ABSTRACT: Mesenchymal stem cells have the potential to secrete a variety of humoral factors, deliver exogenous genes and differentiate into a range of cells. Thus, these cells may serve as carriers for cell and gene-cell therapies. In this paper, we report differences between adipose-derived mesenchymal stem cells (ASCs) and bone marrow-derived mesenchymal stem cells (BSCs) from the viewpoint of gene transfer efficiency. Both ASCs and BSCs were harvested from 5-week-old mice (C57BL/6N). Following three passages in culture, we analyzed the capacity of these cells to differentiate along osteogenic, chondrogenic and adipogenic lineages in vitro. After confirmation of multipotency, these cell populations were studied for gene transfer efficiency, using adenoviral, lentiviral, retroviral and adeno-associated viral (AAV) type 1, 2 and 5 vectors. EGFP was used as the reporter for all vectors. The gene encoding EGFP was transduced into ASCs more easily than into BSCs. Both cell populations were transduced effectively with AAV types 1 and 5, lentiviral and retroviral vectors, but were resistant to transduction with adenoviral and AAV type 2 vectors. In conclusion, found that the AAV types 1 and 5, lentiviral and retroviral vectors deserved further study in both ASCs and BSCs. Moreover, the transduction efficiency of ASCs was greater than that of BSCs, suggesting that the former would be more useful in clinical applications involving gene transduction.

KEY WORDS: Bone Marrow, Fat, Mesenchymal stem cells, Osteogenesis

INTRODUCTION

Given recent advances in stem cell research, the fields of “gene therapy” and “regenerative medicine” have developed rapidly. Stem cells have the potential to secrete a variety of humoral factors, deliver exogenous genes and differentiate into the types of cells required for repair of specific injuries (Asahara et al. 2000; Deans and Moseley 2000; Ogawa 2006; Taguchi et al. 2005). As first reported in 2001 (Zuk et al. 2004), there are a number of advantages to using mesenchymal stem cells (MSCs) derived from adipose tissue (ASCs): they can be harvested non-invasively, handled easily and multiplied effectively. As such, their multipotency and proliferative efficiency does not differ substantially from bone marrow-derived MSCs (BSCs) and in comparison to the harvest of MSCs from other sites, donor morbidity is considerably lower. Since they are less dense than water, mature adipocytes are particularly easy to remove from adipose tissue, requiring only collagenase treatment and centrifugation. In addition, ASCs exhibit significant advantages from the clinical point of view: 1 g of fat can supply sufficient MSCs for treatment purposes, the patient requires only local anesthesia and the donor site wound heals within a week. Moreover, sufficient MSCs can be harvested via liposuction or lipectomy to enable immediate autotransplantation, avoiding the need for ex vivo culture.

In the near future, both ASCs and BSCs may be used as carriers for cell and gene-cell therapies; in this study we examined the gene transfer efficiency of these MSCs.

MATERIALS AND METHODS

Harvesting of ASCs

Five-week-old mice (C57BL/6N) were maintained following the National Research Council’s guidelines for the care and use of laboratory animals. Mice were anesthetized with sodium pentobarbital (0.1 mg/100 g) and shaved. The inguinal fat pads were harvested and washed extensively in phosphate-buffered saline (PBS; Gibco BRL, Grand Island, NY). They were then minced finely and incubated for 1 h on tissue culture plates (10 cm², Becton Dickinson, Franklin Lakes, NJ) containing Dulbecco’s modified Eagle medium (DMEM; Gibco BRL) with 10% fetal bovine serum (FBS; Gibco BRL) and 5% antibiotic-antimycotic (Gibco BRL). The tissue was then rinsed three times for 5 min in PBS, followed by digestion with 0.15% collagenase (Wako, Osaka, Japan) and vigorous
shaking for 20 min at 37 °C in a 50 mL centrifuge tube (Becton Dickinson). Next, an equal volume of DMEM with 10% FBS was added to neutralize the collagenase. The cell suspension was centrifuged at 1300 rpm (260 g) for 5 min and the cell pellet resuspended in control medium (Table 1). Cells were counted using trypan blue, then plated at 10^5 cells per plate (10 cm^2) and maintained in the control medium at 37 °C and 5% CO_2.

**Harvesting of BSCs**

Five-week-old mice (C57BL/6N) were maintained, anaesthetized and prepared for cell harvesting as described above. The femur and tibia were harvested and washed extensively with PBS. The edges of both sides of the bone were then cut with scissors and the bone marrow flushed with PBS into a 50 mL centrifuge tube. The tube was shaken vigorously to break clots and large clots were removed using a 70 µm cell strainer (Becton Dickinson). The cell suspension was then centrifuged at 1300 rpm (260 g) for 5 min and the pellet resuspended in control medium (Table 1). Trypan blue-stained cells were counted and plated at 10^7 cells per plate (10 cm^2) and maintained in the control medium at 37 °C and 5% CO_2.

<table>
<thead>
<tr>
<th>Media</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Dulbecco's modified Eagle Medium <em>10% fetal bovine serum</em> 1% antibiotics-antimycotics*</td>
</tr>
<tr>
<td>Osteogenic</td>
<td>Dulbecco's modified Eagle Medium* 10% fetal bovine serum* 1% antibiotics-antimycotics* 0.1 µM dexamethasone** 10 mM beta-glycerophosphate* 50 µM ascorbic acid-2-phosphate**</td>
</tr>
<tr>
<td>Chondrogenic</td>
<td>Dulbecco's modified Eagle Medium* 1% fetal bovine serum* 1% antibiotics-antimycotics* 10 ng mL^-1 TGF-beta1** 6.25 µg mL^-1 insulin*** 6.25 µg mL^-1 transferrin**</td>
</tr>
<tr>
<td>Adipogenic</td>
<td>Dulbecco's modified Eagle Medium* 1% fetal bovine serum* 1% antibiotics-antimycotics* 1 µM dexamethasone** 10 µM insulin*** 200 µM indomethacin** 500 µM isobuthyl-methylxanthine**</td>
</tr>
</tbody>
</table>

**Gibco BRL, Grand Island, N.Y.**  
**Sigma, St Louis, MO**  
**Wako, Osaka, Japan**

**Confirmation of multipotency**

Following three passages in culture medium, the capacity of ASCs and BSCs to differentiate along osteogenic, chondrogenic and adipogenic lineages in vitro was determined as described previously (Ogawa et al. 2004a; Ogawa et al. 2004b). Differentiation was performed using the inducing media described in Table 1. Following induction, osteogenic differentiation was assessed at 1 and 4 weeks using alkaline phosphatase and von Kossa staining, respectively. Chondrogenic differentiation was assessed 4 weeks after induction using Alcian blue staining at pH 1.0. Both monolayer and micromass cultures were set up for assessment of chondrogenic differentiation, and adipogenic differentiation was determined using Oil Red O staining 2 weeks after the initial adipogenic induction. Following confirmation of multipotency, these cell populations were examined for gene transfer efficiency. Previously, we confirmed cell-specific gene expression in differentiated cells using RT-PCR (Ogawa et al. 2004a; Ogawa et al. 2004b). In this study, we omitted this verification as cell-specific staining has always correlated with the expression of the cell type-specific genes.

**Confirmation of proliferation potency**

After one and three passages in culture, we examined the doubling time of ASCs and BSCs in order to determine their capacity for proliferation over time. At different time points, the total number of cells was determined and the doubling time calculated.

**Gene transfer efficiency**

We examined the gene transfer efficiency of ASCs and BSCs using adenoviral, lentiviral, retroviral and adeno-associated viral (AAV) types 1, 2 and 5 vectors. The lentivirus vector stock was prepared using the SJ-1 HIV1-based lentivirus vector system (Hanawa et al. 2004). In this experiment, the LTR and MSCV leader sequences were used for the expression of EGFP. The MSCV retrovirus vector stock was prepared by transient transfection of HEK 293T cells. This system generated vesicular stomatitis virus G protein (VSV-G)-pseudotyped vector preparations with mean titers on HeLa cells of 6.2 × 10^7 and 8.9 × 10^7 transducing units (TU) mL^-1, respectively.

An adenoviral vector encoding EGFP was generated using the COS-TPC method (Miyake et al. 1996). The EGFP expression unit, which contains the gene encoding EGFP driven by the CAG promoter (Miyazaki et al. 1989), was ligated into the SwaI site of the cosmid pAdexlv. Following two cycles of caesium chloride gradient centrifugation, the average titer of vectors on HEK 293T cells was 1.0 × 10^11 colony forming units (CFUs) mL^-1.

AAV type 2 and pseudotype 1 and 5 vectors were prepared using the AAV Helper-Free System (Stratagene, La Jolla, CA). This system uses a calcium-phosphate precipitation technique to perform triple transfection of HEK 293T cells with pCAEGFPPTN (AAV vector), a packaging plasmid [p5E18RscCl : rep2cap1 (Xiao et al. 1999), pAAV/Ad: rep2cap2 (Samulski et al. 1987), or PackH: rep2cap5 (Hildinger et al. 2001)] and an Ad helper plasmid (pHelper: AAV Helper-Free System, Stratagene). The AAV vectors (pAAV.CAEGFPPTN) are derivatives of psub201 (Samulski et al. 1987), which contains the AAV-type ITRs, the gene encoding EGFP driven by the
CAG promoter and the neoR expression unit from pMC1neo (Thomas and Capecchi 1987). Following harvest, viral purification was performed using preformed iodixanol gradients (Hermens et al. 1999). The titer of AAV vectors was determined using a slot-blot hybridization assay with an EGFP-specific probe. Final titers were 1 × 10^{11} vector mL^{-1}.

The gene encoding EGFP was used as the reporter for all vectors. ASCs and BSCs were infected with all viral vectors for 2 h at 37 °C using the same multiplicity of infection (MOI). Infection with lentiviral and retroviral vectors was performed in the presence of 8 µg mL^{-1} polybrene. EGFP expression was quantified 5 d after infection. ASCs and BSCs from passages 1 and 3 were infected with vector at the same MOI. A FACS Calibur Cytometer (Becton Dickinson) was used to quantify EGFP expression in infected cells. Gene transfer was attempted five times with all vectors and the mean transfer efficiency calculated. In addition, we determined the ability of cells to differentiate following transduction, using the same protocol as described above.

RESULTS

Confirmation of multipotency and proliferation potency

Initially, cultured ASCs were fibroblastic in appearance and BSCs were elliptical (Fig. 1). After two passages, both ASCs and BSCs were spindle-shaped, resembling fibroblasts. Although the doubling time of ASCs was shorter than that of BSCs, the proliferation rate of both MSCs increased between passages 1 and 3 (Fig. 2). As such, the mean doubling times of passages 1 and 3 were 45 and 38 h for ASCs, and 86 and 58 h for BSCs, respectively.

Osteogenesis

Both ASCs and BSCs exhibited changes in cell structure after 5 d in osteogenic media, changing from an elongated, fibroblastic appearance to a rounder, more cuboidal shape. Islands of extracellular matrices were secreted 7 d after osteogenic induction and the cells exhibited endogenous alkaline phosphatase activity (Fig. 3). In addition, the presence of mineralized modular structures was confirmed by von Kossa staining of ASCs (E) and BSCs (F) incubated in osteogenic medium; Day 28. In osteogenic media, both ASCs and BSCs exhibited changes in cell structure after 5d. The cells stained positively for endogenous alkaline phosphatase activity after 1 week and the presence of mineralized modular structures was confirmed by von Kossa staining at 4 weeks.
staining 4 weeks after induction. Positive staining was not observed for cells grown in control media.

Chondrogenesis
Following incubation in chondrogenic media, high-density micromass cultures of both MSCs condensed into small spheroids that were visible to the naked eye within 3 d of induction (Fig. 4). Over the course of 10 d, the number of cartilaginous nodules increased. No nodules were observed in monolayer cultures grown in control media. Both monolayer and micromass cells supplemented with chondrogenic media stained positively for Alcian blue at pH 1.0, indicating the highly-sulfated proteoglycans of cartilage matrices. Positive staining was not observed for cells grown in control media.

FIGURE 4. Chondrogenic differentiation of ASCs and BSCs. ASCs (A) and BSCs (B) incubated in chondrogenic medium; Day 28. Monolayer cultures of ASCs (C) and BSCs (D) incubated in chondrogenic medium and stained with Alcian blue; Day 28. Micromass cultures of ASCs (E) and BSCs (F) incubated in chondrogenic medium; Day 28. Micromass cultures of ASCs (G) and BSCs (H) incubated in chondrogenic medium and stained with Alcian blue; Day 28. In chondrogenic media, high-density micromass cultures of both ASCs and BSCs condensed into small spheroids that were visible to the naked eye within 3 d of induction. Over the course of 10 d, the number of cartilaginous nodules increased. No nodules were observed in the monolayer cultures grown in control media. Both the monolayer and micromass cells cultured in chondrogenic media stained positive for Alcian blue at pH 1.0, indicating the highly-sulfated proteoglycans of cartilage matrices.

Adipogenesis
In adipogenic media, both MSCs exhibited alterations to cell structure after 7 d of culture, changing from an elongated fibroblastic appearance to a rounder shape. Two weeks after induction, we observed lipid-filled cells that stained positively with Oil Red O, an established lipid-specific dye (Fig. 5). Positive staining was not observed for cells grown in control media.

FIGURE 5. Adipogenic differentiation of ASCs and BSCs. ASCs (A) and BSCs (B) incubated in adipogenic medium; Day 14. ASCs (C) and BSCs (D) incubated in adipogenic medium and stained with Oil red O; Day 14. In adipogenic media, both ASCs and BSCs exhibited alterations in cell structure after 7 d, changing from an elongated fibroblastic appearance to a rounder shape. Two weeks after induction, lipid-filled cells were observed and stained positive for the lipid-specific dye Oil Red O.

Gene transfer efficiency
After confirming the multipotency of both ASCs and BSCs, we examined their gene transfer efficiency, performing 5 independent experiments per vector. Each vector contained the gene encoding EGFP and their mean transfer efficiencies are shown in Table 2. These vectors transduced more effectively into ASCs than BSCs. Both cell populations were effectively transduced with lentiviral, retroviral, and AAV types 1 and 5 vectors, whereas they were resistant to transduction with adenoviral and AAV type 2 vectors. Moreover, the transduction efficiency of passage 3 cells was higher than that of passage 1 cells. Thus, the most efficient transfer of these vectors was observed for passage 3 ASCs (Fig. 6). Since we confirmed that these cells retained the ability to differentiate into the three lineages following transduction, we did not investigate gene silencing (unpublished observations).
FIGURE 6  FACS analysis of transduced ASCs (Passage 3). Both ASCs and BSCs were transduced easily with lentiviral, retroviral, and AAV types 1 and 5 vectors, whereas they were relatively resistant to transduction with adenoviral and AAV type 2 vectors. Passage 3 ASCs exhibited the most efficient transduction. The gene encoding EGFP was used as a reporter for all vectors under investigation. The black and green lines indicate mock and EGFP transduced cells, respectively.

TABLE 2. Gene transfer efficiency of ASCs and BSCs

<table>
<thead>
<tr>
<th>Virus vectors</th>
<th>ASCsP1</th>
<th>ASCsP3 (Fig. 5)</th>
<th>BSCsP1</th>
<th>BSCsP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lenti</td>
<td>81.6 ± 6.7 %</td>
<td>91.7 ± 6.1 %</td>
<td>48.2 ± 6.7 %</td>
<td>58.2 ± 5.7 %</td>
</tr>
<tr>
<td>Retro</td>
<td>40.2 ± 3.1 %</td>
<td>68.5 ± 8.1 %</td>
<td>17.8 ± 3.4 %</td>
<td>23.0 ± 3.2 %</td>
</tr>
<tr>
<td>Adeno</td>
<td>10.5 ± 2.0 %</td>
<td>13.5 ± 1.8 %</td>
<td>8.0 ± 1.5 %</td>
<td>12.0 ± 2.1 %</td>
</tr>
<tr>
<td>AAV type1</td>
<td>85.6 ± 7.2 %</td>
<td>86.4 ± 5.9 %</td>
<td>45.4 ± 4.5 %</td>
<td>56.5 ± 7.5 %</td>
</tr>
<tr>
<td>AAV type2</td>
<td>8.0 ± 1.5 %</td>
<td>12.0 ± 2.4 %</td>
<td>10.2 ± 1.7 %</td>
<td>13.8 ± 1.5 %</td>
</tr>
<tr>
<td>AAV type5</td>
<td>40.2 ± 4.5 %</td>
<td>45.7 ± 7.4 %</td>
<td>24.8 ± 4.5 %</td>
<td>36.0 ± 4.2 %</td>
</tr>
</tbody>
</table>

DISCUSSION

Adult stem cells do not have the “true” totipotency of embryonic stem (ES) cells, but the multipotency of mesenchymal stem cells (MSCs) has been confirmed by many studies since they were first described in 1976 (Friedenstein 1976). MSCs secrete a variety of cytokines and growth factors, exhibiting both paracrine and autocrine activity (Caplan and Dennis 2006). These factors suppress the local immune system, inhibiting fibrosis (scar formation) and apoptosis, enhancing angiogenesis, and stimulating mitosis and differentiation of the intrinsic reparative cells or stem cells (Caplan and Dennis 2006). There are few ethical or immunological problems associated with the use of MSCs for autotransplantation, and thus they represent excellent candidates for this process.

Given their ease of harvest and relative abundance, ASCs may be the ideal carriers for cell and gene-cell therapies (Banfi et al. 2000). Although ASCs are now being characterized (Gimble and Guilak 2003; Yoshimura et al. 2006), much remains to be investigated, including the identity and effects of secreted humoral factors. In order to evaluate their potential as gene carriers in gene-cell therapy, we examined the differences between ASCs and BSCs with respect to gene transfer efficiency.

Retroviral vectors are useful for gene transfer and long-term expression in BSCs both in vitro and in vivo, and Lee et al. (1999) have used amphotropic envelope-expressing retroviruses to achieve transduction efficiencies of 80-90%. In 2000, Moska et al. (2000) reported parameters for optimal retroviral transduction of eight species of BSCs for intravenous infusion in a canine model of myeloablative transplantation. Morizono et al. (2003) reported that the lentivirus was useful for gene transduction into ASCs and suggested the possibility of using these cells as gene delivery vehicles. In addition, they observed that the transduction efficiency of human ASCs was greater than that of mouse ES cells. Moreover, Dragoo et al. (2003) reported transfer of a gene encoding a bone morphogenic protein gene into ASCs using an adenoviral vector.

In this study, we tested adenoviral, lentiviral and 3 types of adeno-associated virus (AAV) vectors. There are no reports comparing the transfer efficiencies of various vectors into ASCs and BSCs. Therefore, we examined the differences between these MSCs using the gene encoding EGFP as a reporter. Vectors were transduced more efficiently into ASCs than BSCs. Both cell types were resistant to transduction with adenoviral and AAV type 2 vectors. However, both ASCs and BSCs could be transduced efficiently with lentiviral, retroviral, and AAV types 1 and 5 vectors, suggesting that these vectors should be considered as candidates for further study. Our next investigation will focus on the stability of reporter gene expression in these vectors both in vitro and in vivo.

Many early-passage cells are considered to be in a resting phase and do not proliferate immediately and our doubling time data indicated that the proliferative activity of ASCs is higher than that of BSCs, even during early passages (Fig. 5). This study also demonstrated that the transduction efficiency of passage 3 cells was higher than that of passage 1 cells, suggesting that cells with higher activities can facilitate more efficient transduction. Thus, with respect to transduction efficiency, ASCs may be of greater use than BSCs in clinical applications.

ACKNOWLEDGEMENTS

This paper could not have been written without the guidance of our Head of Department, Professor Hiko Hyakusoku, and Associate Professor Hiroshi Mizuno, Department of Plastic and Reconstructive Surgery, Nippon Medical School. Thanks are also due to my colleagues at the Nippon Medical School and apologies to the many scientists whose work has not been cited due to space limitations.

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