Controlled Secretion of the Anticancer Protein MDA-7 from Engineered Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) have been explored as a “live” carrier of cytokines for targeted cancer therapy, but, in earlier reports in the literature, the secretion process of therapeutic cytokines was not regulated. The purpose of this study was to generate MSCs to conditionally secrete the melanoma differentiation-associated gene-7 (MDA-7) tumor-suppressor protein. To control the secretion of MDA-7 from MSCs, a well-established tetracycline-controlled transcriptional activation system was incorporated into MDA-7 plasmid. MDA-7 gene expression was induced in the engineered MSCs only in the presence of doxycycline, as characterized by quantitative reverse transcription (qRT)-PCR. Enzyme-linked immunosorbent assay (ELISA) also revealed that the MDA-7 protein was secreted from the engineered MSCs only after the cells had been exposed to doxycycline. Both recombinant human MDA-7 protein and the conditioned medium from the engineered MSCs in the presence of doxycycline significantly inhibited tube formation of human umbilical vascular endothelial cells (HUVECs), indicating that our system could be used for targeted, antiangiogenic therapy. Overall, this study provides useful information on the potential use of engineered MSCs for the controlled secretion of therapeutic proteins, in this case MDA-7, for targeted cancer therapy.

Key words controlled secretion; melanoma differentiation-associated gene-7; mesenchymal stem cell; Tet-On system; in vitro

Since traditional cytotoxic anticancer agents often cause adverse effects, it would be ideal to develop a system that enables anticancer agents to be delivered specifically to tumor tissue and to damage cancer cells without affecting healthy tissues. Melanoma differentiation-associated gene-7 (MDA-7), also known as interleukin (IL)-24, is a tumor suppressor gene, which was originally identified from human melanoma cells. The loss of MDA-7 expression correlates well clinically with tumor invasiveness and metastasis. Adenoviral MDA-7 has been reported to show cancer cell-specific cytotoxicity and anticancer activity through different molecular mechanisms. Following successful preclinical studies, a Phase I trial was initiated to determine the safety and therapeutic potential of adenoviral MDA-7 (Ad-MDA-7; INGN 241). However, the clinical development of Ad-MDA-7 has not been evaluated since 2008. This might be attributable to toxicities associated with adenovirus vectors. Virus vectors also require intratumoral injection, which makes it difficult to treat metastatic cancers. Thus, a new carrier system is required to deliver the MDA-7 gene or protein to tumor tissue by systemic administration.

Over the past decade, it has become increasingly clear that mesenchymal stem cells (MSCs) migrate to wound tissues to promote healing or to tumor tissues to support their growth/metastasis by participating in the formation of new blood vessels. To take advantage of their unique characteristics, MSCs have been explored as a new carrier of cytotoxic proteins for targeted cancer therapy. Despite recent extensive efforts, research into MSC carriers is still in its infancy, and the secretion of cytokines in earlier reports was not regulated, which means that therapeutic cytokines were secreted continuously.

Here, we genetically incorporated a tetracycline-controlled transcriptional activation system (the TET system) into MSCs. With this system, gene expression is regulated by the presence or absence of tetracycline/doxycycline. Thus, if successful, this system would allow engineered MSCs to secrete the MDA-7 protein only in the presence of tetracycline/doxycycline. Since MDA-7 has been reported to have an antiangiogenic effect, the intracellular delivery of the MDA-7 gene is not required, which is also a good reason to use MSCs as carriers. The primary goal of this study was to demonstrate controlled secretion of MDA-7 from the engineered MSCs in response to doxycycline, and the second goal was to prove that MDA-7 secreted from the engineered MSCs remains pharmacologically active.

MATERIALS AND METHODS

Cell Culture Mouse mesenchymal stem cells (mMSCs) derived from the bone marrow of Balb/c mice were purchased from Cyagen Biosciences Inc. (Santa Clara, CA, U.S.A.) and cultured in MSC basal medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% glutamine (Cyagen Biosciences). Primary human umbilical vascular endothelial cells (HUVECs) were purchased from Cell Applications (San Diego, CA, U.S.A.) and cultured in endothelial cell growth medium with the supplements provided by the supplier (Lonza Group Ltd., Basel, Switzerland). We used mMSCs and HUVECs up to 13 and 6 passages, respectively, in the experiments. All cells were maintained under a humidified atmosphere of 5% CO₂ at 37°C.

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Preparation of pTetOne-MDA-7 Plasmid  
MDA-7 cDNA (633 bp) was synthesized by Eurofins Genomics K.K. (Tokyo, Japan), and used as a template for cloning. The MDA-7 primers used for PCR amplification were as follows: FW, 5'-CCC TCG TAA AGA ATT CAT GAA TTT TCA ACA GAG GCT GCA A-3' (40 bp); and RV, 5'-GCA GAG ATC TGG ATC CTC AGA GCT TGT AGA ATT TCT GCA T-3' (40 bp). The Tet-One Inducible Expression System (TaKaRa Bio Inc., Shiga, Japan) was used to prepare the pTetOne-MDA-7 plasmid. The pTetOne vector was linearized by EcoRI and BamHI restriction enzymes, and a purified PCR fragment of MDA-7 was incorporated into a linearized TetOne vector using an In-Fusion® HD cloning kit (TaKaRa Bio Inc.). The resultant pTetOne-MDA-7 plasmid was cloned with Stellar® Competent Cells (TaKaRa Bio Inc.). The sequence of the pTetOne-MDA-7 plasmid was verified, and the plasmid was used for subsequent experiments.

Transfection of pTetOne-MDA-7 Plasmid into mMSCs  
mMSCs (5.0×10^4 cells/well) were cultured in a six-well plate, and transfected with pTetOne-MDA-7 plasmid using Xfect™ reagent (TaKaRa Bio Inc.). Five microgram per well of pTetOne-MDA-7 plasmid was mixed well with Xfect™ Reaction Buffer, followed by Xfect™ Polymer in a total volume of 100µL (well), and incubated for 10min. One hundred microliters of nanocomplex solution were added to 1 mL of mMSCs, and the cells were incubated for 4h in a CO₂ incubator at 37°C. After incubation, the medium was replaced by 2mL of fresh medium.

In Vitro Secretion of MDA-7 Protein from Engineered mMSCs  
At 4d post-transfection, the medium was replaced by 1mL of doxycycline (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at various concentrations. After 72h incubation with doxycycline, the supernatants were collected, and MDA-7 was quantitated with a human IL-24 ELISA Kit (Aviva Systems Biology, San Diego, CA, U.S.A.). The incubation medium without doxycycline was used as a control. The cells were also collected for the extraction of total RNA.

RNA Isolation and Quantitative Reverse Transcription (qRT)-PCR  
Total RNA was isolated from the engineered mMSCs using RNasea (QIAGEN, Hilden, Germany), and cDNA was synthesized using the PrimeScript RT Master Mix (TaKaRa Bio Inc.). β-Actin mRNA (ACTB)
was amplified as a control. qPCR was performed using SYBR Premix Ex Taq II (TaKaRa Bio Inc.), Ct values were obtained using an Applied Biosystems® StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific Inc., Waltham, MA, U.S.A.), and relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. The PCR primer sets used were as follows: MDA-7 FW, 5′-TGTCTGATGGCTGAGA-GCTGC-3′; and MDA-7 RV, 5′-AAA GTT GTT GGC GAG TGTGCTG-3′; and ACTB FW, 5′-TGGCTCTAGCAACCACTGAA-3′, and ACTB RV, 5′-GTG GAC AGT GAG GGC AGG AT-3′.

In Vitro Tube Formation Assay

To investigate the extent to which MDA-7 inhibits the tube formation of HUVECs, one ng/mL recombinant human MDA-7/IL-24 (rhMDA-7; PeproTech; Rocky Hill, NJ, U.S.A.) or the supernatant of the mMSC/TetOn-MDA-7, post-72 h-exposure to doxycycline, was used as an incubation medium for HUVECs rather than endothelial cell growth medium. As a control, no rhMDA-7 nor the conditioned medium of the mMSC/TetOn-MDA-7 without doxycycline were used in any experiment. Ten microliters of the conditioned medium of the mMSC/TetOn-MDA-7 without doxycycline were used in the supernatant where MDA-7 was secreted from the mMSC/TetOn-MDA-7 after incubation with 0 or 1000 ng/mL doxycycline for 72 h. Data represent the mean with S.E.M. of three independent experiments ($p<0.05$ vs. no Doxy).

RESULTS

Antiangiogenic Effect of Recombinant Human MDA-7 Protein in Vitro

Endogenous bone marrow MSCs are recruited to tumor tissue to participate in the formation of new blood vessels. An in vitro tube formation assay using HUVECs is a standard method with which to evaluate the antiangiogenic or antiangiogenic effects of exogenous substances. In this study, rhMDA-7 significantly reduced the number of branches (Fig. 1A), the number of loops (Fig. 1B), the number of tubes (Fig. 1C), and the total tube length (Fig. 1D) of HUVECs, like Ad-MDA-7. Representative images of the tube formation of HUVECs are shown in Figs. 1E and F.

Gene and Protein Expression of MDA-7 in Engineered mMSCs

Next, we performed qRT-PCR to assess the controlled gene expression of MDA-7 in the mMSC/TetOn-MDA-7 in the presence or absence of doxycycline. While the mRNA level of MDA-7 was significantly higher in the mMSC/TetOn-MDA-7 at 72 h after exposure to doxycycline at a concentration of 1000 ng/mL, the expression level of MDA-7 was only marginal when the cells were not treated with doxycycline (Fig. 2A). Consistent with the qRT-PCR results, the mMSC/TetOn-MDA-7 secreted MDA-7 protein only in the presence of doxycycline (Fig. 2B). Although doxycycline led to the expression of MDA-7 mRNA in mMSC/TetOn-MDA-7 in a dose dependent manner (Supplementary Fig. 1), which was in line with a typical TET system, only doxycycline at a concentration of 1000 ng/mL secreted the MDA-7 protein, over 72 h, up to the level where rhMDA-7 inhibited the tube formation of HUVECs.

MDA-7 Secreted from Engineered mMSCs Inhibited the Tube Formation of HUVECs

We investigated whether MDA-7 secreted from the mMSC/TetOn-MDA-7 had an inhibitory effect on the tube formation of HUVECs comparable to that of rhMDA-7. MDA-7 secreted from the mMSC/TetOn-MDA-7 inhibited the tube formation of HUVECs (Figs. 3A–D), compared to the conditioned medium of the mMSC/TetOn-MDA-7 with or without 1000 ng/mL doxycycline. Data represent the mean with S.E.M. of five independent experiments ($**p<0.01$ vs. no Doxy).
TetOn-MDA-7 incubated without doxycycline. This indicates that MDA-7 secreted from engineered mMSCs was of equivalent antiangiogenic potency. Representative images of the tube formation also showed that MDA-7 secreted from the mMSC/TetOn-MDA-7 had an inhibitory effect on the tube formation of HUVECs (Figs. 3E, F). As previously reported with an antiangiogenic agent,\textsuperscript{18} the agent that partially inhibited (about 30% decrease from the control) the tube formation of HUVECs \textit{in vitro} successfully achieved tumor growth inhibition \textit{in vivo}, implying a promising future of MSC-based antiangiogenic therapy.

**DISCUSSION**

In this study, we generated the prototype of engineered MSCs that achieve controlled secretion of MDA-7 in response to doxycycline. The successful inducible secretion of MDA-7 from the prototype MSCs could represent a promising start toward the controlled secretion of other cytokines from cell-based carriers. Provided that the dose of doxycycline is optimized to prevent toxicity, but still maximize the secretion of cytokines, the controlled secretion of therapeutic cytokines from cell-based carriers using the TET system could be an intriguing strategy to put into practice. To carry this strategy forward, inevitable challenges must still be overcome. As with other drug carriers, the delivery of MSCs to tumor tissue must be improved. To achieve sufficient intratumoral concentration of MDA-7, which might eventually lead to successful tumor growth inhibition \textit{in vivo}, the administration protocol (the number of cells, repeated administration, etc.) can be adjusted, based on the amount of MDA-7 secreted per cell. Note that up to five million and 400 million MSCs can systemically be administered into mice and human, respectively, without serious adverse events.\textsuperscript{19} If MSCs delivered to tissues other than tumors can be eliminated by natural or artificial selection, adverse effects on other tissues where MSCs could lodge can be expected."

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**Fig. 3. Inhibitory Effect of MDA-7 Secreted from the mMSC/TetOn-MDA-7 on the Tube Formation of HUVECs**

A. Number of branches. B. Number of loops. C. Number of tubes. D. Total tube length (µm). Data represent the results of five independent experiments (*p<0.05 vs. no Doxy). Representative photomicrographs of the tube formation of HUVECs with the conditioned medium of the mMSC/TetOn-MDA-7, incubated without and with doxycycline, are shown in E and F, respectively. Scale bars, 100 µm.
avoided. In addition, engineered MSCs should first accumulate in tumor tissue and then secrete MDA-7; doxycycline should be administered only after the delivery of MSCs to the tumor is confirmed. Thus, the timing of doxycycline administration is critical. Since noninvasive imaging of stem cells has been extensively investigated,\textsuperscript{20,23} image-guided delivery of MSCs should help to determine the optimal timing of doxycycline administration. Furthermore, although our prototype was transfectively engineered, longitudinal success would require stable transfection. Genetic changes in MSCs due to stable transfection might possibly increase the chance of carcinogenic mutation of engineered MSCs. Thus, the system must be evaluated to determine whether the long-term use of MSCs causes carcinogenic mutation. Moreover, we need to consider the immune rejection of MSCs by patients, although the use of autologous MSCs might reduce the chances of such rejection. Overall, despite several challenges, additional improvements to this system should expand the application of MSC-based, targeted therapy. Considering the clinical translatability of MDA-7, as well as the clinical potential of stem cells, this strategy could take existing cancer treatments to the next level.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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