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Adipose Tissue-Derived Mesenchymal Stem Cell-Based Liver Gene Delivery

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Abstract

Back ground/Aims—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 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 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.

Conclusion—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.

Keywords

Alpha 1 antitrypsin deficiency; Adeno-associated virus (AAV); Liver gene therapy; Liver regeneration

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]

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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.[57] Skeletal muscle-directed gene therapy using recombinant AAV (rAAV) vectors has been established and is being tested in clinical studies.[5,30,52-54] 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 pyrrolizidine alkaloid family and efficiently inhibit hepatocyte proliferation.[25,60] 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. [25,60]

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 of 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 has 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 hundred-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 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 [61,66]. 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-MSCs 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 1,000g for 5 min at room temperature. The resulting cell pellet was resuspended in 160mM NH₄Cl (StemCell Technologies Inc, Vancouver, BC), incubated at room temperature for 2 min to

eliminate contaminating red blood cells and filtered through a 100- μ m 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 $1-2 \times 10^6$ cells/100 mm in plastic tissue culture dishes. The non-adherent cell population was poured off after 12-16 hr 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/60mm dish.

Recombinant AAV Vector Construction and Production

Recombinant single-stranded AAV vectors (ssAAV-CB-hAAT) used in this study have been described previously.[51,62] 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 100mM sodium phosphate buffer (pH 7.0). The fixed slides were washed for 10 min in 100mM glycine in PBS (PBS/glycine) and blocked for 1 h in immunofluorescent blocking buffer (IBB) containing 5% bovine serum albumin (BSA), 10% FBS, PBS, 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 fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200, eBioscience, San Diego, 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, 0.5mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, 200 μ M indomethacin, and 10 μ g/ml bovine insulin (all reagents were from Sigma, St. Louis, MO). Adipogenesis was assessed by staining for intracellular lipid droplets with Oil Red O stain (Sigma, St. Louis, 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 μ M dexamethasone, 10mM β -glycerolphosphate, and 50 μ M 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. Luis, MO, dissolved in PBS) at 50mg/kg BW at a two-week interval by i.p. injection to inhibit endogenous liver cell proliferation.[60] Two weeks after receiving the second injection, the mice were partially hepatectomized to remove 70% liver under general anesthesia. In the meantime, 1.6×10^6 AT-MSCs were suspended in 100ul 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 (5µm) were de-paraffinized, rehydrated, and blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 minutes. To detect hAAT expression, tissue sections were incubated with primary antibody, rabbit anti-human AAT (1:800, RDI/Fitzgerald Industries, Concord, MA, USA), for 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 minutes at 37°C, followed by incubation in Trilogy (Cell Marque Corp., Rocklin, CA) for 25 minutes at 95°C. In order to detect albumin expression, antigen retrieval was performed using citrate retrieval for 30 minutes in a steamer. The tissues were incubated with goat anti-mouse albumin (1:5,000, Abcam, Ltd., Cambridge, MA) overnight at 4°C, followed by incubation with biotinylated horse anti-goat (1:200, Vector Laboratories) for 30 minutes. 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 (5µm), deparaffinized in xylene, rehydrated in a 100%, 95%, 70% ethanol series, and rinsed twice in ddH₂O. Slides were pretreated in 0.2N HCL for 30 minutes at room temperature, and then washed twice for 5 minutes in ddH₂O. Antigen retrieval was performed by immersing slides in 1M sodium thiocyanate for 30 minutes at 85°C. The slides were removed from the retrieval solution and rinsed thoroughly in ddH₂O. Sections were digested in 4mg/ml pepsin (Sigma, St. Luis, MO) diluted in 0.9% NaCl (pH2.0) for 11-15 min at 37°C. Digested sections were removed and immediately rinsed in water, equilibrated in 2X 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 minutes in a 75°C water bath and allowed to pre-anneal at 37°C for 45 minutes prior to being applied on a 22 × 22-mm coverslip. Slides were sealed with rubber cement, denatured at 60°C for 10 minutes 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 3 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]

Results

Isolation and Characterization of AT-MSCs

Mouse AT-MSCs were characterized for MSC marker expressions and multilineage differentiation potential. Immunofluorescence staining revealed that the cells isolated from the mouse peritoneal adipose tissue expressed stromal-associated markers CD44, CD90 and CD105 but did not express either hematopoietic markers (CD34 and CD45) or an endothelial marker (CD31). The expression of CD133 was low (Figure 1A). Two weeks after exposure to adipogenic induction medium, intracellular lipid droplets were observed within the adipogenic-differentiated AT-MSCs using Oil Red O staining (Figure 1B). Osteogenic differentiation resulted in extracellular calcium phosphate precipitates as revealed by Alizarin Red S staining (Figure 1B).

AT-MSCs Transduction of rAAV vectors

In order to select our most efficient rAAV vector for AT-MSCs transduction, matched doses of ssAAV1-CB-hAAT, ssAAV8-CB-hAAT, dsAAV1-CMV-hAAT and dsAAV8-DHBV-hAAT vectors were used to infect AT-MSCs. As shown in Figure 2A, ssAAV1-CB-hAAT vector mediated higher transgene expression (hAAT in culture medium) than the other three rAAV vectors at day 9 after transduction. Intriguingly, rAAV1 mediated more than 25-fold higher levels of hAAT expression than rAAV8 in AT-MSCs. Furthermore, double infection of AT-MSCs with ssAAV1-CB-hAAT vector at a 12 hr interval further increased the transgene expression (Figure 2B). Together, the above results clearly demonstrated that ssAAV1-CB-hAAT vector was relatively efficient within the vectors tested and displayed a clear advantage in transgene expression. Therefore, we decided to use ssAAV1-CB-hAAT vector for the following studies.

Liver transplantation of ex vivo Transduced AT-MSCs

We hypothesized that *ex vivo* transduced AT-MSCs carrying ssAAV1-CB-hAAT could serve as a platform for liver expression of hAAT after autologous transplantation. To test this hypothesis, AT-MSCs (1.6×10^6) were infected with ssAAV1-CB-hAAT ($\text{MOI}=5 \times 10^4$) and were transplanted into the liver of MCT-treated and partial-hepatectomized (PHx) recipients (Figure 3A). Recipients were sacrificed 8 week after transplantation. Y-FISH showed the presence of male donor cells in the female recipient liver indicating that transplanted AT-MSCs were able to migrate into the liver from the splenic injection site, engraft into the recipient liver parenchyma, and contribute to liver repopulation (Figure 3C, D). Immunostaining for hAAT showed that about 20% of liver cells were hAAT positive (Figures 4A and 4B). Those hAAT-positive cells were morphologically similar to hepatocytes. To confirm that these cells were behaving as hepatocytes, serial sections of recipient liver were subjected to mouse albumin and human AAT immunostaining, respectively. As shown in Figures 4 E and 4F, most (greater than 90) of the hAAT positive cells expressed mouse albumin. These results suggested that AT-MSCs were able to differentiate into functional hepatocytes. H&E staining showed that liver tissues from both MCT treated mice and MCT plus PHx treated mice were morphologically normal as previously observed (Supplementary Figure 1). [25, 49, 55, 60]

In order to quantify transgene product generated and secreted from engrafted rAAV-transduced AT-MSCs, serum hAAT levels were measured. All animals showed sustained transgene expression throughout the study with an average serum hAAT concentration between 100-250ng/ml (Figure 5A). Importantly, anti-hAAT antibodies were undetectable (Figure 5B), and histopathological examinations for lymphocyte infiltration in the livers showed no difference between control and transplanted animals. These results demonstrate

that AT-MSCs can be used as a platform for liver-directed gene delivery, which can avoid immune response to the transgene product.

In order to test the possibility that AT-MSCs may home to other organs, we performed a separate experiment using GFP as a reporter gene in ssAAV1 and repeated the same transduction and transplantation procedure. GFP is an intracellular protein that is easily detected by immunostaining and can serve as a sensitive marker to track transplanted cells. On the other hand, staining for secreted hAAT expression would cause under estimation, especially in tissues containing few homed cells. As shown in Figure 6, GFP-positive cells were detectable in spleen, lung and bone marrow indicating the multi-organ homing potential of AT-MSC. However, we did not observe GFP positive cells in other tissues (Figure 6).

Discussion

The treatment for hAAT deficiency-related emphysema requires achieving a high level of circulating wild type hAAT (M-hAAT). This requirement has been achieved by recombinant adeno-associated virus (rAAV) mediated gene delivery to skeletal muscle or liver in a mouse model [51-53]. However, the treatment liver disease associated with hAAT deficiency requires decrease mutant AAT (Z-AAT) in the liver. Flotte and his colleagues have recently shown siRNA targeting significantly decreased Z-AAT expression in Z-AAT transgenic mice.[11] Replacement of dysfunctional hepatocytes with genetically modified stem cells holds great potential for a treatment of both emphysema and liver disease. Stem cell-based gene correction followed by autologous transplantation may also eliminate unwanted vector distribution to other organs and an immune response. In the present study, we isolated and characterized mouse AT-MSCs. We showed that these AT-MSCs can be efficiently transduced by a ssAAV1-CB-hAAT vector. Transplantation of rAAV transduced AT-MSCs resulted in long-term transgene expression in the recipient liver and sustained serum levels of the transgene product (hAAT) without eliciting an immune response. These results imply an alternative approach for liver gene delivery. Importantly, our findings in this study have paved a way for Z-AAT gene correction using AT-MSC for the treatment of AAT deficiency-associated liver disease.

It is generally accepted that patients with serum AAT levels below 11 μ M (600 μ g/ml) may develop emphysema. Therefore, the serum level of hAAT obtained from AT-MSCs transplantation in this study requires further improvement to be of therapeutic use (approximately 500-800 μ g/ml).[12] Several possible approaches may be employed to enhance hAAT expression levels. Banas and his colleagues demonstrated that the CD105⁺ fraction of AT-MSCs exhibited a high hepatic differentiation capability.[3] Therefore, enrichment of the stem cell population by isolating CD105⁺ AT-MSCs may increase the total number of hepatocytes derived from donor AT-MSCs and thus enhance hAAT levels in the recipient serum. Similarly, other cell markers may also be used to enrich stem cell populations, such as p75 neurotrophin receptor (p75NTR), which has been used to isolate and collect putative multipotent stem cells from mouse adipose tissue-derived stromal vascular fraction culture cells (ADSVF cells).[63] Secondly, the hepatic-differentiation potential of AT-MSCs could be improved by *in vitro* preconditioning toward hepatocyte-like cells through incubating MSCs with specific growth factors.[3,47] In addition, improvement of vector transduction efficiency and the transplantation of an increased number of transduced AT-MSCs may also raise hAAT levels in the recipient serum. Finally, considering the dilution of episomal rAAV DNA during cell division, site-specific integration rAAV vector system may be exploited to enhance and ensure long-term transgene expression[13],[65]

In order to achieve high levels of transgene expression in AT-MSC, we have tested 4 rAAV vectors with different potential advantages. The selection of AAV serotypes 1 and 8 was based on our previous studies comparing AAV serotypes and liver transduction. [10,51,55,62] In this study, we showed rAAV1 was more efficient for AT-MSC transduction than rAAV8. To test the advantages of using self complementary AAV and a liver specific promoter, we have generated dsAAV8-DHBV-hAAT and dsAAV1-CMV-hAAT vectors. In contrast to previous studies, our study showed dsAAV vectors did not demonstrate superior transgene expression compared to ssAAV vector.[59] This inconsistency in a well-documented behavior of dsAAV might reflect the inferior promoter activities of DHBV and CMV, compared to the CB promoter. The superior activity of CB promoter, noted in our study, even defeated the advantages of dsAAV genome for faster and stronger transgene expression. Further improvement of transgene expression in AT-MSCs may be achieved by optimization of rAAV vectors. Recently developed new serotypes of AAV vectors and mutant AAV vectors (e.g. tyrosine-mutant AAV2) might mediate higher transduction efficiency in AT-MSCs.[66]

AT-MSCs represent an excellent adult stem cell source for regenerative medicine. [24,28,50,67] Because of their self-renewal and multiple lineage differential capability, MSCs have been widely investigated as a cell therapy source for tissue or organ regeneration. MSCs have been utilized in hematopoietic stem cell transplantation to improve stem cell engraftment and hematopoietic reconstitution. After systemic delivery MSCs preferentially migrate to damaged tissue. In addition, MSCs are expected to be a targeting vehicle for cancer therapy because of selective engraftment of intravenously administered MSCs at the site of a tumor.[34,58] Using genetically modified MSCs, therapeutic molecules can be brought to the tumor site in a concentrated fashion thus decreasing systemic adverse side effects. MSCs also execute an immune regulatory role in treating graft-versus-host disease.[26] Although these features give AT-MSC an impressive therapeutic potential, the safety of MSCs usage and the molecular mechanisms underlying the biological effects of MSCs remain elusive.[22,23,56] The application of AT-MSCs for treatment of human diseases remains under development.

In this study, we employed monocrotaline (MCT) to inhibit endogenous hepatocyte division and partial hepatectomy (PHx) to create a demand for liver regeneration. These methods were used in previous studies and did not lead to considerable problems post recovery. [25,35,48,55,60] Consistent with previous studies, we did not observe any adverse effects from the model used in this study. Intrasplenic injection has been commonly used for liver directed cell transplantation in animal models. Although intravenous injection and portal vein injection may serve as alternatives, they can lead to more stem cells homing into non-hepatic organs or result in portal hypertension and hepatic damage, respectively. [19,25,33,41-43] In mouse models, inbred cross-gender transplantation has been commonly used as a model to predict autologous transplantation in large animals or humans. We have used male mice as donors and female as recipients for tracing the donor cells by Y-FISH. Although quantification of donor cells by Y-FISH is technically difficult, this method clearly defined and identified donor cells in the recipient livers and spleens. Future studies focusing on quantifications of donor cells and donor cell derived hepatocytes in the recipient liver will provide important information for the application of this technology.

As expected, we showed that transplanted cells produced detectable transgene product (hAAT) and expressed a hepatocyte marker (mouse albumin). It is interesting that we observed a high degree of co-localization between hAAT and mouse albumin in the recipient liver. It has been reported that AT-MSC expresses low levels of albumin and the expression increases after hepatic differentiation, which supports our observation.[64] Although the detailed mechanisms underlying this phenomenon remain elusive, several

possibilities exist. First, the albumin gene may not be evenly expressed in the endogenous hepatocytes.[39] Therefore, some weak staining areas were observed for mouse albumin. Similarly AAT gene is also unevenly expressed in the liver.[51] Since both albumin and AAT are liver specific genes, it is possible that hAAT overexpression could enhance endogenous mouse albumin gene expression. Secondly, transplanted AT-MSCs may be in the same cell stage of the cell cycle and in similar conditions. Furthermore, newly differentiated hepatocytes may be more metabolically active. A combination of these events could cause donor derived hepatocytes to express mouse albumin evenly in high levels. Nonetheless, these results suggest donor AT-MSCs differentiated into functional hepatocytes.

Consistent with previous studies, we observed multi-organ homing of AT-MSCs.[8] As expected we saw that some cells were retained in the spleen after splenic injection. Since MSCs are the progenitor cells for bone cells, we were also not surprised to find donor cells in recipient bones. It is interesting that AT-MSCs homed to the lung. It is possible that MCT treatment induced lung injury, creating a demand for lung regeneration and thus attracting AT-MSCs to the lung. Although we did not observe donor cells in other organs tested by immunostaining for the transgene product, it is still possible that some AT-MSCs homed to other organs. Human AAT expression derived from coincidentally transplanted donor cells in the bone, spleen and lung may be contributing to the total serum levels of hAAT in the recipients. However, quantification of this participation would be technically difficult. Although the effect of this occurrence on the involved spleen and bone remain elusive, previous studies showed non-hepatic expression of hAAT did not cause problems.[30] In addition, it should be noted that several non-hepatocytic organs and cell types also express AAT e.g. mononuclear phagocytes and neutrophils. [37,44]

Immune responses to transgene products have been a hurdle in gene replacement therapy for treatment of genetic diseases such as hemophilia. In a recent study, we showed that rAAV8 failed to transduce dendritic cells (DCs) and led to no detectable immune response to the transgene product.[31] One remaining question is whether or not the high levels of transgene product from rAAV8 are responsible for this immune tolerance. In our present study, we show that detectable but low levels of transgene product (hAAT) from transplanted cells did not lead to measurable immune response. These results eliminate the above possibility that high levels of transgene product are responsible for immune tolerance, and provide further evidence to support our previous conclusion. It is worth mentioning that the rAAV1 vector used in this study is able to transduce DCs and generate a strong immune response if it is directly administrated into animals [31]. The lack of an immune response in recipient mice suggests that the AT-MSCs used in this study has no DC contamination and that rAAV1 transcytosis did not occur in AT-MSCs after transplantation.[16] Together, these results not only provide additional evidence supporting our hypothesis that a host cell produced foreign gene product is not immunogenic, but also uncovered unique advantages of AT-MSC mediated liver gene delivery. Future studies will focus on the mechanisms underlying the immune tolerance.

In summary, our current study tested the novel approach of using AT-MSCs as a vehicle to carry the wild type AAT gene. We showed 1) AT-MSCs can be transduced by rAAV1 vector; 2) AT-MSCs can serve as a platform for gene delivery to the liver; and 3) AT-MSCs mediated gene therapy can avoid host immune response to the transgene product and thus has a great potential for the treatment of genetic diseases, in which an immune response is unwanted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AT-MSCs	adipose tissue-derived mesenchymal stem cells
AAT	alpha 1-antitrypsin
AAV	adeno-associated virus

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Figure 1. Characterization of AT-MSCs

(A) Expression of cell surface markers in AT-MSCs detected by immunofluorescence staining. (B) Multiple differentiation potential of AT-MSCs. Left, undifferentiated AT-MSCs; Middle, AT-MSCs were cultured for 2 weeks in an adipogenic induction medium and stained with Oil Red O for lipid droplets; Right, AT-MSCs were cultured for 4 weeks in an osteogenic induction medium and stained with Alizarin Red S (pH 4) for calcium phosphates.

Figure 2. Ex vivo AT-MSCs transduction of rAAV vectors

(A) Transgene expression levels from AT-MSCs transduced by four rAAV vectors. Mouse AT-MSCs (passage 3) were seeded into 24-well plate (5×10^4 cells/well, $n=3$) and infected with rAAV-hAAT vector at 1×10^4 particles/cell. The accumulative hAAT in the culture medium was measured by hAAT specific ELISA. Solid triangle, ssAAV1-CB-hAAT vector; Open circle, dsAAV1-CMV-hAAT vector; Open square, ssAAV8-CB-hAAT vector; Cross, dsAAV8-DHBV-hAAT vector; Dashed line, lower limit of quantification (LLOQ). Results from PBS group (negative control) were below LLOQ. (B) Double transduction of AT-MSCs by ssAAV1-CB-hAAT vector. Mouse AT-MSCs (passage 1) were seeded in 24-well (5×10^4 cells/well, $n=3$) and infected with ssAAV1-CB-hAAT vector at 5×10^4 particles/cell. The accumulative hAAT in the culture medium was measured by hAAT ELISA. Triangle, one infection; Circle, two infections at 12 hr interval; dashed line, lower limit of quantification (LLOQ). Results from PBS group (negative control) were below LLOQ.

Figure 3. AT-MSC transplantation

(A) Experimental outline. The newly purified AT-MSCs were infected with ssAAV1-CB-hAAT vector for 2 hours, washed three times with PBS, and transplanted into the recipient livers by intrasplenic injection. (B, C, and D) Detection of donor cells in recipient liver after AT-MSCs transplantation by fluorescence in situ hybridizations for Y-chromosome. Male liver served as positive control (B). Y chromosomes were detected in female recipient mice (C and D).

Figure 4. Immunostaining for hAAT and mouse albumin in recipient liver

(A) Immunostaining for hAAT in a representative liver section from a mouse transplanted with AT-MSCs transduced with ssAAV1-CB-hAAT. (B) High magnification image of A. (C) Liver section from the same animal as in A and B stained by anti-rabbit immunoglobulin G serving as a negative control. (D) Liver section from an untransplanted (but MCT treated) mouse serves as a negative control. (E and F) Detection of coexpression of hAAT and mouse albumin by immunostaining. (E) Liver section from a mouse transplanted with ssAAV1-CB-hAAT vector infected AT-MSCs stained for hAAT (brown). (F) A serial section of a recipient mouse liver (E) stained for mouse albumin (red). (G) Human liver section stained for hAAT serving as a positive control. (H) Liver section from a normal mouse without AT-MSC transplantation was stained for mouse albumin serving as a positive control.

Figure 5. Detection of human alpha 1-antitrypsin (hAAT) and immune response in the recipients AT-MSCs from C57BL/6 mice were infected with ssAAV1-CB-hAAT vector at 5×10^4 particles/cells for 2 h and transplanted into the liver of partially hepatectomized C57BL/6 recipients (1.6×10^6 cells/mouse; $n=3$). (A) Serum levels of hAAT in recipients. Dashed line is the lower limit of quantification (LLOQ). Results from untransplanted mouse (negative control) were below the LLOQ. (B) Anti-hAAT antibodies were detected by ELISA. W1, W4 and W8 are week 1, 4 and 8 after transplantation, respectively. Sera from normal mice served as a negative control (–); sera from hAAT protein injected mice served as a positive control (+). Dashed line is the LLOQ.

Figure 6. Multi-organ homing of transplanted AT-MSCs

AT-MSCs were transduced with ssAAV1-CB-GFP vector and transplanted into female recipients. Tissue samples were collected at 8 weeks after transplantation. Immunostaining for GFP (brown) was performed with tissue sections of organs. Arrowheads indicate the observed GFP staining. Images were viewed at $\times 20$ magnification.