Potent Osteogenesis and Chondrogenesis of CD34-Enriched Mouse Adipose-Derived Stem Cells

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Abstract

Adipose-derived stem cells (ASCs) obtained using a widely reported culture method are actually a heterozygous cell population of the adipose stromal vascular fraction (SVF). This heterogeneity may interfere with the multi-differentiation potential of adipose-derived mesenchymal stem cells (ADMSCs). It is therefore necessary to establish an efficient method for isolating ADMSCs that have high multi-differentiation potential. Several studies indicated that mucosalin (CD34) might be one of the specific markers of ADMSCs. In our previous studies, a CD34-enriched cell population sorted from the pooled SVF had stronger differentiation potential than that of unsorted cells in a hair follicle morphogenesis model. However, it remains unclear whether CD34-enriched cells have stronger potential for other lineage differentiation, particularly osteogenesis and chondrogenesis. In this study, CD34+ cells were harvested and sorted from murine adipose-derived cells and their potentials for osteogenesis and chondrogenesis were examined using engineered bone and cartilage models. The results show that CD34+ cells exhibited stronger potential for bone formation with stronger van Gieson and collagen I staining than those of CD34− cells and an unsorted SVF when seeded on β-tricalcium phosphate. Furthermore, CD34+ cells also exhibited a much stronger chondrogenic potential than those of CD34− cells and an unsorted SVF, with stronger staining of toluidine blue, Safranin-O, and collagen II in a chondrocyte pellet and engineered cartilage. Most importantly, only CD34+ cells could form a cartilage-like lacunae structure. All these results indicate that CD34 may serve as a specific surface marker for enriching ADMSCs in ASCs.

Keywords: Adipose-derived stem cells (ASCs), Adipose-derived mesenchymal stem cells (ADMSCs), Mucosalin (CD34), Cell sorting, Osteogenesis, Chondrogenesis

1. Introduction

Adipose-derived stem cells (ASCs) are considered as an ideal cell source for tissue regeneration because of their high proliferative and multilineage differentiation potential, including that for adipogenic, osteogenic, and chondrogenic lineages [1]. Compared to bone marrow stem cells (BMSCs), ASCs can be harvested more easily with high yield [2]. However, several studies have revealed that BMSCs are stronger in their differentiation potential towards angiogenic, osteogenic, and neurogenic lineages than that of ASCs [2–4], probably because adipose-derived stromal cells are a heterogeneous population containing mesenchymal stem cells (MSCs), vascular endothelial cells, preadipocytes, smooth muscle cells, hematologic lineage cells, and hematopoietic stem cells [5]. It is therefore necessary to isolate MSCs from this mixed cell population in order to enhance the multi-differentiation potential of ASCs.

There is increasing evidence that adipose-derived mesenchymal stem cells (ADMSCs) reside in a perivascular location and that mucosalin (CD34) is a specific surface marker for identifying ADMSCs [6–8]. Our previous studies demonstrated that CD34+ cells sorted from pooled ASCs displayed stronger differentiation potential than that of unsorted cells in a hair follicle morphogenesis model [9]. However, it remains unclear whether CD34-enriched cells have stronger potential than that of pooled cells for other lineage differentiation, particularly osteogenesis and chondrogenesis.

In this study, cells freshly isolated from mouse adipose tissue (stromal vascular fraction (SVF)) were sorted using flow cytometry according to our previously published method [9] to

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produce three subgroups: (1) CD34+ (lineage+, CD45+, CD31+, CD34+) cells; (2) CD34− (lineage−, CD45−, CD31−, CD34−) cells; (3) unsorted SVF cells. These subpopulations were tested for their ability in osteogenesis and chondrogenesis using previously reported models, namely in vivo tissue-engineered bone formation with β-tricalcium phosphate (β-TCP) [10] and in vitro chondrogenic models, including chondrocyte-pellet- and polyglycolic acid (PGA)-fiber-based cartilage engineering [11].

2. Materials and methods

2.1 Animals

6–8 week-old female C57BL/6 mice and 4–6 week-old male athymic nude mice were purchased from Slac Laboratory Animal Center China (Shanghai, China). The Institutional Review Committee of Shanghai Jiao Tong University School of Medicine approved all animal study protocols.

2.2 Cell isolation and flow cytometry sorting

As previously described [9], the pooled population of ASCs (SVF) was isolated from the subcutaneous and parametrical adipose tissues of C57BL/6 mice. The freshly isolated SVF was resuspended in phosphate-buffered saline (PBS) with 1% fetal bovine serum (FBS) for labeling with phycoerythrin (PE)-conjugated rat anti-mouse CD34, PE-Cy5-conjugated rat anti-mouse CD31 and CD45 (1:100 in PBS containing 1% FBS, BD Biosciences, San Jose, CA), and biotin-conjugated rat anti-mouse hematologic lineage cocktail (1:100 in PBS containing 1% FBS, BD Biosciences), followed by a PE-Cy5-conjugated streptavidin secondary antibody (1:1000 in PBS containing 1% FBS, BD Biosciences). The labeled cells were subjected to fluorescence-activated cell sorting (FACS, Beckman Coulter, Brea, CA, USA) and analyzed on a FACSort with CellQuest Pro v5.2.1 software (BD Biosciences). Finally, three sorted cell subpopulations, namely CD34+, CD34− and unsorted SVF cells, were collected and cultured in MesenPRO RS™ Medium (Life Technologies, Carlsbad, CA, USA) at a density of 1 × 10^5 cells/ml in 100-mm tissue culture plates (BD Falcon, BD Biosciences, Bedford, MA) for subsequent experiments.

2.3 In vitro osteogenic induction of β-TCP/cell construct

Cell subpopulations were cultured in a basic culture medium (Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS) or in an osteogenic medium (DMEM containing 10% FBS, 50 µg/ml ascorbic acid, 10 mmol/l β-glycerophosphate, and 10^-8 mol/l dexamethasone, Sigma-Aldrich, St. Louis, MO, USA) for 3 days. Trypsin-released cells were collected and then resuspended in the basic culture medium at a density of 2.5 × 10^4 cells/ml. Then, 100 µl of the cell suspension was seeded on β-TCP scaffolds (Bio-Lu Biomaterials Company China; cylinder diameter: 6 mm, cylinder thickness: 2 mm) and incubated at 37 °C for 4 h. Then, the β-TCP/cell constructs were transferred to a 12-well plate with 1 ml of the basic culture medium or osteogenic medium. β-TCP without cells served as a control. The medium was changed every 2–3 days after cell seeding.

2.4 Scanning electron microscopy examination

As previously described [12], three samples of CD34+ cell/β-TCP constructs were chosen randomly for SEM observation at 1, 3, and 7 days after cell seeding. A β-TCP scaffold without cells served as the control. All specimens were gently rinsed twice with PBS, fixed with 4% paraformaldehyde in PBS (pH 7.4) for 4 h, rinsed twice with tap water, dehydrated in graded concentrations of ethanol (30, 50, 70, 90, and 100% at 10 min each), and then air-dried in a fume hood overnight. The samples were then sputter-coated for 50 s with gold to increase conductivity and imaged at 8–10 kV with scanning electron microscopy (SEM, JEOL JSM-5600LV, Japan).

2.5 In vivo implantation of in vitro-cultured β-TCP/cell constructs

β-TCP/cell construct grafting was performed as previously described [12]. Briefly, male athymic nude mice were anesthetized by intraperitoneal injection of pentobarbital sodium (1.3 mg/kg body weight). All β-TCP/cell constructs were then respectively implanted subcutaneously in the dorsal aspect of the mice (n = 3). After recovery from anesthesia, the mice were caged individually, and then sacrificed at 4, 8, and 12 weeks post-implantation. Samples were harvested and subjected to various studies.

2.6 Histological analysis of tissue-engineered bone implants

As previously described [12], the tissue-engineered bone implants were harvested and fixed at 4, 8, and 12 weeks post-implantation. One part of the samples was fixed in 4% paraformaldehyde in PBS for 24 h and decalcified in 1% ethylene diamine tetraacetic acid disodium (EDTA-Na2) in PBS for 7 days. The tissues were dehydrated through an ethanol series, embedded in paraffin, and then sectioned into 5-µm slices and stained with hematoxylin and eosin (H&E).

The other part of the samples was fixed in 4% paraformaldehyde for 24 h at room temperature, and then dehydrated through an ethanol series, embedded in plastic, and sectioned into 50-µm undecalcified tissue sections using a microtome (Leica SP1600, Germany). The sections were stained with van Gieson staining as previously described to observe bone formation [13].

Images were captured using a microscope (CX31, Olympus, Tokyo, Japan) to assess the volume of new bone formation. The new bone was measured manually using the software Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA). Three samples were included in each group for each time point. Five randomly selected fields of each sample were evaluated for quantitative analysis with the software.

2.7 In vitro chondrogenesis in a chondrocyte pellet model

According to a previously established method [11], freshly sorted CD34+ and CD34− cells and unsorted SVF cells
(0.5 × 10^6 cells/sample) were respectively cultured in chondrogenic medium for 4 weeks to evaluate the chondrogenic potential of the cells. The chondrogenic medium was composed of DMEM containing 0.1 mM dexamethasone, 10 ng/ml BMP-6, 50 ng/ml IGF-1, and 10 ng/ml TGF-β3 (R&D Systems), as previously described [11,14]. As controls, each cell population was cultured in regular culture medium (DMEM containing 10% FBS). The medium was changed every 2-3 days.

2.8 In vitro cartilage engineering

A PGA-fiber-based scaffold was prepared as previously described [11,15,16]. Briefly, PGA unwoven fibers (5 mg) were compressed into a cylinder shape with a 5-mm diameter and a 2-mm thickness and 0.2 ml of 0.1% polyglyactic acid (PLA) diluted in dichloromethane (Sigma) was added to solidify the scaffold shape. FASC-sorted CD34+ and CD34− cells and unsorted SVF cells (5 × 10^6 in 50 μl) were then respectively evenly added into the scaffolds. The cell/scaffold constructs were incubated in vitro for 4 h at 37 °C with 95% humidity and 5% CO2 to allow for complete adhesion of the cells to the scaffolds. The constructs were then cultured in chondrogenic medium for a total of 8 weeks with the medium changed every 2-3 days.

2.9 Histological analysis of tissue-engineered cartilage

According to a previously described method [11,15,16], harvested tissue samples were fixed and embedded in paraffin and sectioned into 5-μm slices. The sections of tissue-engineered cartilage were stained with H&E, toluidine blue, Safranin-O, and anti-collagen II antibody to evaluate tissue structure and cartilage matrix deposition, as previously described [11]. The sections were imaged using a microscope (Olympus) and measured using Image-Pro Plus 6.0 to assess cartilage formation quantitatively. There were three samples in each group for each time point. Five randomly selected fields of each sample were evaluated using the software.

2.10 Quantitative and statistical analyses

The differences among CD34+ cells, CD34− cells, and unsorted SVF cells in new bone area (as determined from H&E and van Gieson staining) and in the size and weight of formed cartilage were analyzed using one-way analysis of variance (ANOVA). When ANOVA indicated significance in the difference among data, multiple comparisons were performed using Newman-Keuls post hoc tests. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1 SEM examination

The morphology of CD34+ cells on β-TCP was observed by SEM at days 1, 3, and 7 after cell seeding. The cells began to adhere on the pore surface of the β-TCP scaffold at day 1 (Figs. 1(b) and 1(f)). At day 3, they appeared to spread on the pore surface of the β-TCP scaffold (Figs. 1(c) and 1(g)). Part of the pore surface was covered by cells after 7 days of culture (Figs. 1(d) and 1(h)). Similar attachment was also found for CD34− cells and unsorted SVF cells (data not shown).

![Figure 1](image1.png)

Figure 1. SEM image of CD34+ cells/β-TCP constructs taken on days (a), 1 day; (b), 3 days; (c), 7 days; and (d), 7 post-cell seeding. β-TCP scaffold without cells served as a control (e), (f). Similar attachment for CD34+ cells and unsorted SVF cells was observed (data not shown). Scale bars = 100 μm.

3.2 Better engineered bone formation by CD34+ cells

There was no significant difference in the gross appearance of harvested tissue-engineered bones among different groups (Figs. 2(a)-2(f)). Histologically, no new bone formed throughout the examination periods in the control group (β-TCP scaffolds without cells) (Figs. 3(a), 3(h), 3(o); Figs. 4(a), 4(h), 4(o)). However, in the experimental group, the decalcified sections stained with H&E and undecalcified sections stained with van Gieson stain showed distinct areas of new bone formation under osteogenically induced conditions. As shown in Fig. 4, the constructs seeded with CD34+ cells displayed immature bone formation in many pores of the implant 4 weeks after implantation (Fig. 4(b)). Moreover, new bone was deposited progressively in the pores of β-TCP scaffolds (Fig. 4(i)); the area of new bone was 154.02 ± 34.90 × 10^3 at 8 weeks after implantation. At 12 weeks, the amount of mature bone increased in the pores of the scaffolds (Fig. 4(p)); the area of new bone increased to 275.39 ± 70.99 × 10^3. No obvious fat tissue was found in the constructs seeded with CD34+ cells of induced groups (Figs. 3(b), 3(i), 3(p) and 4(b), 4(i), 4(p)). CD34+ cell groups under non-induced conditions had weaker osteogenic potential than that of groups under induced conditions.

![Figure 2](image2.png)

Figure 2. Gross view of engineered bone in three cell groups. The tissue-engineered bone implants were harvested at weeks (a): induced, (b): non-induced 4, (c): induced, (d): non-induced 8, and (e): induced, (f): non-induced 12 post-implantation.
Figure 3. Histological analysis (H&E) of tissue-engineered bone implants after decalcification. H&E view of tissue-engineered bone with osteogenically induced (b), (i), (p)) CD34+ cells, (c), (j), (q) CD34- cells, and (d), (k), (r) unsorted SVF cells at 4, 8, and 12 weeks post-implantation. H&E view of tissue-engineered bone with non-induced (e), (f), (s) CD34+ cells, (f), (m), (t) CD34- cells, and (g), (n), (u) unsorted SVF cells at 4, 8, and 12 weeks post-implantation. β-TCP without cells served as a control ((a), (h), (o)). Scale bars = 200 μm. (v) Quantitative analysis of new bone formation in all groups. The data represent the integrated optical density (IOD) of H&E staining of positive area of every group. * represents significant difference compared to that of CD34+ cell group in both induced and non-induced groups at each time point, p < 0.05. Error bar represents standard deviation (n = 3).

Figure 4. Histological analysis of tissue-engineered bone implants by van Gieson staining. van Gieson view of tissue-engineered bone with osteogenically induced (b), (i), (p)) CD34+ cells, (c), (j), (q) CD34- cells, and (d), (k), (r) unsorted SVF cells at 4, 8, and 12 weeks post-implantation. van Gieson view of tissue-engineered bone with non-induced (e), (f), (s) CD34+ cells, (f), (m), (t) CD34- cells, and (g), (n), (u) unsorted SVF cells at 4, 8, and 12 weeks post-implantation, respectively. β-TCP without cells served as a control ((a), (h), (o)). Scale bars = 200 μm. (v) Quantitative analysis of new bone formation in all groups. The data represent the IOD of van Gieson staining of positive area of every group. * represents significant difference compared to that of CD34+ cell group in both induced and non-induced groups at each time point, p < 0.05. Error bar represents standard deviation (n = 3).
osteogenic potential than those of CD34− cells and unsorted SVF cells.

3.3 In vitro chondrogenesis of chondrocyte pellet

It was noted that cell pellets were heavier in the CD34+ cell group than those in the other two groups (Figs. 5(a), 5(f), 5(k) and 5(p)). In addition, much stronger staining of toluidine blue, Safranin-O, and collagen II was observed in the CD34+ cell group, indicating stronger cartilaginous matrix deposition (Figs. 5(c)-5(e), 5(h)-5(j), and 5(m)-5(o)). Furthermore, the lucuna structure was much better developed in the CD34+ cell group (Fig. 5(b)-5(e), 5(g)-5(j) and 5(l)-5(o)).

3.4 In vitro cartilage engineering with sorted cells and PGA/PLA scaffold

Consistent with the results of chondrocyte pellets, after 8 weeks of chondrogenic induction, the tissue-engineered cartilages in the CD34+ cell group (Fig. 6(a)) were larger and heavier (wet weight) than those in the CD34- cell (Fig. 6(b)) and unsorted SVF groups (Figs. 6(c), 6(g) and 6(h)). More importantly, histological analysis revealed better formed cartilage with a lucuna structure in the CD34+ cell group than those in the other two groups, although cartilage formed in only part of the cell-seeded constructs (Fig. 7). Furthermore, stronger staining of toluidine blue, Safranin-O, and collagen II was observed in the CD34+ cell group than those in the other two groups (Fig. 7). These results suggest that CD34+ cells are more potent in their chondrogenic differentiation than are CD34- cells and unsorted SVF cells.

Figure 5. Chondrogenic differentiation of sorted cells in chondrocyte pellet. Gross view of pellets formed by (a) CD34+ cells, (f) CD34− cells, and (k) unsorted SVF cells after 4 weeks of in vitro culture. Bar = 1 mm. (p) Pellets formed by CD34+ cells are heavier than those formed by CD34- cells and unsorted SVF cells. Stronger staining of toluidine blue, Safranin-O, and collagen II was observed in CD34+ cells ((c)-(e)) than in CD34- cells ((h)-(j)) and unsorted SVF cells ((m)-(o)). Bar = 100 μm. Error bar represents standard deviation (n = 3).
Figure 6. Engineered cartilage and characterization. After 8 weeks of chondrogenic induction, (a) CD34+ cell group formed (g) heavier and (h) larger tissue than those of the (b) CD34− cell group and (c) unsorted SVF group. H&E staining overview shows that well-developed cartilage-like histological structure was only observed in certain area of the constructs is observed in the CD34+ cells group (d) but not in the CD34− cells group (e) and unsorted SVF group (f). Scale bars = 250 µm. Error bar represents standard deviation (n = 3).

Figure 7. Histological analysis of cartilage engineered with sorted cells and PGA/PLA scaffold. H&E, collagen II, toluidine blue, and Safranin-O staining demonstrate better lacuna structure formation and more collagen II and glycosaminoglycan (GAG) deposition in ((a), (b), (g), (h), (m), (n), (s), (t)) CD34+ group than those in ((c), (d), (i), (j), (o), (p), (u), (v)) CD34− group and ((e), (f), (k), (l), (q), (r), (w), (x)) unsorted SVF group. Scale bars = 50 µm.

4. Discussion

ASCs, which are routinely isolated from the adipose stromal vascular fraction, contain multiple cell types, including hematologic lineage cells, hematopoietic stem cells, preadipocytes, smooth muscle cells, vascular endothelial cells, and MSCs. Similar to other types of MSCs, ADMSCs are difficult to define due to the lack of specific surface markers.
Although surface makers related to ASCs have been widely studied [17-19], the most specific one for identifying ADMSCs in ASCs is unknown.

Recently, Tang et al. [7] demonstrated that ADMSCs might be vascular pericytes residing in the adipose stromal vascular fraction. Additionally, Rodeheffer et al. [8] demonstrated that MSCs in an SVF pool with multi-differentiation potential could be identified by a series of surface markers, including CD29, Sca-1, and CD34, after the removal of hematologic lineage cells. However, this complicated method of purifying ADMSCs with multiple surface markers is unsuitable for tissue engineering or clinical applications. Therefore, a specific marker for ADMSC identification is important for their application. The results of Lin et al. [20] and Traktuev [21] illustrated that ASCs reside at the perivascular location and that CD34 is abundantly expressed in all blood vessels in adipose tissue. Several other studies reported similar results [22,23]. Our previous study demonstrated that only CD34+ cells participate in hair follicle morphogenesis by contributing to the dermal sheath formation [9]. The above results suggest that CD3 is a relatively specific marker for the enrichment and purification of ADMSCs.

Engineered bone formation and engineered cartilage formation are typical models used to examine the multi-potent differentiation of isolated MSCs [10,12,24,25]. Bone engineering based on porous β-TCP ceramics is a common tool for examining the osteogenesis of MSCs [26-29]. Regarding chondrogenesis, constructing cartilage-like tissue in vitro using seed cells and a three-dimensional biodegradable scaffold is an effective tool for testing the chondrogenic differentiation of MSCs [11,16,30]. Thus, the two models were used in this study. As shown in this study, in vivo osteogenesis using isolated cells and β-TCP demonstrated better bone formation by CD34+ cells than those obtained using CD34− cells and unsorted SVF cells, as determined from H&E and von Gieson staining (Figs. 3 and 4). Similarly, in vitro chondrogenesis using a pellet formation model and an in vitro cartilage engineering model demonstrated that CD34+ cells better form engineered cartilage than do CD34− cells and unsorted SVF cells, as determined from H&E staining and the staining of toluidine blue, Safranin-O, and collagen II (Figs. 5, 6 and 7).

In addition to the verification of the multi-potent differentiation of CD34+ cells via tissue formation models, their multi-differentiation potential was investigated via an in vitro assay of induced differentiation. Results reveal that upon osteogenic induction, CD34+ cells expressed higher gene levels of runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and osteocalcin (OCN), and also exhibited stronger staining of AKP and Alizarin Red (data not shown) when compared to CD34− cells and unsorted SVF cells. Similarly, after chondrogenic induction, CD34+ cells expressed higher gene levels of collagen II, SRY-related high-mobility group-box gene 9 (SOX9) and aggrecan, and revealed stronger staining of toluidine blue and collagen II (data not shown).

Collectively, the results of potent osteogenesis and chondrogenesis reported here, potent hair morphogenesis in our previous study [9], and other reports [7,8,20,21,22,23] indicate that CD34 enriched cells may represent true ADMSCs, which can be an excellent cell source of engineered tissue formation.

5. Conclusion

This study demonstrated that adipose-derived CD34+ cells possess better potentials for chondrogenic and osteogenic differentiation than CD34− and unsorted SVF cells, indicating that CD34 may serve as a specific surface marker for enriching ADMSCs.

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