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RESEARCH ARTICLE

Mesenchymal stem cells as vehicles for targeted delivery of anti-angiogenic protein to solid tumors

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Abstract

Background Inhibition of tumor-induced angiogenesis may restrict tumor growth and metastasis. Long-term systemic delivery of angiogenic inhibitors is associated with toxicity, as well as other severe side-effects. The utility of cells as vehicles for gene therapy to deliver therapeutic molecules has been suggested to represent an efficient approach. Mesenchymal stem cells (MSCs) exhibit a tropism to cancer tissue, and may serve as a cellular delivery vehicle and a local producer of anti-angiogenic agents.

Methods In the present study, we attempted to assess production of the transgene, α1-antitrypsin (AAT), in lentivirus-transduced human MSCs and its cytotoxicity against human umbilical cord vein endothelial cells (HUVEC). The secreted protein from these effector cells was determined by an enzyme-linked immunosorbent assay. The cytotoxicity of hMSCs that overexpress the human AAT gene against HUVEC was evaluated with an apoptotic assay.

Results Lentivirus-transduced hMSCs produced functional AAT and displayed much higher cytotoxicity against HUVEC than untransduced hMSCs. Moreover, AAT secreted from transduced hMSCs significantly inhibited HUVEC proliferation compared to untransduced hMSCs. The data obtained demonstrate for the first time that genetically modified hMSCs released abundant and functional AAT that caused obvious cytotoxicity to HUVEC.

Conclusions hMSC may serve as an effective platform for the targeted delivery of therapeutic proteins to cancer sites. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords angiogenesis; α1-antitrypsin; cancer gene therapy; mesenchymal stem cells; umbilical cord vein endothelial cells

Introduction

Conventional treatments, such as surgery, radiotherapy and/or chemotherapy have made tremendous advances in fighting malignant tumors; however, malignances continue to display a poor prognosis. New trials of chemotherapeutic agents in an orthotopic model or new agents targeting specific molecules in signaling pathways have been attempted [1]. Angiogenesis is a promising target in the treatment of cancer and, over the last 15 years, major progress has been made in the development of therapies targeting tumor-induced angiogenesis [2]. Inhibition of tumor-induced angiogenesis may restrict tumor growth and metastasis, and potentially lead to tumor regression.

One critical step in tumor growth and invasion is the proteolytic process of the extracellular matrix environment. The degradation of the extracellular

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matrix enables migration and invasion of malignant cells. The regulation of proteolysis by an inhibitor, such as serpins, could theoretically lead to the control of angiogenesis [3]. Serine proteases are a large and conserved proteolytic family that may facilitate tumor growth, invasion and metastasis. A large number of studies support the notion that proteases play an important role in the progression of malignant tumors. Therefore, the expression of protease inhibitors is considered to be rational and effective in controlling tumor progression [4]. Among the anti-angiogenic factors that could limit the progression from a small cluster of malignant cells to a growing tumor, serpins including pigment epithelium derived factor (PEDF) and several active serpins or their fragments, such as maspin, kallistatin antithrombin and α1-antitrypsin (AAT), hold an important position. AAT, a major inhibitor of human serine proteases in serum, is mainly produced not only by the liver, but also by extrahepatic cells, including neutrophils and some types of cancer cells [4–6]. Several studies have shown that AAT and AAT-derived peptides are effective in suppressing cancer progression and in treating other angiogenesis-dependent disorders [7]. Huang *et al.* [7] showed that, in addition to its functioning as a serine protease inhibitor, AAT is a naturally occurring inhibitor of angiogenesis, and is active at nanomolar concentrations. Systemic administration of AAT or an AAT fragment devoid of serpin activity delayed tumor progression and reduced microvessel density by inducing apoptosis of endothelial cells in short-term experiments [7]. These findings suggest AAT-derived peptides or mimetics may be potentially useful as anti-angiogenic agents in treating cancer and angiogenesis-dependent diseases.

Anti-angiogenic therapy is likely tumoristatic, and long-term delivery of angiogenic inhibitors may be required for successful cancer treatment [8]. Production of functional proteins could be quite expensive, and repeated administration will be unaffordable for patients with chronic conditions. Moreover, long-term systemic delivery of angiogenic inhibitors, such as AAT, is associated with toxicity and other adverse effects that limit the overall efficacy and wide use. Cell-based gene therapy in which cells are used as a vehicle for delivery of anti-angiogenic molecules offers an opportunity for patients to become their own source of production (i.e. an endogenous factory for the production of anti-angiogenic protein) [9]. The utility of cells as a vehicle for the delivery of toxic and immunostimulatory genes or anti-angiogenic molecules has been suggested in several studies [10–12]. This approach may overcome the extensive metabolism and toxicity associated with some biologic agents, and could serve as a versatile tool for manipulating the extracellular milieu of malignant cells [13]. The choice of human mesenchymal stem cells (hMSCs) as a cellular vehicle was based on their tumor homing capacity, and on the observation that intravenously administered hMSCs do not engraft in healthy organs [11–13]. MSCs engrafted in tumors may act as precursors for stromal cells, and serve as a cellular vehicle for the delivery and local production of biologically active agents [10–12]. Moreover, the advantages of using stem cells for local cancer therapy include: ease of harvest, ability to be highly proliferative *in vitro*, and high transfection efficiency.

In the present study, we attempted, for the first time, to evaluate the cytotoxicity of lentivirus-transduced hMSCs that overexpress a sustained level of AAT against human umbilical cord vein endothelial cells (HUVEC) as an *in vitro* angiogenic model. The data obtained show that hMSCs possess key properties to secrete anti-angiogenic proteins allowing them to function as a cellular vehicle for cancer gene therapy, and that the release of functional AAT led to decreased proliferation of HUVEC as a result of enhanced apoptosis.

Materials and methods

Chemicals and reagents

Dulbecco's modified eagles medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS) and trypsin-ethylenediamine tetraacetic acid (EDTA) solution were obtained from Gibco BioCult (Paisley, UK). Mesencult and Stem Span medium was purchased from Stem Cell Technology (Sheffield, UK). Antibodies for flow cytometric assays were obtained from Dako (Carpinteria, CA, USA). Antibodies against Ki-67 and proliferating cell nuclear antigen (PCNA) were purchased from Abcam Inc. (Cambridge, MA, USA). Kits of RNA extraction, cDNA synthesis and hot-start polymerase chain reaction (PCR) master mix were purchased from Qiagen (Valencia, CA, USA). Alizarin red staining and Oil red staining kits were purchased from Sigma Aldrich Co (St Louis, MO, USA). An enzyme-linked immunosorbent assay (ELISA) kit for AAT quantification was obtained from Geneway Co (San Diego, CA, USA). The lentiviral transfer construct containing a green fluorescent protein (GFP) expression cassette and the puromycin-resistance gene was purchased from Open Biosystems Co. (Huntsville, AL, USA). Lentiviral envelop pMD2G plasmid coding for the broad range vesicular stomatitis virus envelope (VSV-G) envelope and the packaging psPAX2 plasmid were obtained from Addgenes Co (Cambridge, MA, USA).

hMSC isolation and culture

Bone marrow (5 ml) was aspirated under local anaesthesia from several healthy donors undergoing bone marrow harvest for allogeneic transplantation at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran. Such sampling was only carried out after obtaining an informed consent from individuals in accordance with the guidelines of the Medical Ethics Committee, the Ministry of Health of IR Iran. Bone marrow mononuclear cells were isolated by Ficoll density centrifugation and were cultivated in low-glucose DMEM containing 10% FBS and 2 mM L-glutamine, 1 U/ml of penicillin/streptomycin in a polysterene plastic culture flask. Non-adherent cells were

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removed by medium change every 3 or 4 days, and hMSCs were selected based on plastic adherence. When hMSCs reached 70–90% confluence, they were harvested using 0.25% trypsin-EDTA and subcultured at an initial seeding density of 5×10^4 cells/cm^2 in Mesencult medium supplemented with 15% FBS, L-glutamine, and antibiotics. Cultivation of hMSCs was carried out at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. For all experiments, hMSCs were cultivated up to the fourth passage for lentiviral transduction.

Culture of HepG2, HEK 293T cells and HUVEC

HEK 293T, HUVEC and human HepG2 hepatoma cells were obtained from the Pasteur Institute of Iran Tehran. The HEK 293T cells and HUVEC were maintained in DMEM supplemented with 10% FBS, L-glutamine and antibiotics, HepG2 cells were maintained in MEM medium supplemented with the same supplements as HUVEC.

Phenotypic characterization of bone marrow-derived hMSCs

After *ex vivo* expansion, human bone marrow-derived MSCs were subjected to flow cytometric analysis to confirm the exclusion of hematopoietic cell contamination and the expression of MSC markers by the use of surface antigens: CD73, CD44 (H-CAM), CD166 (ALCAM, activated leukocyte cell adhesion molecule), CD105 (endoglin, SH2), as well as the hematopoietic membrane antigens: CD34 (hematopoietic precursor cells) and CD45 (leukocyte common antigen). Cells at 20×10^5 were harvested 14 days after culture and were labeled separately with fluorescein isothiocyanate-conjugated mouse anti-human CD44, CD73, CD105, CD34 and phycoerythrin-conjugated CD166 and CD45. Isotype-matched irrelevant monoclonal antibodies were used as negative controls. For antibody staining, cells were incubated with specific antibodies in PBS containing 1% bovine serum albumin in the dark for 30 min at 4 °C. After washing, cells were resuspended in PBS and analyzed with a Coulter Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the results were analyzed with Win MDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

Adipogenic and osteogenic differentiation of hMSCs

To ascertain that culture-expanded hMSCs cells were multipotent, their phenotype and mesodermal differentiation potential upon exposure to mesenchymal supportive conditions, such as specific osteogenic and adipogenic agents, were examined. The hMSCs at 3×10^3 were incubated in the adipogenic DMEM medium supplemented with 10% FBS, 1.7 μM insulin, 1 μM dexamethasone, 200 μM indomethacin and 500 μM isobutyl methylxathine for 14 days. The cells were fixed with 4% paraformaldehyde, and fat droplets within differentiated adipocytes derived from hMSCs were stained with an oil red O method [14].

The potential of hMSCs to differentiate into osteogenic lineages was performed as previously described [15]. hMSCs were incubated at 3×10^3 cells/cm^2 in an osteogenic MEM medium containing 10% FBS, 50 μM ascorbate-phosphate, 1 μM dexamethasone and 10 μM β-glycerophosphate for 2 weeks. To assess osteogenic specification, differentiated cells were fixed and stained histochemically for calcium deposition using the Alizarin red staining kit, and examined under a phase contrast microscope (TE-2000; Nikon, Tokyo, Japan).

Generation and preparation of lentiviral constructs

The transfer plasmid contains the puromycin-resistance gene and a GFP expression cassette under the control of the cytomegalovirus (CMV) promoter that leads to the production of the AAT-GFP fuse gene. The human AAT cDNA (1.4 kb) was derived from pReciever MO2 (GeneCopoeia, Rockville, MD, USA) by PCR, and ligated into the *Xho*I and *Mlu*I sites of the PLEX GFP transfer vector. The fragment subcloned into the transfer plasmid was confirmed by nucleotidase digestion and the cycle sequencing method. The envelop pMD2G plasmid, which codes the broad range VSV-G envelope, the packaging psPAX2 plasmid and the transfer plasmid, were amplified in DH5α *Escherichia coli*, extracted and purified using Qiagen isolation kits.

Production and titration of lentiviral vector encoding the human AAT gene

The lentiviral vector was generated in HEK 293T cells by calcium phosphate-mediated transfection of the three plasmids [16]. HEK 293T cells (5×10^6) were transfected with 21 μg of the transfer plasmid encoding the AAT gene, 10.5 μg of the envelop pMD2G plasmid and 21 μg of the packaging psPAX2 plasmid. Medium was removed approximately 14–16 h post-transfection, and refreshed with 10 ml of pre-warmed virus collecting medium. Medium was collected on days 2 and 3 post-transfection. The supernatant was separated by centrifugation (5 min at 7000 ***g***), filtered through a membrane with a 0.4-μm pore size, and then concentrated ten-fold using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The unit consisting of a filter of 100 000 molecular weight cut-off is an easy way to concentrate lentiviral vectors by up to ten- to 20-fold. Titer of GFP-expressing lentiviral vector was determined by infection of HEK 293T cells using serial dilutions in a six-well plate. Serial dilutions of the viral stock were added to HEK 293T cell culture. After 72 h, GFP-expressing cells (4×10^5) were quantified by flow cytometry for each dilution to determine transducing units (TU) per milliliter.

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Lentiviral vector transduction

hMSCs at 2×10^4 cells/cm^2 in a 24-well culture plates were transduced with ten-fold concentrated lentiviral vectors (2.8×10^7 TU/ml). A single round of transduction was carried out at various multiplicities of infection (MOI 5–50) in the presence of polybrene (8 μg/ml). Six hours after transduction, virus-containing medium was removed and replaced with 1.0 ml of pre-warmed Mesencult medium supplemented with 10% FBS. GFP expression was visualized under a fluorescence microscope (TE-2000) and GFP-positive cells were further analyzed by flow cytometry 3 days after transduction. Transduced cells were maintained permanently in medium containing 1 μg/ml puromycin to select puromycin-resistant clones that are GFP-positive and express the AAT gene.

Reverse transcriptase (RT)-PCR analysis of human AAT gene expression in lentivirus-transduced hMSCs

The extraction of total RNA and generation of cDNA were performed using the RNeasy Mini Kit and QuantiTect Reverse Transcription kit (Qiagen) in accordance with the manufacturer's instructions. Then the PCR reaction was performed as described previously [17]. Briefly, PCR reactions were performed with 1 μM of forward and reverse primers in a total volume of 20 μl including 1 μl of cDNA. In an initial denaturation step of 5 min at 95 °C followed by 32 PCR-cycles consisting of 45 s of denaturation at 94 °C, 45 s of annealing at 55 °C, and 1-min extension at 72 °C, with a final extension step of 7 min at 72 °C. Human β-actin was used as a housekeeping gene control. The identification of the AAT transcripts was confirmed by sequencing after RT-PCR amplification.

Adipogenic and osteogenic differentiation of hMSCs after transduction

To evaluate whether lentiviral transduction altered the differentiation properties of hMSCs *in vitro*, the potential of transduced cells to differentiate along osteogenic and adipogenic lineages was examined as described above.

Analysis of AAT content in culture medium by ELISA

A quantitative ELISA kit with a detection range of 7.5–500 ng/ml was used to measure human AAT levels in culture medium. The concentration of AAT secreted from lentivirus-transduced hMSCs and nontransduced cells co-cultured with HUVEC was measured at 3-week intervals using purified human AAT protein as a standard. AAT levels in samples were determined from a standard curve prepared by a series of dilutions of human AAT (Sigma Aldrich).

Anti-elastase activity of human AAT protein

To confirm that AAT protein produced by transduced hMSCs is biologically functional, its activity was determined by an elastase inhibition assay using EnzChek Elastase Assay Kit (Molecular Probes, Inc., Carlsbad, CA, USA) in accordance with the manufacturer's instructions. To optimize the reaction condition, we first used AAT purified from human plasma. The optimal condition was 1:1 molar ratio of enzyme and substrate. Transduced and nontransduced MSCs were maintained in serum-free conditions for 48 h before anti-elastase assay, and cell culture medium was collected. All values were corrected for background fluorescence, and expressed by subtracting the value derived from the no-enzyme control. The relative elastase activity is expressed as a percentage of elastase activity without an inhibitor.

Cytotoxicity assay by *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

The cytotoxicity of transduced hMSCs against target HUVEC was assessed in a co-culture system of these two cell types. hMSCs and HUVEC were mixed to obtain ratios of HUVEC/hMSCs at 0:1, 1:1, 1:2, 1:3 and 1:4. The number of target HUVEC was 5×10^3, and they were co-incubated with lentivirus-transduced MSCs for 7 days at 37 °C. Then, apoptosis of HUVEC cells was determined with an *in situ* TUNEL assay kit in accordance with the manufacturer's instructions. Using this method, apoptotic nuclei were stained in dark brown with chromogen 3,3-diaminobenzidine (DAB). Apoptotic cells in ten random fields were counted and expressed as percentage of total cells. Transduced hMSCs were GFP-positive, which helps distinguish them from dead HUVEC.

Cytotoxicity assay by transwell inserts

To evaluate the cytotoxicity of AAT protein secreted from lentivirus-transduced hMSCs, HUVEC at 5×10^3 were seeded in the lower wells of 24-transwell plates with porous inserts (0.4 μm pore size), whereas hMSCs that persistently express human AAT were plated on the upper wells of the transwell plates with increasing cell numbers (ratios of HUVEC/hMSCs were 0:1, 1:1, 1:2, 1:3 and 1:4). After 7 days of co-culture in transwells, apoptosis of HUVEC in the lower wells was determined with an *in situ* TUNEL staining assay, as described above.

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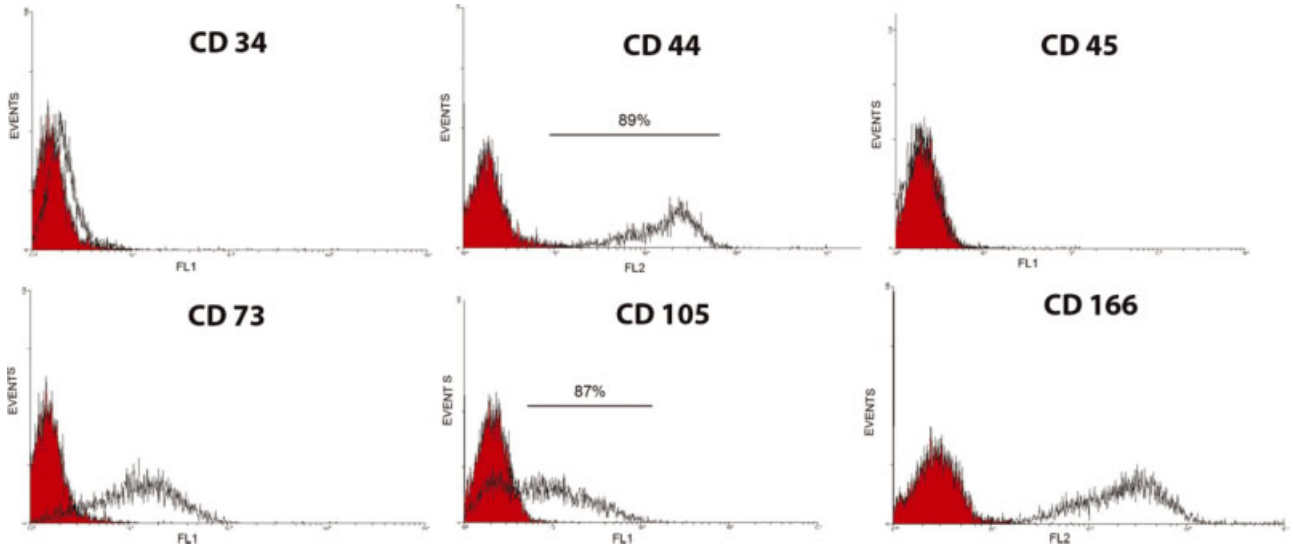

Figure 1. Phenotypic characterization of hMSCs by flow cytometric analysis. Flow cytometric analysis was performed for specific cell surface markers of MSCs and hematopoietic markers 14 days after isolation. hMSCs were positive for CD44, CD73, CD105 and CD166, but were negative for CD34 and CD45

Proliferation assay

HUVEC were seeded in lower wells at 5×10^3 cells per well and incubated overnight at 37 °C to allow them to adhere to the plates. Transduced hMSCs were grown in upper wells of the same transwell plates in increasing ratios (ratios of HUVEC/hMSCs were 0:1, 1:1, 1:2, 1:3 and 1:4). Untransduced MSCs were used as a control. Seven days after co-culture, HUVEC were fixed in 4% PBS-buffered paraformaldehyde (pH 7.4) and post-fixed with ethanol/acetic acid (2:1, v/v) at −20 °C. The immunocytochemical staining against human Ki-67 and PCNA was preformed as described previously [18]. The images were visualized under a Nikon fluorescent microscope and recorded with a digital camera. The percentage of positive nuclear staining was calculated based on total cell numbers in three high power fields.

Statistical analysis

All experiments were repeated for at least three times and each condition was analyzed in triplicate. Data are presented as the mean ± SD for quantitative variables, and summarized by absolute optical density and percentages for categorical variables. One-way variance (ANOVA) analysis was used to determine whether there was a significant difference in the number of viable cells among four test groups. The Newman–Keuls test was employed to make multi comparisons between given groups after ANOVA had rejected the null hypothesis that the four groups were from the same population. For statistical analysis, SPSS, version 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used. $p \leq 0.05$ (two-tailed) was considered statistically significant.

Results

Characterization of hMSCs

Expression of hMSC markers, such as CD73, CD44, CD105 and CD166, as well as CD34 and CD45, was examined by flow cytometry 14 days after culture. Culture-expanded hMSCs were positive for CD73, CD44, CD105 and CD166 but negative for CD34 and CD45 expression (Figure 1). This surface marker expression profile is characteristic of hMSCs. The differentiation of hMSCs into adipogenic cells was demonstrated by positive oil red O staining 4 weeks after culturing in adipogenic medium. Alizarin red staining confirmed the presence of calcium deposits, a characteristic of the osteogenic lineage in a differentiated cell population, 2 weeks after being cultured in osteogenic medium (Figure 2). hMSCs cultured in a standard condition served as a control, and were negative for both types of staining. These findings demonstrate that bone morrow-derived hMSCs were able to differentiate into mesodermal cell types, such as adipocyte-like or oesteocyte-like cells.

Transduction of hMSCs with a lentiviral AAT-GFP vector

Purified hMSCs from passage 4 were transduced initially with a lentiviral vector encoding the AAT-GFP fusion gene at approximately 2.8×10^7 viral particles per milliliter and transduction conditions were optimized with different MOI. Transduced cells were quantified 72 h after transduction by counting GFP-positive cells using a FACS Calibur system (Becton Dickinson, Bohemia, NY, USA), and analyzed for GFP expression by WinMDI 2.8 software

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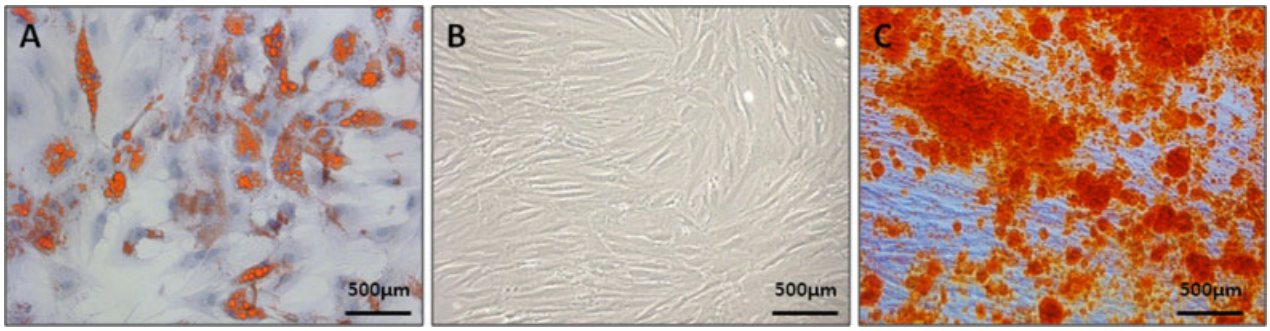

Figure 2. Transdifferentiation of hMSCs along adipocyte and osteoblast lineages. Differentiated MSCs were positive for (A) oil red-staining for adipogenic differentiation and (C) Alzarian red staining for osteogenic differentiation after a 2-week culture in differentiation medium. (B) Undifferentiated MSCs as a control group were negative for both staining

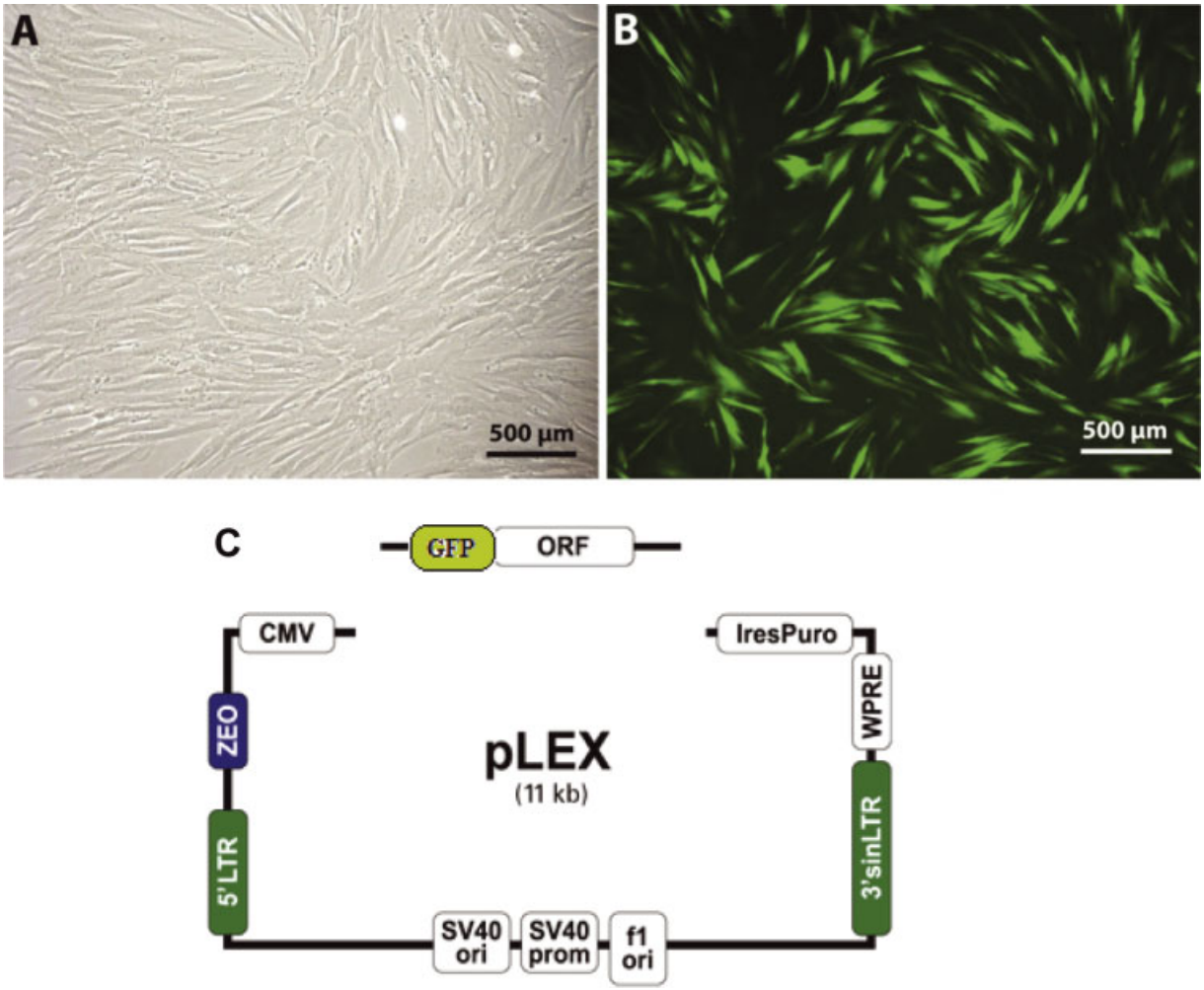

Figure 3. Transduction of hMSCs with a lentiviral AAT-GFP vector and visualization of AAT-GFP expression in hMSCs. GFP-positive hMSCs were visualized under a fluorescent microscope 72 h after transduction (A) Untransduced cells. (B) MSCs transduced with AAT-GFP lentiviral vector. (C) Schematic illustration of lentiviral transfer plasmid, which contains the puromycin-resistance gene and a GFP expression cassette under the control of the CMV promoter. The human AAT cDNA (1.4 kb) was ligated into *Xho*I and *Mlu*I sites of the PLEX GFP transfer vector to form an AAT-GFP fusion gene

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(Scripps Institute, La Jolla, CA, USA). The GFP positivity was found to be close to 93.6% at an MOI of 35 (Figure 3).

Quantification of AAT-GFP fusion gene expression

AAT-GFP expression in hMSCs was visualized using an inverted fluorescence microscope (Figure 3B). The expression of the AAT gene in hMSCs was further confirmed using RT-PCR. HepG2 cells were used as a positive control and untransduced hMSCs as a negative control (Figure 4A). The concentration of AAT secreted from hMSCs into medium was gradually increased. A significantly elevated AAT level was detected in culture medium at a ratio of 1:4 at day 7 compared to a 1:1 ratio ($p < 0.05–0.01$) (Figure 4C).

Anti-elastase activity of AAT protein

To confirm that AAT protein produced by transduced hMSCs is functionally active, the AAT inhibition of elastase activity was evaluated. Culture medium from cells transduced with lentiviral AAT vectors had the capacity to inactivate human neutrophil elastase in contrast to medium supernatants from non-transduced cells. AAT protein secreted by transduced hMSCs had the same anti-elastase activity as purified human AAT protein. As shown in Figure 4B, AAT secreted from transfected hMSC inhibited elastase activity similar to serum free medium mixed with the same amount of purified hAAT. These data indicate that AAT produced by transduced hMSCs is biologically functional and as equally active as purified human AAT.

Adipogenic and osteogenic differentiation of hMSCs after transduction

After the phenotypic characterization of hMSCs and their differentiation potential along mesenchymal cell lineages, we further examined whether these cells still possessed the defined characteristics of MSCs *in vitro* after transduction of lentiviral AAT vector. Accordingly, lentivirus-transduced hMSCs were differentiated into the adipogenic and osteogenic lineages following the same protocol as that used for untransduced hMSCs. The multipotency of transduced hMSCs was demonstrated by being able to differentiate along the osteogenic and adipogenic lineages, respectively, under specific differentiation conditions. Again differentiated hMSCs were positive for the calcium-specific Alizarin red marker and fat droplets. hMSCs cultured in normal medium served as a control, and were negative for both staining procedures (data not shown). Thus, transduced hMSCs displayed the same differentiation potential as untransduced cells.

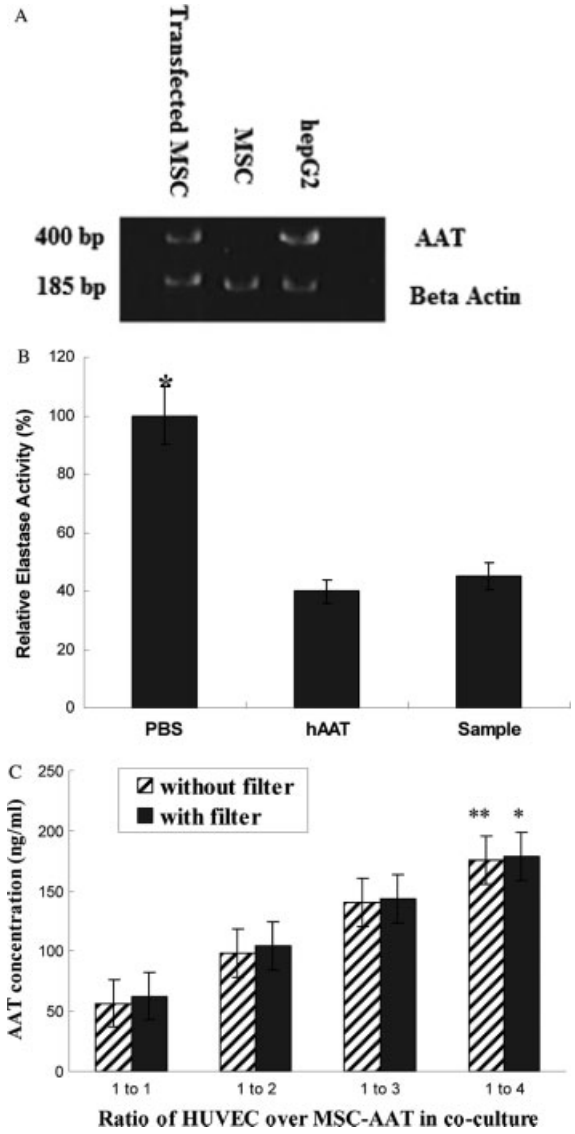

Figure 4. Quantification of human AAT-GFP fusion gene expression in transduced hMSCs. (A) RT-PCR analysis of hAAT in transduced hMSCs. HepG2 cells were used as a positive control and untransduced MSCs as a negative control. (B) Anti-elastase activity of hAAT from transduced MSCs and hAAT purified from human plasma. The relative activity is shown as a percentage of decrease in elastase activity without the inhibitor (as 100%). (C) ELISA analysis of AAT concentration in culture medium from AAT-producing MSCs in co-culture with HUVEC. A significantly higher concentration of AAT was detected in medium when the ratio of HUVEC/AAT-producing MSCs was reduced. Bars indicate the SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to E/T ratio at 1:1 of AAT-producing hMSCs against HUVEC

Cytotoxicity of lentiviral AAT-GFP vector-transduced MSCs against HUVEC

The death of target cells (HUVEC) cultured with effector cells (hMSCs-AAT) was evaluated at day 7 with a TUNEL assay (Figure 5A). These lentivirus-transduced hMSCs were GFP-positive. This allowed us to distinguish hMSCs from dead HUVEC after *in situ* TUNEL staining. As shown in Figure 5B, the percentage of dead HUVEC

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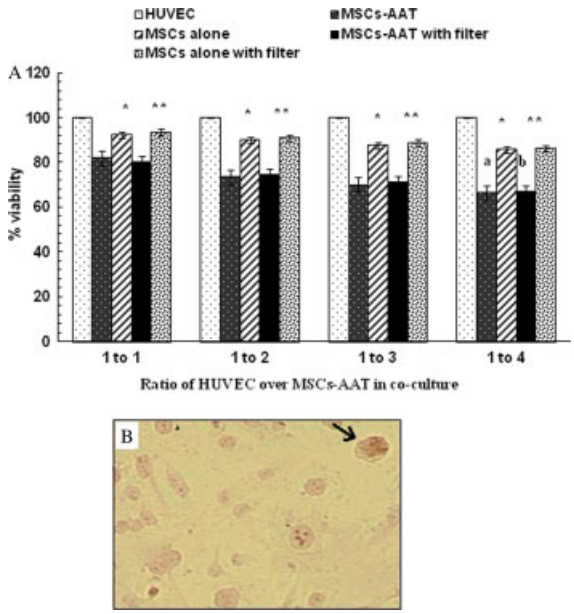

Figure 5. Cytotoxic effect of lentiviral AAT-GFP vector-transduced MSCs against HUVEC. (A) The percentages of the survival target cells (HUVEC) cultured with effector cells (AAT-producing MSCs) and untransduced MSCs at ratios of 0:1, 1:1, 1:2, 1:3 and 1:4 were analyzed 7 days after co-culture. Bars indicate the SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to untransduced controls. 'a' and 'b' indicate $p < 0.05–0.01$. The viability was decreased as the number of transduced MSCs was increased compared to the ratio at 1:1 in culture with or without filter insert. (B) Images of apoptotic cells co-cultured with AAT-producing hMSCs. An *in situ* apoptosis detection kit was employed to stain apoptotic cells after fixation. The nuclei of DAB-positive cells are shown in brown

was significantly increased ($p < 0.05–0.01$) when a co-culture ratio of HUVEC/MSCs was decreased from 0:1, 1:1, 1:2, 1:3 to 1:4; whereas untransduced hMSCs did not show any toxicity against HUVEC. These results indicate that AAT produced by hMSCs exerted a cytotoxic effect against target cells, and a higher number of transduced hMSCs secreted more AAT, and exhibited profound cytotoxicity against HUVEC. To confirm that the cytotoxicity was attributed to the release of the soluble AAT protein by hMSCs, untransduced hMSCs or transduced hMSCs were grown in the upper well of transwell plates, and target (HUVEC) cells were grown in the lower well. The increase in cytotoxicity caused by transduced hMSCs was demonstrated in an effector/target cell ratio-dependent pattern ($p < 0.05$) (Figure 5A). This finding confirmed that increased HUVEC death was caused by soluble AAT protein released from transduced hMSCs.

Effects of AAT-producing hMSCs on HUVEC proliferation *in vitro*

We investigated whether co-culturing HUVEC with AAT-producing hMSCs inhibits HUVEC growth. HUVEC were co-cultured with transduced or untransduced hMSCs in increasing numbers. After the co-culture, HUVEC were stained with primary antibody against Ki-67, which is a nuclear protein that is strictly associated with cellular proliferation, and PCNA, which is expressed in the nuclei during the DNA synthesis phase of the cell cycle. Compared to HUVEC cultured alone, HUVEC in co-culture with AAT-producing hMSCs displayed a significantly reduced rate of positive Ki-67 and PCNA staining ($p < 0.05$); whereas, HUVEC in co-culture with untransduced hMSCs did not show any inhibition in growth, as indicated by similar rates of positive immunostaining staining of either Ki-67 or PCNA ($p > 0.05$) (Figure 6). These data suggest that AAT-producing hMSCs inhibited the proliferation of HUVEC when they were in co-culture with AAT-producing hMSCs.

Discussion

Malignancies acquire certain properties that facilitate tumor progression. Proteases and protease inhibitors are known to play an important role in promoting or blocking tumor invasion and metastasis [19]. Studies have showed an increased proteolytic activity in different malignancies such as lung cancer, which may contribute to tumor progression and metastasis [3,4]. It has been shown that several serpins, such as PEDF, mapsin, AAT, etc., act as inhibitors of angiogenesis [7,20–23]. Serpins interfere with angiogenesis by reducing extracellular matrix degradation through the inhibition of serine proteases. In solid tumor tissues, such as lung cancer, colon adenocarcinoma, etc., a strong negative correlation exists between local levels of AAT and larger tumor size or poor prognoses [24,25].

Tumor growth and angiogenesis are often associated with an intense inflammatory response. Tumor cells secrete growth factors and cytokines that are chemoattractants for macrophages and polymorphonuclear leukocytes. Most of the neutrophil-induced tumor-promoting effects are attributed to their ability to release proteases, such as elastase, cathepsin G and protease-3, which may lead to the activation of matrix metalloproteinases (MMPs) that mediate tumor cell invasiveness [26,27]. Therefore, AAT may exert an inhibitory effect on these proteases and reduce the degradation of extracellular matrices through its anti-protease activity and inhibition of MMP activity.

In this context, it is rational to consider using AAT as a potential anti-angiogenic agent in the treatment of cancer. However, the delivery of the functional protein to the tumor site is challenging. To face this challenge, gene therapy combined with cell therapy offers the possibility for patients to produce the protein by delivering genetically engineered cells (i.e. an endogenous factory for anti-angiogenic protein production) [9].

Previous studies have demonstrated that systemically administered hMSCs home to a tumor site, preferentially survive and proliferate in the presence of malignant cells, and become incorporated into the tumor architecture as stromal fibroblasts [11]. The microenvironment of solid tumors is similar to the environment of injured/stressed

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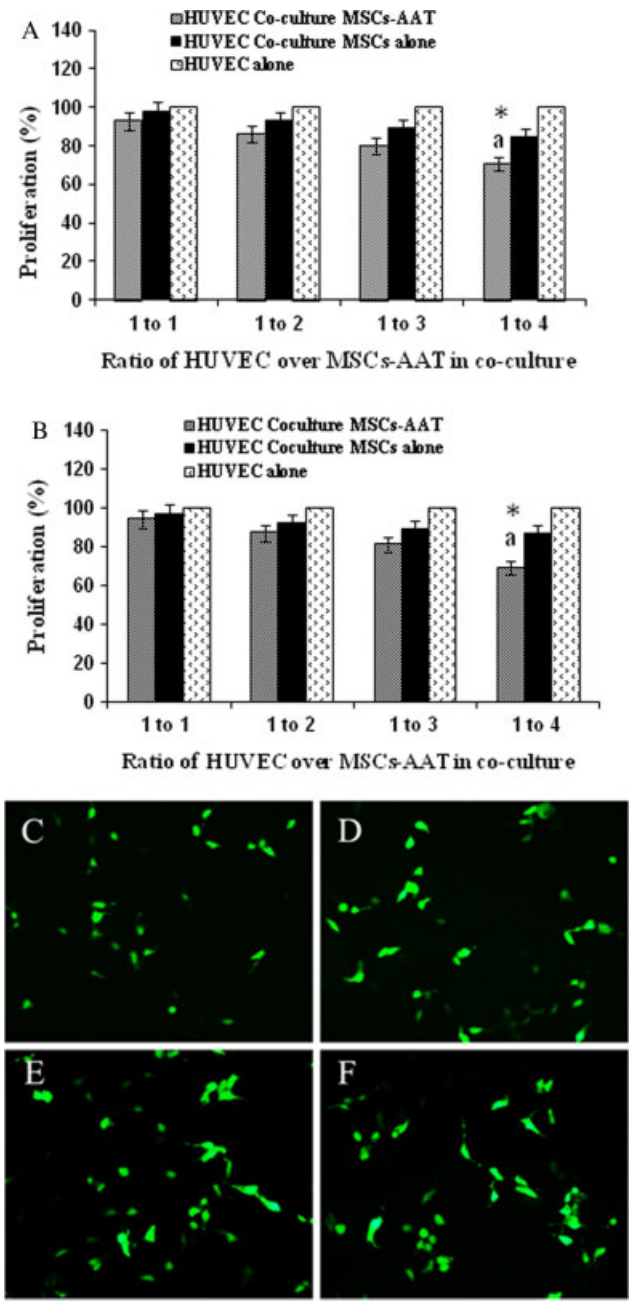

Figure 6. Effects of AAT-producing hMSCs on HUVEC proliferation. The proliferation of HUVEC was inhibited by AAT released from transduced hMSCs. Compared to HUVEC cultured alone, HUVEC cells in co-culture with transduced hMSC displayed a significantly decreased cell number ($p < 0.05$), when they were stained for Ki-67 (A) and PCNA (B). $^{*}p < 0.05$ compared to untransduced MSCs. (C–F) Representative micrographs of immune histotochemical staining of Ki-67 and PCNA in HUVEC. HUVEC co-cultured with transduced (C, D) and untransduced (E, F) hMSCs for 7 days. Immune histotochemical staining of HUVEC co-cultured with hMSCs-AAT (ratio 1:4) for Ki-67 (C) and PCNA (D) or with untransduced hMSCs for Ki-67 (E) and PCNA (F) showed that the the density of positive nucleus staining for Ki-67 and PCNA was higher when they were co-cultured with untransduced hMSCs compared to those co-cultured with AAT-secreting hMSCs

tissue, and therefore it is conceivable to speculate that solid tumors may provide a permissive environment for the engraftment of administrated hMSCs [1,13,28]. On the basis of this speculation, we hypothesized that hMSCs may be used as a gene carrier for AAT production in cancer therapy via inhibiting angiogenesis. In this context, gene therapy for delivering the AAT gene employs hMSCs as a targeting vehicle, and this strategy may potentially be feasible for the development of a novel therapeutic approach against solid and malignant tumor. On the other hand, given the fact that AAT functions physiologically as a protease inhibitor and anti-angiogenic factor, its effects may be enhanced; at the same time, its systemic toxicity could be reduced, when it is produced locally by transduced hMSCs with the human AAT gene.

In the present study, we evaluated the synthesis and release of AAT by lentiviral transduction in hMSCs derived from bone marrow donors, and determined the *in vitro* cytotoxicity of AAT as an anti-angiogenic protein against HUVEC in a co-culture system. This co-culture system is commonly used for physiological and pharmacological investigations, such as angiogenesis. We found that transduced hMSCs produced a significant amount of functional AAT protein as indicated by an increased level of AAT in culture medium and its inhibition of elastase activity. We also observed that after co-culturing transduced hMSCs with HUVEC for 7 days, transduced hMSCs exerted significant cytotoxic effects on HUVEC. By contrast, untransduced hMSCs did not show any toxicity (Figure 5A).

In an attempt to determine whether the soluble proteins, such as AAT, secreted from hMSCs contributed to cytotoxicity against HUVEC, we conducted an assay using transwell inserts. We were able to verify that AAT released from hMSCs was responsible for the enhanced cytotoxicity against HUVEC because an increased release of soluble AAT proteins was found in a lower ratio (1:4) of HUVEC over hMSCs in the co-culture system (Figure 5A). To examine the effect of human AAT secreted from hMSCs on HUVEC proliferation, immunostaining of Ki-67 and PCNA was conducted in HUVEC co-cultured with transduced hMSCs. As shown in Figure 6, AAT released from hMSCs inhibited HUVEC proliferation in a cell number ratio-dependent fashion. These data suggest a potential role of AAT released from hMSCs, as a direct inhibitor of angiogenesis when hMSCs home to a tumor site. Therefore, AAT released from hMSCs could be therapeutically effective in inhibition of angiogenesis.

In the present study, we have demonstrated that lentivirus-transduced hMSCs were able to synthesize and release functional AAT, which is responsible for the inhibition of proliferation and enhanced cell death of HUVEC in a co-culture system. Our study points to a new possibility that AAT-producing hMSCs may serve as a cellular vehicle for the delivery and local production of anti-angiogenic agents. This approach may overcome the extensive metabolism and toxicity associated with the systemic administration of biological agents and anti-angiogenic proteins, and could serve as a versatile tool for manipulating the extracellular milieu of malignant cells.

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