PTEN-mRNA engineered mesenchymal stem cell-mediated cytotoxic effects on U251 glioma cells

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Abstract. Mesenchymal stem cells (MSCs) have been considered to have potential as ideal carriers for the delivery of anticancer agents since the capacity for tumor-oriented migration and integration was identified. In contrast to DNA-based vectors, mRNA synthesized in vitro may be readily transfected and is mutagenesis-free. The present study was performed in order to investigate the effects of phosphatase and tensin homolog (PTEN) mRNA-engineered MSCs on human glioma U251 cells under indirect co-culture conditions. PTEN-bearing mRNA was generated by in vitro transcription and was transfected into MSCs. The expression of PTEN in transfected MSCs was detected by immunoblotting, and the migration ability of MSCs following PTEN-bearing mRNA transfection was verified using Transwell co-cultures. The indirect co-culture was used to determine the effects of PTEN-engineered MSCs on the viability of U251 glioma cells by luminescence and fluorescence microscopy. The synthesized PTEN mRNA was expressed in MSCs, and the expression was highest at 24 h subsequent to transfection. An enhanced migration rate was observed in MSCs transfected with PTEN mRNA compared with non-transfected MSCs (P<0.05). A significant inhibition of U251 cells was observed when the cells were cultured with conditioned medium from PTEN mRNA-engineered MSCs (P<0.05). The results suggested that anticancer gene-bearing mRNA synthesized in vitro is capable of being applied to a MSC-mediated anticancer strategy for the treatment of glioblastoma patients.

Introduction

Gliomas are recognized as highly malignant and intractable cancers and are the most common type of primary brain tumors throughout the world (1). The complex cellular composition, diffuse invasiveness and capacity to escape conventional therapies have been challenging and have hampered progress towards an effective treatment (2,3). A major obstacle limiting the effectiveness of conventional therapies for gliomas and other types of cancer, is the lack of tumor specificity (4,5). Therefore, exploring the therapeutic strategies that specifically target tumor tissues is critical.

The mesenchymal stem cell (MSC)-mediated strategy has demonstrated considerable potential for the development of clinically meaningful patient-tailored anticancer therapies (6-9). The tumor-directed migration and incorporation of MSCs have been demonstrated in a number of preclinical studies in vitro, using Transwell migration assays, and in vivo, using animal tumor models (10-12). Directed at the tumor microenvironment, engineered MSCs are able to express or release anticancer substances that may constantly act on the adjacent tumor cells (13). As a direct attacker, an anticancer gene that was pre-engineered on MSCs plays a critical role for the action in this system (14). Plasmid DNA transfection and viral vector transduction are widely used in gene therapy-associated studies (15-19). These methodologies have the potential hazards of genomic recombination or insertional mutagenesis, which is a significant problem when this technology is applied to gene therapy (20,21). Therefore, an approach that carries anticancer genes using MSCs, without genetic modification and without risks for clinical applications, may be desired for anticancer medicine. Recently, a novel stabilized mRNA construct has become a more attractive alternative to the most commonly used DNA-based plasmid (pDNA) (22). Synthesized mRNA in vitro has been
Phosphatase and tensin homolog (PTEN) functions as the important negative regulator of PI3K-AKT-mTOR pathway in controlling cells apoptosis (27-29). The activity of PTEN is lost by deletions, mutations or promoter methylation at a high frequency in numerous human cancers (30). Therefore, restoring PTEN function in cancer cells may break down the PTEN mutation-dependent cancer cell growth, termed onco-gene addiction, and demonstrates considerable promise for cancer therapy. The present study describes an experimental strategy that inhibits malignant glioma U251 cells using a conditioned medium (CM) from PTEN-engineered MSCs through the use of in vitro synthesized PTEN mRNA. The strategy has initiated the application of advantageous PTEN mRNA in the field of cancer research. The novel synthetic modified PTEN mRNA possesses great potential for use as an effective therapeutic candidate for glioblastoma patients.

Materials and methods

Cells and culture conditions. MSCs were isolated from healthy human pancreatic ductal tissue and expanded ex vivo as previously described (31). Briefly, human pancreases were obtained with full informed consent from adult heart-beating cadaver organ donors through the organ procurement program at the British Columbia Transplant Society (Vancouver, Canada). A total of 5 pancreatic ductal tissue samples were collected between July 2011 and November 2011 at Vancouver General Hospital (Vancouver, Canada). The pancreatic ductal tissue taken from the Ricordi® Chamber (Biorep Technologies, Inc., Miami, FL, USA), which was used for islet isolation, was utilized as the starting material for MSC isolation. The human glioma U251 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) to be used as target cells in the present study. U251 cells were maintained as suggested by ATCC, and the culture conditions were kept consistent with those of the MSCs. In order to track the viability in a timely manner, the U251 cells were pre-labeled with luciferase using the pGL4.51[luc2/CMV/Neo] vector (Promega Corporation, Madison, WI, USA), according to the manufacturer’s protocol.

In vitro synthesis of PTEN mRNA and MSC transfection. The in vitro transcription template construction and RNA synthesis is schematized in Fig. 1. The oligonucleotide sequences used in the construction of in vitro synthesized PTEN mRNA. The strategy has initiated the application of advantageous PTEN mRNA in the field of cancer research. The novel synthetic modified PTEN mRNA possesses great potential for use as an effective therapeutic candidate for glioblastoma patients.

Table I. Primers for in vitro synthesized PTEN mRNA.

| PTEN mRNA         | Forward primer | Reverse primer                  |
|-------------------|----------------|--------------------------------|
| PTEN ORF          | F1: ATGAAGTTTCCTTTCTTACAATTTCC | R1: TCAGACTTTTTGTAATTGTGATGTT |
|                   | F2: CGCGGATCCGAGATCTGTTAGTTTCTTCC | R2: CCGCTCAGATCGACTTTTGTAATTT |
| 5’ Splint         |                |                                |
| 3’ Splint         |                |                                |
| 5’ UTR            |                |                                |
| 3’ UTR            |                |                                |
| Ligation product  |                |                                |
| Tail PCR          |                |                                |

PTEN, phosphatase and tensin homolog; mRNA, messenger RNA; ORF, open reading frame; UTR, untranslated region; PCR, polymerase chain reaction.
spin columns (Thermo Fisher Scientific) and quantified using NanoDrop (Thermo Fisher Scientific). RNA transfection was performed using TransIT-mRNA (Mirus Bio, Madison, WI, USA). RNA was diluted in Invitrogen Opti-MEM basal media (Thermo Fisher Scientific), and the Boost reagent (Mirus Bio LLC, Madison, WI, USA) and TransIT-mRNA were added sequentially according to the manufacturer’s protocol. Subsequent to 2 min incubation at room temperature (RT), the RNA–lipid complexes were delivered to culture media in the culture plates. The plates were then returned to the incubator and PTEN expression was analyzed 12, 24 and 36 h later using western blot analysis.

Viability assay of tumor cells in indirect co-culture. The cytotoxic effects of PTEN-engineered MSCs (MSCPTEN) on the proliferation of malignant glioma cells were assessed under indirect co-culture conditions. The luciferase gene transfected U251 cells were plated at a density of 5,000 cells/well in a Falcon 96-well plate (BD Biosciences, Franklin Lakes, NJ, USA) in 100 µl of minimal essential medium (MEM) on day 0. The cells were cultured using CM that was collected previously (32). U251 cells were placed on the plastic surface of 24-well plates (1x10⁵ cells/well). On the following day, the wild-type MSCs and MSCPTEN in serum-free media were seeded onto the 8 µm microporous membranes of the Transwell insert (Corning Incorporated, Corning, NY, USA). MSCs were incubated for 48 h at 37°C and in a 5% CO₂ atmosphere. The inserts were washed with phosphate buffered saline (PBS) and the upper surface of the membrane was scraped gently using a cotton swab to remove non-migrated cells. Then, the membranes were fixed with 95% ethanol (Sangon Biotech Co., Ltd., Shanghai, China) and stained using 0.1% crystal violet (Sangon Biotech Co., Ltd.). The average number of migrated cells was assessed by counting five randomly selected microscopic fields at x100 magnification. All experiments were repeated at least three times. To detect the migration status of the MSCs with normal cells, U251 glioma cells were replaced with native MSCs on the plastic surface of the 24-well plates.

Immunoblotting. Immunoblotting was used to detect the cellular expression of MSCs. Briefly, the MSCs were washed with PBS three times and collected using cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were incubated on ice for 30 min. The protein concentration was determined using bicinchoninic acid protein assay reagents (Beyotime Institute of Biotechnology), according to the manufacturer’s protocol. Equal amounts of protein (50 µg/sample) were loaded on each lane and separated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gel and electro-transferred to nitrocellulose membranes (Beyotime Institute of Biotechnology). The membrane was incubated in blocking buffer for 1 h at RT and then incubated overnight at 4°C with monoclonal rabbit anti-mouse PTEN primary antibodies (cat. no. 217702; dilution, 1:1,000; R&D Systems, Inc., Minneapolis, MN, USA). The blots were rinsed with Tris-buffered saline and Tween-20 (EMD Millipore, Billerica, MA, USA) three times, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. A0216; dilution, 1:1,000; Beyotime Institute of Biotechnology) for 60 min and detected by chemiluminescence using ECL Hyperfilm (EMD Millipore). The CM samples were filtered through a 0.22 µm membrane (EMD Millipore) and equally concentrated using a 10,000 molecular weight cut-off (catalog no., 42406; EMD Millipore). Then, PTEN was detected in the CM.

Fluorescence microscopy. The cell viability was also detected using an Invitrogen LIVE/DEAD Viability/Cytotoxicity assay kit (Thermo Fisher Scientific), according to the manufacturer’s protocol, but with a slight modification (10). Briefly, a total of 1x10⁵ U251 cells were plated onto 24-well plates in 500 µl of MEM on day 0. The media were replaced with 50 or 100% CM on day 1. On day 4, the cultures were washed twice with PBS. Freshly prepared working solution containing 1µM calcein acetoxymethyl and 2 µM ethdium homodimer-1 (Thermo Fisher Scientific) was then added directly to the cultures, with 250 µl solution added per well on 24-well plates, and the wells were incubated at RT for 10 min in the dark. The images were captured using a fluorescence microscope (IX71; Olympus)
Corporation, Tokyo, Japan) and the associated analysis was performed using ImageJ 1.45 k software (National Institute of Health of USA, Bethesda, MD, USA).

**Statistical analysis.** Numerical data were expressed as the mean ± standard error. Statistical differences between the means for the various groups were evaluated using Prism 4.0 software (GraphPad software, San Diego, CA, USA) using the Student’s t-test. \( P < 0.05 \) was considered to indicate a statistically significant difference.

**Results**

**PTEN-bearing mRNA synthesis and expression in MSCs.** Anticancer gene-bearing mRNA was constructed as illustrated in Fig. 1A. In order to make the PTEN product secretory (\( _{s} \)PTEN), a leading sequence that corresponded to an 18 amino acid segment (sequence, MKFPSQLLLLLF-GIPGM) was introduced into the DNA template. A transacting activator of transcription (TAT, sequence, YGRKKRRQRRR) was inserted into the PTEN template, to act at the transmembrane (Fig. 1B). The \( _{s} \)PTEN DNA template was cloned from previously constructed pDsRed1-TAT-PTEN vectors (Fig. 2Aa) (33) and the PCR product of the \( _{s} \)PTEN sequence was then inserted into the pcDNA 3.3-TOPO TA vector. The inserted DNA sequence was confirmed by an analysis of the colony using PCR (Fig. 2Ab), restriction enzyme digestion (Fig. 2Ac) and sequencing (data not shown). The correct plasmid insert was excised by restriction enzyme digestion (using BamHI and XhoI; Takara Biotechnology Co., Ltd., Dalian, China) and used to template tail PCR (Fig. 2Ad). As shown in Fig. 2B, the expression of PTEN was clearly enhanced in transfected MSCs. The endogenous PTEN was observed in control MSCs with a slightly varied size, the maximum expression of transfected PTEN mRNA was observed at 24 h post transfection, and subsequently decreased at 36 h. The \( _{s} \)PTEN was also detectable in the corresponding CMs.

**Effect of mRNA transfection on MSC migration.** MSC migration was analyzed using the Transwell system. Subsequent to 48 h of co-culture, a considerable number of cells migrated across the microporous membrane towards the U251 cells (Fig. 3A). However, few cells migrated towards the normal MSC control cells (Fig. 3B). Notably, the transfection of PTEN-mRNAs significantly enhanced the migration of
Figure 3. *In vitro* migratory ability of MSCs. (A) The migratory ability of wild-type MSCs and MSC\textsuperscript{PTEN} toward U251 glioma cells using a Transwell co-culture system. Representative photomicrographs of the stained membrane indicates migrated cells (magnification, x100). (B) The migration of MSCs was tested using wild-type MSCs as control cells (magnification, x100). (C) Summary of three independent experiments was presented as mean ± standard error of the mean. The cell number of each filter membrane was obtained from the average of five randomly selected microscopic fields. *P*<0.05 vs. wild-type MSCs. MSCs, mesenchymal stem cells; PTEN, phosphatase and tensin homolog; MSC\textsuperscript{PTEN}, PTEN-engineered MSCs.

Figure 4. Assessment of U251 cell viability using bioluminescence determination. (A) Representative measurement of luminescence intensity. Cell culture medium is indicated on the left side of the graph. CM ratios and time points are labeled at the top and bottom of the images respectively. The luminescence intensity of each well was determined by IVIS Spectrum System, 10 min subsequent to adding D-luciferin. Luminescence scale: Color scale: Min = 6.31x10\textsuperscript{6}; Max = 1.19x10\textsuperscript{8}. (B) Summary of U251 cell viability. U251 cells were co-cultured with (a) CM\textsubscript{control} and (b) CM\textsubscript{PTEN}. The relative cell viability is represented as luciferase activity. Data are presented as mean ± standard error of the mean. *P*<0.05 vs. control (0%) at the same time point. CM, conditioned media; PTEN, phosphatase and tensin homolog; CM\textsubscript{control}, CM collected from the cultures of native MSCs; CM\textsubscript{PTEN}, CM collected from the cultures of PTEN-engineered MSCs.
MSCs towards the U251 glioma cells compared with control MSCs (Fig. 3C).

**Effect of PTEN-engineered MSCs on the viability of U251 glioma cells.** The indirect co-culture was used to determine the effects of PTEN-engineered MSCs on the viability of U251 glioma cells by luminescence. As shown in Fig. 4A, the intensity of luminescence in U251 cells decreased with the increased incubation time and CM ratio. All test results are summarized in Fig. 4B. Starting at a low CM ratio of 25%, the cells incubated with CM_{PTEN} revealed significant cell death at day 6 (47.7%; P<0.05). However, significant cell death (P<0.05) started to appear at day 3, at a CM ratio of 100%. The ratio of CM_{PTEN}-induced cell death at day 3 was not statistically different from day 0.

**MSC<sup>PTEN</sup>-mediated U251 cell death in indirect co-cultures.** CM-induced U251 cell death was also examined on day 4 by fluorescence microscopy (IX71; Olympus Corporation) subsequent to LIVE/DEAD staining. Two CM ratios, 50 and 100%, were used in this component of the study. As shown in Fig. 5, the cell death was proportionally associated with CM_{PTEN}. The dose-dependent cell death indicates that MSC<sup>PTEN</sup>-derived PTEN is an important mediator responsible for U251 cell death during indirect co-culture. Marked cell death was not detected in the CM obtained from the native MSCs in the current experimental conditions.

**Discussion**

Malignant glioma is one of the most challenging cancers to treat successfully, mainly due to the particularity of its location and biological characteristics, including the infiltrative nature, resistance to apoptosis, propensity for recurrence and resistance to conventional therapies (3). Since the identification of the tumor-oriented migration capacity of MSCs, the application of
specific anticancer gene-engineered MSCs has demonstrated potential for the development of targeted therapy of malignant gliomas (34-36). While MSCs may carry anticancer genes with various viral or plasmid vectors, these methods may not be used in clinical applications (37).

Synthetic mRNA in vitro is a novel technique that presents a safe, efficient strategy for gene therapy (25). The use of synthetic mRNA completely removes the risk of modifying the host genome (38-39), and the safety of synthetic mRNA has been demonstrated in clinical studies (40). Therefore, the synthetic mRNA-based strategy is a simple and non-integrating approach for the application of specific anticancer gene-engineered MSCs.

In the present study, the tumor cell-oriented migration of MSCs, which were engineered with DNA-based anticancer gene expression vectors, was investigated using the xCELLigence system in pancreatic cancer cells and glioma DBTRG cells (33,42). In order to make the strategy clinically practical, the effects of synthesized mRNA transfection on the migration ability of MSCs were examined in the present MSC-mediated anticancer study. As demonstrated in Fig. 2B, the synthesized PTEN mRNA was expressed in MSCs with the peak activity at 24 h following transfection. The expression is consistent with a previous study, which used a DNA-based vector in the same cell model (33).

In the present study, the tumor cell-oriented migration of MSCs, which were engineered with DNA-based anti-cancer gene expression vectors, was investigated using the xCELLigence system in pancreatic cancer cells and glioma DBTRG cells (33,42). In order to make the strategy clinically practical, the effects of synthesized mRNA transfection on the migration ability of MSCs was detected. As shown in Fig. 3, the migration of MSCs was not obstructed by PTEN mRNA transfection. Notably, the migration of MSCs towards the U251 cells was significantly increased by transfected PTEN mRNA. The underlying mechanisms require additional studies in the future. The results of the present study suggest that synthesized mRNA may be applied to the MSC-mediated anticancer strategy.

To the best of our knowledge, the present study is the first demonstration that synthetic modified PTEN mRNA engineered MSC-mediated cytotoxic effects on glioma cells. In the present study, the cytotoxic effects of CM from MSC PTEN on U251 cells were examined using a luminescence technique and fluorescence microscopy. Under the current indirect co-culture conditions, U251 cells were sensitive to CM PTEN (Fig. 4). The significant cytotoxicity was observed on day 6 and at a CM ratio of 50%. The LIVE/DEAD assay possessed the advantage of being straightforward, and reflected the intact status of the detected cells at any time point. As shown in Fig. 5, dose-responsive death of U251 glioma cells revealed the anticancer effect of MSCs that were transfected with PTEN-mRNAs. However, the drawback of the LIVE/DEAD assay is that it only applies to the cells that remain on the culture surface during staining. The detached cells, the majority of which are dead cells, are not included in the assessment. Multiple assessments and in vivo experiments are required in additional studies.

In the present study, a safe and efficient strategy of mRNA-based gene transfer was successfully applied in the MSCs-mediated cytotoxicity of tumor cells. The approach of MSC-mediated gene therapy caused a dual-targeted killing effect to the tumor, mainly due to the tropism characteristics of MSCs and engineered anticancer agents. mRNA-based gene transfer may also kill tumor cells locally and consistently. The methodology used in the present study also holds the potential to use the MSCs of the patients and to switch the tumor attackers corresponding to the individual condition. In conclusion, the present study provides an essential foundation for additional in vivo and potentially clinical anticancer studies using synthesized mRNAs.

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