Research Article

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Adipose tissue-derived mesenchymal stem cell-based liver gene delivery

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Background & Aims: The adipose tissue represents an accessible, abundant, and replenishable source of adult stem cells for potential applications in regenerative medicine. Adipose tissue-derived mesenchymal stem cells (AT-MSCs) resemble bone marrow-derived mesenchymal stem cells (BM-MSCs) with respect to morphology, immune-phenotype, and multiple differentiation capability. In the present study, we investigated the feasibility of AT-MSC-based liver gene delivery for the treatment of alpha 1-antitrypsin deficiency.

Methods: Mouse AT-MSCs were transduced by rAAV vectors and transplanted into the mouse liver.

Results: We showed that AT-MSCs can be transduced by recombinant adeno-associated viral vector serotype 1 (rAAV1-CB-hAAT). After transplanting to the mouse liver, *ex vivo* transduced AT-MSCs expressed the transgene product, human alpha 1-antitrypsin (hAAT). Importantly, serum levels of hAAT were sustained and no anti-hAAT antibody was detected in any recipients.

Conclusions: These results demonstrated that AT-MSCs can be transduced by rAAV vectors, engrafted into recipient livers, contribute to liver regeneration, and serve as a platform for transgene expression without eliciting an immune response. AT-MSC-based gene therapy presents a novel approach for the treatment of liver diseases, such as AAT deficiency.

Introduction

Alpha 1-antitrypsin (AAT) deficiency is a genetic disorder, which leads to an accumulation of mutant AAT in hepatocytes and a reduction in serum levels of this protein [15]. Consequently, this mutation causes an increased risk of developing pulmonary emphysema and severe forms of liver disease [7,45]. Protein replacement therapy is the only available treatment for AAT deficiency-associated lung disease [56]. Skeletal muscle-directed gene therapy using recombinant AAV (rAAV) vectors has been established and is being tested in clinical studies [5,30,51–53]. For AAT deficiency-associated liver disease, no effective therapy is available except liver organ transplantation, which is hampered by a shortage of donor organs. The development of a safe and efficient therapy is needed.

Hepatocyte transplantation has been applied in metabolic liver disease as a valuable alternative to whole-organ transplantation [18]. Transplantation of non-liver cells such as bone marrow cells has also demonstrated the feasibility and efficacy of generating hepatocytes [4,6,36,38]. However, liver directed cell transplantation is challenging in animal models because endogenous hepatocytes are capable of proliferating and thus compete with transplanted cells during liver regeneration. Retrorsine and monocrotaline (MCT) are members of the pyrrolizidine alkaloid family and efficiently inhibit hepatocyte proliferation [25,59]. Partial hepatectomy (PHx) creates an acute demand for liver regeneration. Consequently, long-term, near-total liver replacement by exogenous hepatocytes can be achieved in recipient rats treated with a combination of PHx and retrosine or MCT [25,59].

Mesenchymal stem cells (MSCs) are a heterogeneous population of plastic-adherent, spindle-shaped, and fibroblast-like cells, which can be extensively expanded *in vitro* while retaining their multi-lineage differentiation potential, which includes osteogenesis, chondrogenesis, and adipogenesis [8,40]. In addition to differentiation into its native derivatives of mesenchymal tissues, MSCs also have the potential to differentiate into hepatocytes *in vitro* and *in vivo* [1,2,27]. Traditionally, MSCs have been isolated from bone marrow aspirates with a low yield, which have limited their application [8]. Recent studies have shown that MSCs can also be isolated from adipose tissue [20,46]. There is little or no difference between BM-MSCs and AT-MSCs [14,24]. Since adipose tissue is abundant and the frequency of MSCs in adipose tissue is a 100-fold higher than that of bone marrow [24], adipose tissue may represent an ideal autologous stem cell source allowing for repeat access, replenishment, easy isolation, and minimal patient discomfort.

Adeno-associated virus (AAV) is a linear single-stranded DNA parvovirus [21]. Because of the lack of pathogenicity, low risk of insertional mutagenesis, multitude of available serotypes, and

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Abbreviations: AT-MSCs, adipose tissue-derived mesenchymal stem cells; AAT, alpha 1-antitrypsin; AAV, adeno-associated virus.

broad tissue tropisms, rAAV vector has been widely used in gene therapy applications [17,32]. Recent improvements of rAAV vectors, including self-complementary or double-stranded AAV (ds AAV) vectors, chimeric rAAV vectors, and mutant rAAV vectors, have greatly enhanced the transduction efficiency of rAAV vectors and created more opportunities for their application [60,65]. In the present study, we investigated the feasibility of AT-MSC-based liver gene delivery for the treatment of alpha 1-antitrypsin deficiency.

Materials and methods

AT-MSC isolation and culture

AT-MSCs were isolated from peritoneal adipose tissue excised from the abdominal region of 6- to 8-week-old male C57BL/6 mice. Adipose tissue was enzymatically digested with 0.075% collagenase (type I; Sigma–Aldrich, St. Louis, MO) in PBS for 1hr at 37 °C with gentle agitation. The collagenase was inactivated with an equal volume of DMEM (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT), and the infranatant was centrifuged at 1000*g* for 5 min at room temperature. The resulting cell pellet was resuspended in 160 mM NH_4Cl (StemCell Technologies Inc., Vancouver, BC), incubated at room temperature for 2 min to eliminate contaminating red blood cells and filtered through a 100-μm nylon mesh strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove debris. The resulting AT-MSC-containing cell pellet was collected by centrifugation as described above, resuspended in a DMEM/10% FBS medium, and plated at 1–2 × 10^6 cells/100 mm in plastic tissue culture dishes. The non-adherent cell population was poured off after 12–16 h of culture. Adherent cells were washed with PBS and cultured in DMEM/10% FBS medium for expansion. When the cells reached 70–80% confluence, they were harvested with 0.25% trypsin–EDTA and reseeded at 1.0 × 10^5 cells/60 mm dish.

Recombinant AAV vector construction and production

Recombinant single-stranded AAV vectors (ssAAV-CB-hAAT) used in this study have been described previously [50,61]. Briefly, plasmid ssAAV-CB-hAAT contained full length AAV2 ITRs, and hAAT cDNA driven by cytomegalovirus enhancer/chicken-β-actin (CB) promoter. In order to achieve high levels of hAAT expression in AT-MSCs, we generated and tested two double strand AAV (dsAAV) vectors. In these vectors, smaller promoters (shorter in length) were used. Duck hepatitis B virus (DHBV) promoter was used in dsAAV-DHBV-hAAT [29]. The CMV promoter was used to generate dsAAV-CMV-hAAT. These vectors were packaged into AAV serotype 1 or 8 capsids as described previously [9].

Immunofluorescent staining of AT-MSCs

Cells at passage 3 were plated onto glass chamber slides for a day, and then fixed for 15 min in 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.0). The fixed slides were washed for 10 min in 100 mM glycine in PBS (PBS/glycine) and blocked for 1 h in immunofluorescent blocking buffer (IBB) containing 5% bovine serum albumin (BSA), 10% FBS, PBS, and 0.1% Triton X-100. The cells were subsequently incubated for 1 h in IBB containing the following anti-mouse monoclonal antibodies: CD31, CD34, CD44, CD45, CD90, CD105, and CD133 (1:100, eBioscience, San Diego, CA). Next, the cells were washed extensively with PBS/glycine and incubated for 1 h in IBB containing a fluoroisothiocyanate (FITC)-conjugated secondary antibody (1:200, eBioscience, San Diago, CA). All slides were then washed in PBS/glycine and mounted with glass coverslips using DAPI Vectorshield (Vector, Burlingame, CA).

Adipogenic differentiation of AT-MSCs

AT-MSCs at passage 3 were seeded in six-well plates and grown to 100% confluence for differentiation studies. Adipogenic differentiation was induced by culturing cells in adipogenic medium for 2 weeks with medium changes twice weekly. Adipogenic medium consisted of DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 200 μM indomethacin, and 10 μg/ml bovine insulin (all reagents were from Sigma, St. Luis, MO). Adipogensis was assessed by staining for intracellular lipid droplets with Oil Red O stain (Sigma, St. Luis, MO).

Adipogenic differentiation of AT-MSCs

AT-MSCs at passage 3 were seeded in six-well plates and grown to 100% confluence for differentiation studies. Osteogenic differentiation was induced by culturing cells in osteogenic medium for 4 weeks with medium changes twice weekly. Osteogenic medium consisted of DMEM supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbate-2-phosphate (all reagents were from Sigma, St. Louis, MO). Osteogenesis was assessed by staining for calcium depositions with Alizarin Red S (pH 4) stain (Sigma, St. Louis, MO).

AT-MSCs transplantation

Adult female C57BL/6 mice received monocrotaline (MCT, Sigma, St. Louis, MO, dissolved in PBS) at 50 mg/kg BW at a two-week intervals by i.p. injection to inhibit endogenous liver cell proliferation [59]. Two weeks after receiving the second injection, the mice were partially hepatectomized to remove 70% of the liver under general anesthesia. In the meantime, 1.6 × 10^6 AT-MSCs were suspended in 100 μl saline and transplanted into the remaining liver immediately after PHx by intrasplenic injection into the inferior tip of the spleen using a 30-gauge needle. To aid in the coagulation process, the splenic injection site was ligated using sterile absorbable surgical suture (Ethicon, Inc., Somerville, NJ). All mice were sacrificed at 8 weeks after transplantation for tissue examination.

Immunohistochemistry for human AAT and mouse albumin

Organ tissues were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. For hAAT and albumin immunostaining, tissue sections (5 μm) were de-paraffinized, rehydrated, and blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 min. To detect hAAT expression, tissue sections were incubated with primary antibody, rabbit anti-human AAT (1:800, RDI/Fitzgerald Industries, Concord, MA, USA), overnight at 4 °C. Staining was detected using ABC-Rabbit-HRP and DAB kits (Vector laboratories, Burlingame, CA). Antigen retrieval was performed in Digest-All™ (trypsin) (Zymed® Laboratories, Carlsbad, CA) for 5 min at 37 °C, followed by incubation in Trilogy (Cell Marque Corp., Rocklin, CA) for 25 min at 95 °C. In order to detect albumin expression, antigen retrieval was performed using citrate retrieval for 30 min in a steamer. The tissues were incubated with goat anti-mouse albumin (1:5000, Abcam, Ltd., Cambridge, MA) overnight at 4 °C, followed by incubation with biotinylated horse anti-goat (1:200, Vector Laboratories) for 30 min. Staining was developed using the Vectastain ABC-Alkaline Phosphatase kit (Vector Laboratories) with Vulcan Fast Red (VFR) chromagen (Biocare Medical, Concord, CA).

Y-chromosome fluorescence in situ hybridization

NBF fixed, paraffin embedded liver tissue samples were sectioned (5 μm), de-paraffinized in xylene, rehydrated in a 100%, 95%, 70% ethanol series, and rinsed twice in ddH_2O. Slides were pretreated in 0.2 N HCl for 30 min at room temperature, and then washed twice for 5 min in ddH_2O. Antigen retrieval was performed by immersing slides in 1 M sodium thiocyanate for 30 min at 85 °C. The slides were removed from the retrieval solution and rinsed thoroughly in ddH_2O. Sections were digested in 4 mg/ml pepsin (Sigma, St. Luis, MO) diluted in 0.9% NaCl (pH 2.0) for 11–15 min at 37 °C. Digested sections were removed and immediately rinsed in water, equilibrated in 2× SSC and dehydrated through a graded ethanol series. Three μl mouse Y-paint probe (FITC) diluted in 12 μl hybridization buffer (Cambio Ltd.; Cambridge, UK) was denatured for 10 min in a 75 °C water bath and allowed to pre-anneal at 37 °C for 45 min prior to being applied on a 22 × 22-mm coverslip. Slides were sealed with rubber cement, denatured at 60 °C for 10 min and hybridized overnight at 37 °C in a Hybrite oven (Vysis, Downers Grove, IL). After hybridization, coverslips were removed and the sections were washed in three changes of 50% Formamide/2 × SSC, followed by 2 × SSC, and 4 × SSC + 0.1% Igepal (NP-40) at 46 °C for 7 min each. Slides were air dried at RT in the dark and mounted with DAPI Vectorshield (Vector Laboratories, Burlingame, CA).

ELISAs for hAAT and anti-hAAT antibodies

Human AAT and anti-hAAT antibodies were detected by ELISA as previously described [31].