p53 gene therapy of human osteosarcoma using a transferrin-modified cationic lipidosome

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Abstract
Gene delivery via transferrin receptors, which are highly expressed by cancer cells, can be used to enhance the effectiveness of gene therapy for cancer. In this study, we examined the efficacy of p53 gene therapy in human osteosarcoma (HOSM-1) cells derived from the oral cavity using a cationic lipidosome supplemented with transferrin. HOSM-1 cells were exposed to transferrin-liposome-p53 in vitro, and the growth inhibition rate, expression of p53 and bax, and induction of apoptosis were measured 48 hours later. Treatment of HOSM-1 cells with transferrin-liposome-p53 resulted in 60.7% growth inhibition. Wild-type p53 expression and an increase in bax expression were observed following transfection with transferrin-liposome-p53, and 20.5% of the treated HOSM-1 cells were apoptotic. In vivo, the HOSM-1 tumor transplanted into nude mice grew to 5 to 6 mm in diameter. Following growth of the tumor to this size, transferrin-liposome-p53 was locally applied to the peripheral tumor (day 0) and then applied once every 5 days for a total of six times. During the administration period, tumor growth did not occur, and the mean tumor volume on the last day of administration (day 25) was 10.0% of that in the saline control group. These results suggest that p53 gene therapy via cationic lipidosome modification with transferrin is an effective strategy for treatment of osteosarcoma. [Mol Cancer Ther 2005;4(4):625–31]

Introduction
Osteosarcoma is mainly treated by surgical excision or chemotherapy, but new therapies for the tumor are being developed because the prognosis of unresectable or recurrent cases is quite poor. Gene therapy is one of the most promising strategies among these therapies, but few clinical trials of gene therapy for osteosarcoma have been done to date (1). It has been assumed that mutation of tumor suppressor genes participates in the development and progression of malignant tumors, including osteosarcoma (2), and it is anticipated that introduction of normal tumor suppressor genes into osteosarcoma cells may result in tumor growth inhibition.

The p53 gene is a tumor suppressor gene that has a regulatory function associated with DNA damage in cells. Hence, the status of the gene has an effect on the growth of cancer cells, apoptosis, and sensitivity to anticancer agents (3, 4). Mutation of the p53 gene has been frequently observed in malignant tumors (5) and is found in 24% to 42% of osteosarcomas (6, 7). It has been reported that tumors with p53 mutations show resistance to chemotherapy, and the prognosis of such tumors is poor (8). Consequently, gene therapy studies aimed at introduction of a wild-type p53 gene into cancer cells have been conducted for lung cancer (9), breast cancer (10), esophageal carcinoma (11), prostate cancer (12), and colorectal cancer (13).

Both viral and nonviral vectors have been used as carriers in gene therapy approaches. Viral vectors show high efficacy of gene transfer, but it has also been pointed out that they may be biohazards and have immunogenicity concerns (14, 15). In contrast, nonviral vectors, such as cationic liposomes, can be safely administered to humans, because they have lower toxicity and immunogenicity than viral vectors (16). Nonviral vectors do, however, have the disadvantage of low efficacy of gene transfer; therefore, improvement of gene transfer using cationic liposomes is important if such nonviral vectors are to be used clinically.

The level of expression of transferrin receptors on malignant tumor cells is higher than that on normal cells (17). Cheng first reported a method to improve the efficacy of gene transfer by binding of transferrin (the ligand for the transferrin receptor) to a cationic lipidosome (18). Subsequently, transferrin-modified cationic lipidosome systemic gene therapy has been attempted in vitro and in vivo for different tumor cells and is effective (19–21). However, to date, there are no reports of its use in osteosarcoma.

In this study, we did in vitro and in vivo introduction of a p53 gene into a transferrin receptor–expressing osteosarcoma cell line using a transferrin-modified cationic lipidosome and studied the subsequent growth inhibition and induction of apoptosis.

Materials and Methods
Cells and Culture Conditions
The human osteosarcoma cell line HOSM-1, which is derived from the mandible, was established in our
laboratory (22). HF is a normal fibroblast cell derived from human gingiva. The cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C under 5% CO2 in a humidified atmosphere. Both cell lines were tested for Mycoplasma and confirmed to be negative.

**Animals and Tumor Model**

Four- to 5-week-old, 19 to 22 g female BALB/cAJcl nude mice (Nihon Clea, Osaka, Japan) were used and housed at the Institute of Laboratory Animals, Faculty of Medicine, Mie University (Tsuj, Japan). HOSM-1 cells (1 x 106) were inoculated s.c. in the back of nude mice. After the tumor size reached between 5 and 6 mm in diameter, treatment (see below) of the animals was initiated (day 0).

**Plasmids**

The plasmid pSVp53 contains the lacZ gene under control of the SV40 promoter (Promega, Madison, WI). The p53 expression plasmid pcGSP53 containing wild-type human p53 cDNA under control of a cytomegalovirus promoter was purchased from Invitrogen. The plasmid pcDNA3.1/β-Gal was used as an empty plasmid without the p53 gene (Invitrogen).

**Identification of Mutations in the HOSM-1 Cells**

Genomic DNA was extracted from HOSM-1 cells, and exons 4 to 9 of the p53 gene were amplified as described previously (23). Exons 8 and 9 of the p53 gene were amplified using the following primers: forward 5'-TTCGGAAGCAAGGT-3' and reverse 5'-AGGCATTGAAGTCATGGA-3'.

**Preparation of the Transferrin-Liposome-DNA Complex**

Dioleoyl trimethylammonium propane (Avanti Polar Lipid, Alabaster, AL) and dioleoyl phosphatidylethanolamine (Avanti Polar Lipid) were mixed at a 1:1 molar ratio. A lipid film was prepared with a rotary evaporator, and after addition of distilled water, the liposomes were vortexed vigorously for 2 minutes. Transferrin (ion-saturated holotransferrin; Sigma, St. Louis, MO) was dissolved in HBSS (Invitrogen). Transferrin (25 μg) in 100 μL HBSS and 100 μL liposomes (100 μmol/L) were mixed in a polypropylene tube and incubated for 15 minutes at room temperature. Then, Opti-MEM (100 μL, Invitrogen) containing 2 μg plasmid DNA was added and gently mixed. After 15-minute incubation, RPMI (700 μL) was added to the tube. The final DNA/liposome/transferrin ratio was 1:10:12.5 (μg/μmol/μg).

**In vitro Transfection with the Transferrin-Liposome-pSVβ Complex**

Cells were plated on 24-well plates (1 x 104 per well) 24 hours before transfection. After the cells were washed with PBS, transfection medium (1 mL) containing the transferrin-liposome-pSVβ complex was added to each well for a 5-hour incubation period. The transfection medium was then replaced with fresh culture medium containing 10% fetal bovine serum. After culturing for 48 hours, β-galactosidase expression was measured by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining and β-galactosidase activity. Transfected cells were washed with PBS and fixed with 2% formaldehyde plus 0.2% glutaraldehyde for 5 minutes at room temperature. The fixed cells were washed twice with PBS and exposed to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in PBS containing 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L MgCl2. Cell and Molecular Technologies, Phillipsburg, NJ) at 37°C overnight. After washing, cells stained blue were counted in five random fields under a microscope to determine the percentage of blue cells per 200 cells. β-Galactosidase activity was measured with a β-Galactosidase Enzyme Assay System (Promega) according to the manufacturer’s protocol.

**Cytotoxic Assay**

Cells were seeded on 96-well plates at a density of 1 x 104 cells per well. After culturing for 24 hours, the cells were treated with seven different agents (100 μL) as follows: (a) RPMI only (as the control), (b) transferrin-liposome, (c) pcGSP53 alone, (d) liposome-pcDNA3.1/Gs complex, (e) transferrin-liposome-pcDNA3.1/Gs complex, (f) liposome-pcGSP53 complex, or (g) transferrin-liposome-pcGSP53 complex. After culturing for 5 hours, the cells were washed with PBS and the medium was replaced by RPMI supplemented with 10% fetal bovine serum. After culturing for another 48 hours, the number of viable cells was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results for each agent were compared with those for the control.

**Treatment of Tumor Xenografts in Nude Mice**

The tumor diameter reached 5 to 6 mm in all mice by 7 to 9 days after HOSM-1 cell transfer. The animals were divided into seven groups with five mice per group, and each group was treated with one of the following: (a) saline, (b) transferrin-liposome, (c) pcGSP53 alone, (d) liposome-pcDNA3.1/Gs complex, (e) transferrin-liposome-pcDNA3.1/Gs complex, (f) liposome-pcGSP53 complex, and (g) transferrin-liposome-pcGSP53 complex. The transferrin-liposome-pcGSP53 complex contained 125 μg transferrin, 100 nmol liposome, and 10 μg pcGSP53. Each agent (100 μL) was injected along the tumor margin using a 27-gauge needle. Different sites were used for each injection such that the injection points formed a circle that surrounded the tumor. The initial day of administration was defined as day 0. Administration was then repeated five times at 5-day intervals such that day 25 was the final day of administration. The tumor volume was measured during the administration period and for 25 further days after day 25.

**Western Blotting**

Cells were washed twice with cold PBS and lysed at 4°C for 30 minutes in buffer (100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 20 μg/mL aprotinin, 20 μg/mL leupeptin). Insoluble material was removed by centrifugation at 4°C for 30 minutes at 16,000 x g, and protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). The protein contents of the cell lysates were adjusted to 4 mg/mL. The cell lysates were mixed by boiling in SDS sample buffer with reducing...
 agents and applied to 10% to 15% SDS-polyacrylamide gradient gels. The proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) using the PhastSystem (Amersham Biosciences, Piscataway, NJ). Blotted membranes were blocked with 10% dried milk in TBS plus 0.1% Tween 20 for 1 hour at room temperature and incubated for 1 hour with antibodies specific for p53, bax (mouse monoclonal anti-human antibodies, Oncogene Research Products, Boston, MA), or transferrin receptor (mouse monoclonal anti-human antibodies, Zymed Laboratories, Inc., South San Francisco, CA) diluted 1:1,000 in TBS plus 0.1% Tween 20. After incubation, the membranes were extensively washed in TBS plus 0.1% Tween 20 and incubated with anti-mouse IgG horseradish peroxidase– conjugated secondary antibody diluted 1:10,000 in TBS plus 0.1% Tween 20. Band detection was carried out using an enhanced chemiluminescence system (Amersham Biosciences).

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

Apoptosis-related DNA fragmentation was detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using an Apop-Tag Plus Peroxidase In situ Apoptosis Detection Kit (Intergen, Oxford, United Kingdom). Cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) at a density of 1 × 10⁵ cells per well. After culturing for 24 hours, the cells were treated with one of the above agents (100 μL) for 5 hours. Another 48 hours later, the cells were fixed by treatment with 1% paraformaldehyde for 10 minutes. The fixed cells were washed twice with PBS and preserved in precooled ethanol plus acetic acid (2:1, v/v) for 5 minutes. After washing twice with PBS, an equilibration buffer was added to the cells. Subsequently, the cells were incubated at 37°C for 1 hour with working-strength terminal deoxynucleotidyl transferase enzyme that contained digoxigenin-labeled dUTP. The reaction was stopped by addition of prewarmed stop/wash buffer. After washing twice with PBS, an anti-digoxigenin antibody fragment carrying a conjugated peroxidase was added and the mixture was incubated in a humidified chamber for 30 minutes at room temperature. The peroxidase enzyme location in the cells was detected with 3,3′-diaminobenzidine, which is a substrate for the enzyme.

Statistical Analysis

The entire experiment was done in triplicate and the data were analyzed using Student’s t test. Ps < 0.05 were considered statistically significant.

Results

Mutation of the p53 Gene in HOSM-1 Cells

The presence of a p53 mutation in HOSM-1 cells was determined by sequencing of exons 4 to 9. This procedure confirmed that a mutation was present in one allele in codon 306, which was changed from CGA to TGA, thereby creating a stop codon in exon 8.

Expression of Transferrin Receptor

Transferrin receptor expression in HOSM-1 cells was compared with that in normal fibroblast HF cells using Western blotting. As shown in Fig. 1, bands of ~95 kDa, corresponding to the molecular weight of transferrin receptor, were identified for both HOSM-1 and HF cells, but transferrin receptor expression in HOSM-1 cells was higher than in HF cells.

Transfection Efficiency with the Transferrin-Liposome-pSVβ Complex

Transfection efficiency using the transferrin-liposome or the unmodified liposome was evaluated by measurement of the β-galactosidase activity and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. The β-galactosidase activity in HOSM-1 cells transfected with the liposome-pSVβ complex was 35.6 ± 9.0 mU/mg protein, whereas that following transfection with the transferrin-liposome-pSVβ complex was 151.0 ± 12.1 mU/mg protein. β-Galactosidase activities of cells transfected with medium alone, transferrin-liposome alone, and pSVβ alone were all below the detection limit. In the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining assay, 17.0 ± 2.2% of cells treated with liposome-pSVβ and 31.0 ± 4.0% of cells treated with transferrin-liposome-pSVβ were stained blue.

Cytotoxic Effects

The numbers of viable HOSM-1 cells relative to the control in liposome-p53-treated cells and transferrin-liposome-p53-treated cells were significantly lower than those found after other treatments (P < 0.05). Growth inhibition rates of 43.6% and 60.7% were observed for cells treated with liposome-p53 and transferrin-liposome-p53, respectively, and the growth of transferrin-liposome-p53-treated cells was significantly inhibited compared with that of liposome-p53-treated cells (P < 0.05; Fig. 2). In HF cells, each treatment had little effect on cell survival.

Expression of p53 and bax

As shown in Fig. 3, expression of exogenous wild-type p53 was detected in liposome-p53-treated cells (lane 4) and...
transferrin-liposome-p53-treated cells (lane 5) but not in cells that underwent other treatments. Furthermore, the expression of p53 following transferrin-liposome-p53 treatment was slightly higher than that following liposome-p53 treatment. On the other hand, decreased expression of endogenous mutated p53 was observed in transferrin-liposome-p53-treated cells compared with that in the control group. We also evaluated the expression of bax, which is a target gene in the p53-dependent apoptosis pathway. Bax expression increased distinctly in cells treated with transferrin-liposome-p53, but little change was observed in the liposome-p53-treated cells.

**Growth Inhibitory Effect of Transferrin-Liposome-p53 on Tumors Transplanted into Nude Mice**

On day 0, each group of mice had an average tumor volume in the range 60 to 70 mm³. During the administration period, the tumors in the saline group continued to grow and had increased by 5.9 times to an average of 418.7 mm³ on day 25. Tumor growth was also seen in the liposome-p53 group during the administration period, with an increase in tumor volume of 1.6 times to an average of 118.0 mm³ on day 25; this, nonetheless, reflected relative growth inhibition compared with the saline group. In contrast, during administration of transferrin-liposome-p53, the tumor gradually reduced in size, with the tumor volume significantly decreased by 36.6% from an average of 66.1 mm³ on day 0 to 41.9 mm³ on day 25. Continued observation from completion of administration (day 25) until day 50 revealed an even steeper tumor growth curve in the saline group and a growth rate in the liposome-p53 group comparable with that in the saline group, with the tumor volume on day 50 reaching 2,218.9 and 926.1 mm³ in the saline and liposome-p53 groups, respectively. In contrast, the tumors in the transferrin-liposome-p53 group only began to grow after day 35 and at a considerably slower rate compared with the other groups, with the volume on day 50 being no greater than 186.4 mm³, 8.4% of that in the saline group (Fig. 4).

**TUNEL Assay**

*In vitro*, TUNEL-positive cells that stained brown were found in each treatment group. The percentage of TUNEL-positive cells in a field, which is called the apoptotic index, was 3.3 ± 0.45% in the control but was markedly increased to 12.9 ± 1.6% in liposome-p53-treated cells and 20.5 ± 1.7% in transferrin-liposome-p53-treated cells (Fig. 5). *In vivo*, the tumor was extirpated 48 hours after completion of the final administration (day 27) and observed by the TUNEL assay. TUNEL-positive cells were sparse in the physiologic saline group but were particularly prevalent in the periphery of the tumor in the liposome-p53 or transferrin-liposome-p53 groups in proximity to the local injection site. For more central regions of the tumor, no differences in TUNEL-positive cells were noted between these groups and the saline group (Fig. 6).

**Discussion**

The purpose of this study was to confirm whether transferrin-liposome-mediated p53 gene therapy is effective against growth inhibition of osteosarcoma with p53 mutations. Due to transferrin binding to the transferrin receptor, the efficacy of gene transfer with a transferrin-modified liposome increased by 3.7 times and the growth inhibition effect increased by 1.4 times compared with use of the unmodified liposome. The expression of exogenous wild-type p53 was higher following transfection with transferrin-liposome-p53 compared with transfection with unmodified liposome-p53. Therefore, the increase in the efficacy of p53 gene transfer and the subsequent increase in p53 expression seemed to have an effect on enhancement...
of HOSM-1 growth inhibition. The results of TUNEL staining suggested that this growth inhibition effect is produced by induction of apoptosis through p53. Cells are directed to cell cycle termination or apoptosis by increased p53 expression, and the transcriptional activity of the bax gene is directly associated with the p53-dependent apoptosis pathway (24). Bax activation is maintained in the presence of wild-type p53 activity, but, as shown in HOSM-1 cells carrying a mutation in codon 306 of p53, apoptosis via bax is markedly reduced when p53 function is diminished (25). The introduction of exogenous wild-type p53 enhanced bax expression in HOSM-1 cells, and induction of apoptosis by transferrin-liposome-p53 treatment occurred through a bax-dependent pathway. Slight bax expression was also seen in HOSM-1 cells in the control group, suggesting that the cells have a p53-independent pathway through which bax can be regulated (26). It has also been reported that other genes, such as PIG3 (27), Noxa (28), and p53 AIP1 (29), can act as target genes in p53-induced apoptosis. However, previous reports (30, 31) have confirmed an increase in bax expression after p53 gene therapy, and this is consistent with our results. Therefore, we conclude that bax plays an important role in the antitumor effect elicited by p53 gene therapy.

It has been noted previously that osteosarcomas with p53 mutations show resistance to chemotherapy and that their prognosis is poor (32, 33). Hence, studies of p53 gene therapy for osteosarcoma have been implemented (34). Many such studies have used viral vectors, and growth inhibition in vitro has been reported to be ~60% (34). In the current work, we obtained a growth inhibition rate for HOSM-1 cells of 60.7% following transfection with transferrin-liposome-p53. Nonspecific growth inhibition by viral infection may be a consequence of gene therapy using viral vectors, but the nonspecific growth inhibition of HOSM-1 cells caused by transfection with transferrin-liposome was only ~10% (Fig. 2). Furthermore, transferrin-liposome alone showed no cytotoxicity against normal gingiva-derived fibroblasts.

The in vivo antitumor effect of transferrin-liposome-p53 on the HOSM-1 tumor was considerably higher than expected based on the in vitro results. Treatment with liposome-p53 inhibited tumor growth during the administration period but allowed tumor growth at a rate similar to that in the saline group after completion of administration, whereas growth after completion of administration of transferrin-liposome-p53 was relatively low. In the transferrin-liposome-p53 group, the tumor...
The greatest benefit of the transferrin-liposome system is that it can be formulated by simply mixing each component with the plasmid DNA. First, the slightly negatively charged transferrin and the cationic liposome are electrostatically associated, and no chemical modification is necessary. The formulation can then be made by introduction of the negatively charged plasmid DNA, which will bind to the cationic liposome. In addition, it seems that the transferrin not only increases the uptake of DNA into cells but also plays an indirect role in enhancement of gene expression after uptake of the transferrin-liposome-DNA complex. The transferrin receptor is bound to the transferrin-liposome-DNA complex within cells, and transferrin may facilitate the escape of DNA from the endosome, because endosome escape is a normal physiologic process for transferrin and its receptor complex, thus making more DNA available for gene expression (18).

Binding of negatively charged transferrin to a cationic liposome reduces the overall charge of the assembly compared with the cationic liposome alone, and most of the complexes formed between this assembly and plasmid DNA are of neutral charge (37). Therefore, it is possible that a transferrin-modified cationic liposome might have a smaller amount of encapsulated plasmid DNA compared with a cationic liposome alone and that the transferrin-liposome-DNA complex might be less adhesive to the surface of cells. However, Xu et al. have reported that the amount of plasmid DNA bound to a transferrin-liposome is similar to that bound to an unmodified liposome. Regarding cell adhesiveness, it is thought that the transferrin-liposome-DNA complex is compressed to less than a third of an unmodified liposome-DNA complex, making it more susceptible to endocytosis and overcoming the effects of the reduced charge (37).

The sensitivity of carcinomas to anticancer agents (34) and radiosensitivity (19, 31) is increased by p53 gene therapy, so the effect of the therapy described in this study is likely to be further increased by combination with anticancer agents or radiotherapy. Regarding the transferrin-liposome-p53 administration schedule, administration once every 5 days inhibited tumor growth during the administration period and until 10 days after completion of administration, suggesting that long-term maintenance administration about once weekly may stop tumor growth; that is, it may induce “tumor dormancy.” Hence, a future
challenges will be to investigate the antitumor effects of long-term administration of transferrin-liposome-p53 as well as to examine survival and safety in tumor-bearing mice.

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