Mesenchymal stem cells modified to express lentivirus TNF-α Tumstatin45–132 inhibit the growth of prostate cancer

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Abstract

Mesenchymal stem cells (MSCs) are a potential novel delivery system for cell-based gene therapies. Although tumour necrosis factor (TNF)-α has been shown to have antitumour activity, its use in therapy is limited by its systemic toxicity. For the present study, we designed lentivirus-mediated signal peptide TNF-α-Tumstatin45–132-expressing mesenchymal stem cells (SPTT-MSCs) as a novel anticancer approach. We evaluated the effects of this approach on human prostate cancer cells (PC3 and LNCaP) by co-culturing them with either SPTT-MSCs or supernatants from their culture medium in vitro. The antitumour effects and possible mechanisms of action of SPTT-MSCs were then determined in PC3 cells in vivo. The results showed that efficient TNF-α-Tumstatin45–132-expressing MSCs had been established, and demonstrated that SPTT-MSCs inhibited the proliferation of and induced apoptosis in prostate cancer cells and xenograft tumours. As would be expected, given the properties of the individual proteins, the TNF-α-Tumstatin45–132 fusion exerted potent cytotoxic effects on human prostate cancer cells and tumours via the death receptor-dependent apoptotic pathway and via antiangiogenic effects. Our findings suggest that SPTT-MSCs have significant activity against prostate cancer cells, and that they may represent a promising new therapy for prostate cancer.

Keywords: mesenchymal stem cells • TNF-α • Tumstatin • prostate cancer • gene therapy

Introduction

Prostate cancer is the most commonly diagnosed cancer in men in the western world and the second leading cause of cancer death [1]. Despite recent advances in early detection and new therapeutic approaches, the occurrence of metastatic disease due to androgen independence and resistance to existing treatments is common [2–5]. New therapies that can limit the local advancement of primary tumours and distant metastases are urgently needed for prostate cancer.

The increase in knowledge about the molecular biology of prostate cancer and genetic engineering have made gene-based cancer therapy attractive. Unfortunately, the development of an efficient, safe, and reliable gene delivery system (a critical step) has so far limited the successful implementation of gene therapy. However, recent studies have shown the feasibility of using mesenchymal stem cells (MSCs) as a cellular vehicle for gene therapy [6–10]. MSCs have greater potential to contribute to the population of stromal cells than fully differentiated fibroblasts. This allows the development of therapeutic strategies that are based on the local production of tumoricidal biological agents by MSCs transduced to express specific genes of interest. Demonstrating this capacity, MSCs have previously been shown to exert antitumour effects when transduced with adenoviral vectors expressing a variety of transgenes [11–18]. Nevertheless, transduction of MSCs with adenoviral vectors is relatively inefficient compared with lentiviral gene transfer, as a result of the limited expression of the cellular coxsackie and adenovirus receptor on these cells [6, 11]. As a result of this inefficient transduction, the therapeutic transgene is diluted over time, limiting the use of adenoviral vectors to deliver gene therapy via MSCs. In contrast, lentivirus (LV)-based gene transfer compares favourably with other gene
transfer methods for introducing genes into MSCs [19]. In particular, lentiviral vector systems have been shown to efficiently transduce both dividing and non-dividing cells, and are less prone to transcriptional silencing than retroviral systems, making lentiviral systems the ideal approach for gene delivery into MSCs [20, 21].

Tumour necrosis factor (TNF-α), an inflammatory cytokine, is cytotoxic to tumour cells, and also disrupts tumour blood vessels. However, the clinical use of TNF-α as an antitumour drug is hampered by its severe systemic toxicity [22]. Another anticancer molecule with anti-angiogenic activity, Tumstatin, exerts its effects on the tumour vasculature via its binding to \( \alpha V \beta 3 \) on endothelial cells and the tumour vascular endothelium, which shows increased expression of \( \alpha V \beta 3 \). The region of Tumstatin spanning amino acids 54–132 is responsible for the observed in vitro and in vivo anti-angiogenic activity of the protein [23]. Luo et al. explored the possibility of fusing Tumstatin45–132 with human TNF-α in the hope of generating a specifically targeted, bi-functional protein for cancer therapy. In that study, the Tumstatin45–132-TNF-α fusion protein inhibited endothelial cell proliferation to a greater extent than TNF-α alone, and led to a similar level of apoptosis against L929 mouse fibroblast cells [24].

In the present study, we describe the potential of using genetically modified MSCs that constitutively express TNF-α-Tumstatin45–132 to inhibit the proliferation of prostate cancer cells in vitro and reduce xenograft tumour growth in vivo. We also examined the mechanisms of action of the approach.

Materials and methods

Cell culture

MSCs were isolated from bone marrow of 8-week-old Wistar rats and cultured as previously described [25, 26]. PC3, LNCaP and ECV304 cells were cultured in Dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). Human umbilical vein endothelial cells (HUVEC) cells were generously provided by Dr. E. Tabengwa (University of Alabama at Birmingham, Birmingham, AL, USA) and cultured in MG199 containing 10% FBS.

Cloning and transfection

The signal peptide TNF-α-Tumstatin45–132 (SPTT) fragment was generated by PCR amplification using pBV220-TNF-α-Tumstatin445–132 (kindly provided by Dr. Y. Luo, The Affiliated Anhui Provincial Hospital of Anhui Medical University, Hefei, People’s Republic of China) as a template. The primers used were SPTT-S (5’-GCGGATCCATGTGGCTGCAGAGCCTGCT-3’) and SPTT-A (5’-GCGAATTCGACATCTCTCTGACGACAGGACCTGCAAACTGTCCTG66GTGCGACTGTCGCTGCGACTGTCGCTGCTCAGACGTTCACCTCGAG-3’) and SPTT-A (5’-GCGAATTCGACATCTCTCTGACGACAGGACCTGCAAACTGTCCTG66GTGCGACTGTCGCTGCGACTGTCGCTGCTCAGACGTTCACCTCGAG-3’). The signal peptide is underlined in the sense primer. A BamH I restriction site (codons in bold) was introduced into the 5’ end of the SPTT-S primer, and an EcoRI restriction site (codons in bold) was introduced into the 5’ end of the SPTT-A primer. The SPTT fragment was then subcloned into LV transfer vector FUGW (F, HIV-1 flame sequence; U, human polyubiquitin promoter; G, green fluorescent protein; W, woodchuck hepatitis virus post-transcriptional regulatory element) by BamH and EcoRI double digestion. Recombinant lentiviral particles were produced in 293T cells by transient cotransfection involving a three-plasmid expression system [27], and MSCs were transacted as described previously [28]. For FACS analysis, transduced MSCs were trypsinized and analysed on a FACScalibur instrument, and data were analysed using CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). Expression levels of SPTT were verified by ELISA and Western blotting analysis.

Cell growth curves, ex vivo differentiation and DNA contents of transduced MSCs

The growth curve analysis and DNA quantitation of transduced MSCs were performed as described previously [29]. In addition, cultured transduced MSCs were tested for the ability to differentiate into osteogenic and adipogenic lineages using a previously described protocol [29] with modifications. Briefly, the transduced and parental MSCs were plated at a density of 3 × 10³ cells per well in either osteogenic media (supplemented with 100 mM β-glycerophosphate, 50 mg/l ascorbic acid, 4 mg/l bFGF and 10⁻⁸ M dexamethasone; Sigma, St. Louis, MO, USA) or adipogenic media (supplemented with 10 μg/ml insulin, 10⁻⁶ M dexamethasone; Sigma) for up to 3 weeks. The conditioned media were changed every 3–4 days. Evidence of osteogenic differentiation was determined by alkaline phosphatase (ALP) staining, whereas adipogenic differentiation was monitored using oil red 0 staining for the appearance of intracellular lipid inclusion vacuoles.

Cell viability assay

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay as described previously [30]. Briefly, PC3, LNCaP and ECV304 cells were seeded in 96-well plates at 3 × 10³ cells per well, and were incubated at 37°C for 24 hrs. Supernatants from normal MSCs, and LV-EGFP- and LV-SPTT-transduced MSCs were added to the plates, which were then returned to the incubator for 2 days. Untreated cells served as a control. MTT (5 mg/ml) was added to each well for the last 4 hrs of treatment. The reaction was stopped by the addition of dimethyl sulphoxide, and the optical density was determined at 570 nm on a multiwell plate reader (Bio-TEK, Winooski, VT, USA). Background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean for each experiment was calculated. Results were expressed as a percentage of the control, which was considered to be 100%.

Cell proliferation assay

The effects of the infusion protein SPTT on cell proliferation were determined by BrdUrd incorporation assay (Oncogene, La Jolla, CA, USA), following the manufacturer’s protocol. Cells were seeded in 96-well plates (6 × 10³ cells per well) and incubated with supernatants from normal MSCs, LV-EGFP- transduced MSCs or LV-SPTT-transduced MSCs for 24 hrs. BrdUrd was added to the medium 8 hrs before termination of the experiment. The BrdUrd incorporated into cells was determined by anti-BrdUrd antibody, and absorbance was measured at dual wavelengths of 450/540 nm with an OPTImax microplate reader (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed in triplicate. Results were expressed as a percentage of the control, which was considered to be 100%.
Detection of apoptosis

Cells in early and late stages of apoptosis were detected using an Annexin V-FITC apoptosis detection kit from Bio-Vision (Mountain View, CA, USA), according to the manufacturer’s protocol. In brief, 2–3 × 10^5 cells were exposed to the supernatants from normal MSCs, LV-EGFP- transduced and LV-SPTT-transduced MSCs and incubated for 48 hrs prior to analysis. The samples were analysed using a Becton-Dickinson FACS Calibur instrument (Ex = 488 nm; Em = 530 nm). Cells that were positive for Annexin V-FITC alone (early apoptosis) and Annexin V-FITC and PI (late apoptosis) were counted. All samples were assayed in triplicate. Results were expressed as a percentage of the control, which was considered to be 100%.

In vitro co-culture of prostate cancer cells with MSCs

PC3 cells (5 × 10^4 per well) were cultured either alone or with EGFP-MSCs or SPTT-MSCs in six-well plates (1 × 10^4 cells per well) for 72 hrs. Cells were then trypsinized, counted, and fixed with 70% ethanol overnight. The relative numbers of MSCs (diploid cells) and PC3 cells (aneuploid cells) were determined after labelling the cells with propidium iodide (Sigma) using ModFit software (Becton-Dickinson).

Endothelial tube formation assay

The endothelial tube formation assay was performed following the manufacturer’s protocol (BD Biosciences, Franklin Lakes, NJ, USA). Matrigel was added (100 μl) to each well of a 24-well plate and allowed to polymerize. A suspension of 2 × 10^4 HUVEC cells was seeded into each well. The cells were then treated with conditioned medium from either EGFP-MSCs or SPTT-MSCs. Control cells were incubated with medium alone. Cells were incubated for 24–48 hrs at 37°C and viewed using a microscope. The cells were then photographed and the number of tubes was counted.

Animals and treatments

Six-week-old male Balb/c nu/nu mice (Laboratory Animal Center of Shanghai, Academy of Sciences, China) were injected with 2 × 10^6 PC3 cells in 200 μl PBS. After 2 weeks, subcutaneous tumours had reached approximately 50 mm^3, and were directly injected with 2 × 10^6 MSCs that had been transduced with LV-EGFP (MOI 25) or LV-SPTT (MOI 25). The transduced MSCs were trypsinized and washed three times with PBS before their in vivo use. Two additional groups of mice bearing PC3 xenografts were injected with PBS or non-transduced MSCs as controls. Three animals per group were analysed for tumour growth. The MSCs were injected three times at weekly intervals. The growth of the tumours was followed for one month. Tumour measurements in two dimensions were performed with calipers twice weekly and tumour volumes were calculated using the formula: volume = width^2 × length × 0.5. The animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Western blotting

MSCs were transduced with LV-EGFP and LV-SPTT at a MOI of 25. After 72 hrs, the transduced cells were collected and lysed with RIPA buffer (Cell Signaling, Boston, MA, USA). PC3 cells co-cultured with MSCs, EGFP-MSCs and SPTT-MSCs at a 2:1 ratio for 48 hrs in a transwell system were also collected and lysed with RIPA buffer. After centrifugation at 13,000 rpm for 15 min. at 4°C, the supernatant was removed and kept for analysis. Total cellular protein concentrations were assessed using a Bio-Rad protein assay kit (Hercules, CA, USA). Aliquots containing identical amounts of protein were fractionated by SDS-PAGE, then transferred to methanol pre-activated PVDF membranes (Millipore, Termecula, CA, USA). Membranes were blocked and sequentially incubated with primary and secondary antibody, then bands of the proteins of interest were visualized using the ECL plus system from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Immunohistochemistry

Animals were killed approximately 4 weeks after treatment, and the tumour tissues were harvested, and tumour weights were compared. Paraffin sections (5-mm thick) were made from the harvested tissue. Tissue sections were de-waxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol before washing the slides in water. Slides were incubated in citrate buffer (pH 6.0) for 20 min. in a steamer for antigen retrieval. Nonspecific binding was blocked by incubation in 5% (v/v) normal serum. The primary antibodies used in the following dilutions: proliferating cell nuclear antigen (PCNA, 1:100; CD31, 1:200 and TNF-α, 1:2000. Following binding of the primary antibodies, slides were incubated with the respective secondary antibodies conjugated to horseradish peroxidase. The bound antibody was visualized using the peroxidase-based Vectastain Elite ABC Kit (Boster, Wuhan, China). The substrate reaction was stopped by washing the slides in running water. Finally, the slides were lightly counterstained in haematoxylin.

TUNEL staining

Apoptotic cells were visualized using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay. The TUNEL procedure was performed with an in situ cell death detection kit (Boster) according to the manufacturer’s instructions. TUNEL+ apoptotic tumour cells were counted in 10 consecutive fields at 40× magnification in the tissue sections.

PCR and RT-PCR

Genomic DNA was isolated from the transduced MSCs by phenol-chloroform extraction and PCR was performed to detect the integration of the LV genome using primers specific for the ubiquitin promoter of the transfer vector. Total RNA was extracted from MSCs, and mock- and SPTT-transduced MSCs using Trizol (Invitrogen) according to the manufacturer’s instructions. Equal quantities of RNA were subjected to reverse transcription followed by PCR using primers specific for SPTT and Oct4 as described in Table 1. β-actin was used as an internal control.

Statistical analysis

All data were expressed as means ± S.D. SPSS software was used to analyse the data using the Student’s t-test. All reported P-values are two-tailed, and those <0.05 were considered statistically significant.
Results

Efficient lentiviral transduction of MSCs

The MSCs were transduced with recombinant LV at a multiplicity of infection (MOI) of 25, and were found to efficiently express either the reporter gene (EGFP) or the SPTT target gene. The MSCs transduced with LV-EGFP showed evidence of a green fluorescent signal in almost all cells (Fig. 1A-I, II), and FACS analysis revealed that 96.8% of all cells expressed the transgene (Fig. 1A-III). To confirm the expression of the transgene, MSCs transduced with LV-EGFP and LV-SPTT were harvested, and protein lysates were prepared 72 hrs after transduction. Western blotting indicated that the SPTT protein was detectable in the cells transduced with LV-SPTT (Fig. 1A-IV). To determine the transduction efficiency of LV-SPTT in MSCs, the cells were harvested three days after transduction, fixed on slides, and subjected to immunohistochemistry using a human TNF-α/H9251 antibody. The results indicated that the LV-EGFP-transduced MSCs were negative for the transgenic SPTT TNF-α/H9251 protein (Fig. 1A-V), whereas more than 90% of the MSCs transduced with LV-SPTT were positive for SPTT (Fig. 1A-VI). PCR results showed that the ubiquitin promoter gene was detectable in the genomic DNA of both LV-EGFP and LV-SPTT-transduced MSCs, that the SPTT gene was only detectable in the LV-SPTT-transduced MSCs, and that both the cells were still positive for Oct4, a stem cell marker (Fig. 1B).

Table 1
Table 1 Primer sequences for the amplification of target genes and β-actin

| Gene   | Primer sequence (5’-3’) | Product size | Annealing temp. |
|--------|-------------------------|--------------|-----------------|
| Ubiquitin | For: GTCTTGAGGCTTCTGCTAAT | 414 bp       | 59°C            |
|         | Rev: AACCAGAGCTTCAGCTACTT |              |                 |
| SPTT    | For: ATGGGCTGACAGCCCTGCTG | 813 bp       | 63°C            |
|         | Rev: TCGGCGATCGGAGACCTTC  |              |                 |
| Oct4    | For: ATACAGAGGCCGATGG    | 397 bp       | 60°C            |
|         | Rev: GTGCATAGTCGCTGTTGA  |              |                 |
| β-actin | For: CACGAAACTACCTTCAACTCC | 600 bp      | 60°C            |
|         | Rev: CATACTCCTGTGGCTGATC |              |                 |

Stem cell properties of MSCs are not affected by LV transduction or transgene expression

As shown in Fig. 1C, EGFP-MSCs and SPTT-MSCs exhibited virtually identical growth curves (Fig. 1C, upper panel) and cell cycle profiles (Fig. 1C, lower panel) compared to those of untransduced parental cells. Analysis of DNA content showed that all of the MSCs had a high number of cells in the G1 phase, and a lower number in the S phase. The virally transduced MSCs retained their normal differentiation potential. Three weeks after initial lentiviral transduction, the EGFP-MSCs, SPTT-MSCs, and non-transduced parental MSCs were induced with adipogenic or osteogenic conditioned media. Cells that had differentiated into the adipogenic lineage were indicated by positive staining with oil Red O for intracellular fat droplets, whereas cells of osteogenic lineage stained positive for ALP (Fig. 1D).

SPTT inhibits the growth of prostate cancer cells and endothelial cells in vitro

ELISA experiments showed that up to 250 pg/ml of the SPTT protein could be detected in the supernatant of SPTT-MSCs between 3 and 4 days after transduction (Fig. 2A), which amounted to approximately 4 × 10⁻⁴ pg/cell. MTT assays demonstrated that the viability of human prostate cancer (PC3 and LNCaP) and endothelial (ECV304) cells was decreased by 51.6% (P < 0.004), 57.8% (P < 0.02) and 16.2% (P < 0.002), respectively (Fig. 2B–D). In contrast, the supernatants of normal MSCs and mock-transduced MSCs did not affect the growth of any of the cell lines. Demonstrating the activity of the fusion protein, only SPTT was able to decrease the viability of ECV304 endothelial cells, whereas TNF-α alone did not lead to any significant change (Fig. 2D).

SPTT decreases the proliferation of prostate cancer cells in vitro

We then examined the effects of SPTT on prostate cancer cell proliferation. Treatment with the fusion resulted in anti-proliferative effects in both PC3 and LNCaP cells (Fig. 3A and B), with a decrease in the proliferation rate by 46.6% (P < 0.001) and 50.8% (P < 0.001) in PC3 and LNCaP cells, respectively. The supernatants of normal MSCs and mock-transduced MSCs did not affect the proliferation of either cell line.

Induction of apoptosis by SPTT

We next determined whether SPTT induced apoptosis in inducing apoptosis of prostate cancer cells. PC3 and LNCaP cells were treated with conditioned media from SPTT-MSCs for 48 hrs, and apoptosis was analysed by Annexin V-FITC/PI staining. The apoptosis rate of prostate cancer cells significantly increased following
exposure to conditioned media from SPTT-MSCs (PC3 15.2%; LNCaP 25.2%). In contrast, the conditioned media from MSCs (PC3 6.0%; LNCaP 9.2%) and EGFP-MSCs (PC3 6.2%; LNCaP 10.0%) did not cause any major increase in apoptosis, showing levels comparable to control cells (PC3 5.7%; LNCaP 9.6%) (Fig. 3C and D).

**Co-culture with SPTT-MSCs inhibits the growth of PC3 cells in vitro**

We next examined the therapeutic potential of MSCs as cellular vehicles for production of SPTT using a co-culture system with PC3 cells under *in vitro* conditions. When the two cell lines were co-cultured, the SPTT-MSCs directly inhibited the growth of the malignant prostate cells (Fig. 4A), whereas co-culture with mock-transduced MSCs did not have this inhibitory effect.

**Inhibition of angiogenesis by SPTT in vitro**

The ability of SPTT to block angiogenesis *in vitro* was evaluated using the endothelial tube formation assay. When HUVEC cells are cultured on matrigel, they rapidly align and form hollow tube-like structures. Compared to cells cultured with control media (Fig. 4B-I)
and conditioned media from EGFP-MSCs (Fig. 4B-II), the conditioned media of SPTT-MSCs significantly inhibited endothelial tube formation (Fig. 4B-III). The extent of tube formation after treatment with conditioned media from SPTT-MSCs was less than half of that observed in the group treated with media from EGFP-MSCs (Fig. 4C).

LV-SPTT-transduced MSCs inhibit the growth of PC3 xenografts in mice

Given its potent in vitro activity, we wanted to know whether MSCs transduced with LV-SPTT would also have antitumour activity in vivo. Mice bearing PC3 xenograft tumours were injected peritumourally with parental (non-transduced) MSCs, EGFP-MSCs or SPTT-MSCs. Representative tumours harvested at the conclusion of the experiment are shown in Fig. 5A. Although the tumour mass was markedly smaller in the mice treated with SPTT-MSCs, the LV-EGFP-transduced MSCs did not lead to any significant difference in tumour size or weight compared to the mice treated with PBS or the control MSCs (Fig. 5A and B). The differential effects of the control MSCs and SPTT-MSCs were further illustrated by the tumour growth curves (Fig. 5C). All of the tumours in the animals treated with PBS, non-transduced MSCs, or LV-EGFP-transduced MSCs grew to about six times their original size. In contrast, the tumours treated with the LV-SPTT-transduced MSCs only grew by a factor of 2.0.

LV-SPTT-transduced MSCs decrease tumour cell proliferation, promote apoptosis and decrease tumour vascularity

Immunohistochemical examination of the expression of PCNA revealed that the number of proliferating tumour cells was significantly lower in the tumours from mice in the LV-SPTT-transduced MSCs group compared with the groups treated with PBS, MSCs
The use of recombinant proteins for the treatment of cancer is often limited by their short half-life or excessive toxicity. More
Both highly efficient transduction of MSCs and efficient expression of the antitumour transgenic protein are needed in order to use the MSCs for therapeutic applications. Although many approaches have previously been tested on MSCs, including adenoviral vectors, adeno-associated viral vectors, and retroviral vector-based systems, most of these were unsuitable due to their relatively low transduction efficiency or unstable transgene expression [19]. We have recently observed highly efficient transduction of MSCs using a LV system. The transduction efficiency of MSCs reached up to 96.8%, and the transduced MSCs persistently expressed the transgene for more than 6 months while maintaining their capacity for self-renewal, differentiation, and survival after transplantation (data not shown). This efficient, safe, and reliable gene delivery system provides a platform for MSC-based gene therapy, and was the basis for the present study.

Prostate cancer is one of the most common malignant diseases and is the most lethal urological malignancy. The acquired drug resistance of tumour cells and cumulative side-effects of the cytotoxic agents used to treat advanced prostate cancer present serious clinical obstacles. It is necessary to explore new therapeutic strategies that can either enhance the effects of existing antitumour therapies or that exhibit potent activity against tumours and low cytoxicity to normal tissues. MSC-based gene therapy may present just such an approach.

A large number of animal studies have shown that TNF-α has potent antitumour activity; however, the clinical use of TNF-α as an anticancer drug is hampered by its severe systemic toxicity. In addition, several malignant cell lines and most normal cells (including endothelial cells) are resistant to TNF-α. However, the combination of TNF-α with other molecules, and tumour-targeted delivery of TNF-α, still represent alternative approaches for its use as cancer therapy [24, 39–41]. Angiogenesis is a major pathogenic step in the process of tumour growth and metastasis, and tumour angiogenesis has become an important area in cancer treatment. Tumstatin, an endothelial cell-specific protein, has been shown to suppress tumour growth in human renal carcinoma (786O) and prostate carcinoma (PC3) in mouse xenograft models, and to induce endothelial cell-specific apoptosis [42–44]. Therefore, combining TNF-α with Tumstatin may provide a new therapeutic molecule for prostate cancer that is directly cytotoxic and can decrease angiogenesis.

In the present study, we established a LV-SPTT-transduced MSC line that efficiently expressed a human TNF-α-Tumstatin45–132 fusion protein. MTT assays indicated that the LV-SPTT-transduced MSCs inhibited the growth of prostate cancer cells (PC3 and LNCaP) and endothelial cells (ECV304) in vitro. Because recombinant human TNF-α alone was not able to suppress the proliferation of ECV304 cells, this suggests that the inhibitory effect of SPTT on ECV304 cells was due to the Tumstatin45–132 domain of the fusion protein. We found that the growth inhibitory effect was due to both suppression of proliferation and induction of apoptosis by SPTT. Proliferation and cytotoxicity assays indicated that the TNF-α-Tumstatin45–132 fusion protein retained both its TNF-α-like activities (demonstrated by its cytoxicity to PC3 cells), as well as its Tumstatin45–132-like activity.
indicates that the fusion of TNF-α and Tumstatin45–132 by a linker amino acid does not interrupt the anticancer activities of the individual proteins. The activity of the SPTT-MSCs was further demonstrated in the PC3 xenograft prostate cancer model. Based on our observations, we suggested that the inhibition of tumour growth by LV-SPTT-transduced MSCs occurred through both induction of apoptosis and inhibition of proliferation. To confirm the mechanisms of action of the LV-SPTT-transduced MSCs, we evaluated the expression of apoptotic effector caspases-8 and −3 in PC3 cells treated with the SPTT-MSCs or their culture medium. The cleavage of caspases-8 and −3 increased following exposure to the SPTT-MSCs, but not to any other MSCs, providing evidence to confirm our hypothesis. An up-regulation of the pro-apoptotic/anti-apoptotic protein (Bax/Bcl2) ratio also supported our observed effects on apoptosis. Because ERK and Akt signalling plays a key role in the cell growth and survival of a variety of cell types, we then evaluated the changes in ERK-1/2, p-ERK-1/2, Akt and p-Akt in vitro and in vivo after treatment with SPTT-MSCs to further examine the mechanism(s) of action of the approach. Decreased phosphorylation of ERK-1/2 and Akt was observed both in vitro in cells co-cultured with the SPTT-MSCs and in vivo in xenograft tumours, suggesting that the antitumour effects may be at least partially mediated through the effects on

Fig. 5 SPTT-MSCs suppress tumour growth in vivo. (A) Tumours from animals in the SPTT-MSCs-treated group were smaller than those treated with the controls. (B) The tumours from mice treated with SPTT-MSCs weighed much less than those from mice treated with the control MSCs or PBS. (C) Tumour growth curves showed that treatment with SPTT-MSCs suppresses tumour growth. Results reflect the average of three animals per group (*P < 0.05 compared to mock-transduced cells).

Fig. 6 Immunohistochemical analysis of proteins related to cell proliferation, apoptosis, and the formation of neovasculature. For immunohistochemical analysis of tumour cell proliferation, apoptosis and microvessel density, tumours were paraffin embedded and sectioned. (A) Staining was performed with anti-PCNA antibody to assess proliferation, while TUNEL staining was performed to examine apoptosis, and a CD31 antibody was employed to detect endothelial cells, representing sites of vascularization. Slides were lightly counterstained with haematoxylin. (B) Quantitative analysis of proliferation (PCNA) and apoptotic (TUNEL) indices were calculated by counting positive cells in 10 random fields at 40× magnification. The number of proliferating tumour cells decreased, whereas the number of apoptotic cells increased in the group treated with SPTT-MSCs. Blood vessel counts were determined by counting the number of vessels in 10 randomly chosen areas of CD31 stained sections (40×). The number of CD31+ cells was decreased in the group treated with SPTT-MSCs (*P < 0.05 compared to mock-transduced MSCs).
In contrast, we did not observe any changes in the expression or phosphorylation status of NF-κB, IKK-β, IκB-α, or JNK proteins, indicating that these pathways are not affected by the TNF-α portion of the protein, and that the fusion protein does not have anti-apoptotic effects through these pathways (as TNF-α alone has been shown to do).

In addition to the effects on cell apoptosis and proliferation, the fusion protein has also been shown to decrease angiogenesis in both the in vitro endothelial tube formation assay and in the xenograft model, which contributes to the molecule’s antitumour activity (Fig. 7C presents a cartoon illustrating the potential mechanisms of action of the SPTT-MSCs). Thus, the LV-transduced MSCs exert antitumour effects via several mechanisms of action: induction of apoptosis, inhibition of proliferation, and reduction in angiogenesis. It may also be possible to combine these transduced cells with conventional approaches to achieve an even greater decrease in tumour growth, angiogenesis and metastasis.

In conclusion, our results indicate that LV-SPTT-transduced MSCs can inhibit the proliferation and growth of prostate cancer cells (both androgen receptor-positive and negative cell lines) and tumours, as well as decrease their blood supply by inhibiting angiogenesis. Our findings provide a basis for future pre-clinical and clinical studies of MSC-based gene therapy for prostate cancer.

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