. Effect of FADD gene transfer under the hTERT promoter on subcutaneous tumors in nude mice. U373-MG tumors were established subcutaneously in nude mice. The hTERT promoter plasmids with FADD cDNA (hTERT/FADD, 10 μg) or with luciferase gene (hTERT/luciferase, 10 μg) were directly injected with 2 μg of carrier lipid into the tumor every 24 h for 7 days. Six mice were used in each treatment group. (A), Tumor volume after the treatment for 7 days was determined with calipers. Values represent the means ± SD of the percentage of increase in tumor volume. p<0.001 compared to controls. (B), In situ end-labeling of DNA (TUNEL assay) and FADD expression in subcutaneous tumors. After the treatment for 7 days, the mice were sacrificed and the subcutaneous tumors were removed, frozen, and sectioned on a cryostat. The tumor specimens were counterstained with methyl green following the TUNEL staining (a, b). To detect the expression of FADD protein, immunohistochemical staining was performed (c, d). The consecutive sections from treated tumors were used for TUNEL and FADD stainings. Bars, 50 μm.

showed normal morphology for adherent cells, spindle shape, both in U373-MG and MRC5 cells (Fig. 3B, a and c). With the hTERT/FADD construct, however, many cells with X-Gal-positive staining showed rounded, condensed shape which is characteristic with apoptosis in U373-MG cells while MRC5 cells remained intact (Fig. 3B, b and d, respectively). Apoptotic morphology in U373-MG cells was confirmed with Hoechst 33342 staining of the nuclei (data not shown). Furthermore, transfection with hTERT/FADD was performed on two more malignant glioma cell lines and cultured astrocytes to confirm that the cytotoxic effect depends on the expression of hTERT of the cell. Percentage of apoptotic cells in X-Gal-positive cells were 26.6, 23.6, 47.2, 40.0 and 0.0 for U373-MG, A172, GB-1, TEN and MRC5 cells, respectively (Fig. 3C). These data show that the hTERT/FADD construct induced apoptosis in a significant portion of transfected cells.
It should be emphasized that apoptotic cell death took place selectively in hTERT-positive malignant glioma cells, but not hTERT-negative cells.

Effect of the hTERT/FADD treatment on tumor cells in vivo. Since results in vitro are not always substantiated in vivo, we examined if the hTERT/FADD construct gives enough anti-tumor effect in the animal model. U373-MG cells were injected subcutaneously in nude mice. When the tumor volume reached 40 to 60 mm³, treatment with the hTERT/FADD or hTERT/luciferase construct was initiated. Each construct mixed with a cationic lipid was injected directly to the tumor every 24 h for 7 days and tumor volume was compared between the two groups. Tumors treated with the hTERT/FADD construct reduced the volume by 45.0% while those treated with the hTERT/luciferase construct increased the volume by 17.0% (Fig. 4A). The treatment with the hTERT/FADD construct inhibited tumor growth significantly (p<0.0001). Slices of the removed tumors were stained to detect apoptosis or the FADD protein. As shown in Fig. 4B, the tumor treated with the hTERT/FADD construct showed TUNEL-positive, apoptotic cells (Fig. 4B, b) while few positive stained cells were detected in the control tumor (Fig. 4B). Immunohistochemical reactivity against FADD antibody was detected only in the tumor treated with the hTERT/FADD construct. These results indicate that the anti-tumor effect of the hTERT/FADD construct was mainly due to apoptosis induced by FADD expression.

Discussion

We have demonstrated that the FADD gene was successfully and specifically expressed in hTERT-positive malignant glioma cells using the hTERT promoter system. Apoptosis was induced in transplanted malignant glioma cells while hTERT-negative fibroblasts and astrocytes were spared. The in vitro studies revealed apoptosis in significant portion of malignant glioma cells after the transfection with the hTERT/FADD construct. Twenty-four to 47% of transfected cells in malignant glioma cells showed apoptotic morphology 48 h after transfection. Since the assay was performed only at one time point, the extent of cell death was underestimated due to the following reasons. Cells which died within 48 h after transfection might have been already detached from the culture plate and could not be detected. And there might be more cells induced to die more than 48 h after transfection. Therefore, to estimate what percentage of transfected cells actually die, we may need to detect apoptotic cells at many time points. Furthermore, the hTERT/FADD construct significantly inhibited tumor growth in vivo, due to induction of apoptosis, at least in part. Staining of tumor samples for TUNEL and FADD was only for one time point, which is after a 7-day treatment. Although animals are athymic, nude mice still possess macrophages and microglia in the immune system. It is possible that tumor cells which underwent apoptosis within 7 days after the initiation of the treatment might have been phagocytosed and/or removed. To examine this possibility, we may need to monitor the extent of cell death during the whole course of the treatment.

Telomerase is an RNA-protein complex which plays an important role in cancer cells. Development of a PCR-based assay to detect telomerase activity made it convenient to screen tissue samples for telomerase activity. A vast majority of malignant gliomas express the enzyme. The incidence of telomerase activity in GBMs for three different studies on a large group of 40 or more tumors ranges from 72.3 to 89% (17,18,30). On the contrary, normal brain tissues do not have telomerase except for early fetal period (20,21). Therefore, this exclusive expression of telomerase in malignant glioma makes the hTERT/FADD system theoretically attractive as a novel targeted therapy. There are some reports utilizing the promoter of hTERT to kill tumor cells selectively. Cytotoxic genes used to induce apoptosis were caspase-8 (14), BAX (31), and rev-caspase-6 (32). These studies showed that the therapeutic gene was successfully expressed in targeted tumor cells and killed them effectively. It is of interest to investigate which of these genes or FADD used in this study has the strongest effect or whether they work to a similar extent against tumor growth.

Engelhard classified gene therapy strategies against cancer into 5 categories: i) gene-directed enzyme prodrug (suicide gene) therapy; ii) gene therapy designed to boost the activity of the immune system against cancer cells; iii) oncolytic virus therapy; iv) transfer of potentially therapeutic genes into cancer cells; and v) antisense therapy (33). The present study belongs to the fourth category, transfer of therapeutic genes. One of the most challenging issues toward a clinical trial is to increase transduction efficacy of the therapeutic genes. In order to improve the efficacy, continuous delivery using an osmotic pump, microsphere or polymer may be useful particularly for malignant brain tumors. In conclusion, the present study demonstrated that the FADD gene transfer using the hTERT promoter was successful in expressing the FADD protein and inducing apoptosis in hTERT-positive malignant glioma cells. On the other hand, normal cells lacking hTERT were spared from cell death. Since anti-tumor effect was observed also in the animal model, the hTERT/FADD construct may become a promising novel therapy against malignant gliomas.

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References


