Attenuation of Trauma-induced Osteoarthritis by Repetitive Intra-articular Administration of Peripheral Blood Derived Mesenchymal Stem Cells

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Abstract

Background: Osteoarthritis (OA) is a chronic joint disease, characterized by articular cartilage degradation, subchondral bone hardening, and inflammation of the whole synovial joint. There is no pharmacological treatment in slowing down OA progression, leading to costly surgical interventions eventually. Cell therapy using chondrocytes or progenitor cells from different sources has been reported in clinical trials for OA management with some success, but outcomes are varied. Peripheral blood derived mesenchymal stem cells (PB-MSCs) are promising cells owing to their easy collection, superior migration, and differentiation potentials. In the current study, we evaluated the effect of intra-articular administration of PB-MSCs on the progression of OA in mice.

Methods: C57BL/6J mice (8-10 weeks old male) were subjected to destabilization of the medial meniscus surgeries (DMM) on their right joints following protocols as previously reported. The mice after DMM were randomly treated with saline (vehicle control), PB-MSCs, or adipose tissue derived MSCs (AD-MSCs) (n = 7 per group). The mice treated with sham surgery were regarded as sham controls (n = 7). PB-MSCs and AD-MSCs were harvested and cultured according to previous published protocols, and pre-labeled with BrdU for 48 h before use. PB-MSCs or AD-MSCs (5×10^5 cells/mouse; passage 3~5) were injected into the right knee joints thrice post-surgery (except sham surgery group). The mice were euthanized at 8 weeks post-surgery and knee joint samples were collected for micro-CT and histological examinations.

Results: PB-MSCs administration significantly reduced hardening of subchondral bone comparing to vehicle controls. Safranin O staining showed that PB-MSCs treatment ameliorated degeneration of articular cartilage, which is comparable to AD-MSCs treatment. The expression of catabolic marker MMP13 was significantly reduced in articular cartilage of PB-MSCs-treated groups comparing to vehicle controls. Co-expression of BrdU and Sox9 were detected, indicating injected PB-MSCs differentiated towards chondrocytes in situ. Reduced level of IL-6 in the peripheral sera of PB-MSCs- and AD-MSCs-treated mice was also determined.

Conclusions: Repetitive administration of PB-MSCs or AD-MSCs halted OA progression through inhibiting cartilage degradation and inflammation. PB-MSCs may become a promising cell source for cartilage tissue repair and alleviation of OA progression.

Background

Osteoarthritis (OA) results from degradation of articular cartilage is one of the leading global causes of pain and immobility [1, 2]. Cartilage remains one of the most difficult tissues to heal due to its limited intrinsic repair capacity [3]. One reason of its poor self-repair capacity is the avascular property of cartilage. Another important reason is lack of enough chondroprogenitor cells within the cartilage.

Mesenchymal stem cells (MSCs) are diverse subsets of self-renewing and multipotent stromal cells that exist in almost all postnatal organs and tissues that gives rise to mature osteoblasts, chondrocytes,

adipocytes, and marrow stromal cells required for skeletal development, homeostasis, and repair [4–7]. Stem cell-based therapy has become an emerging regime for cartilage repair since loss of articular chondrocyte function is a major contributing factor in the pathogenesis of OA [8, 9]. The chondrogenic differentiation of MSCs is a multi-step tightly regulated process, which is characterized by a strong interdependence of cell shape, cytoskeletal organization, and the onset of chondrogenic gene expression [10]. MSCs derived from various adult tissues and organs have been intensely investigated on cartilage repair in preclinical studies and clinical trials [11–17]. Among those tissues, adipose tissue derived MSCs (AD-MSCs) or bone marrow derived MSCs (BM-MSCs) are most intensively investigated [18, 19]. However, invasive operations are required in the procedures of harvesting AD-MSCs or BM-MSCs, which cause pain, bleeding, and even infection to the patients [20–22]. Peripheral blood derived circulating mesenchymal stem cells (PB-MSCs) have emerged as an important cell source for tissue regeneration owing to their proliferation and differentiation potential, and only minimal invasive procedures required during cell harvesting procedures. Recently, our studies found that PB-MSCs may represent an ideal alternative source of MSCs within peripheral blood with potent multi-differentiation potential [20, 21, 23]. We thus hypothesized that PB-MSCs could be used as an alternative source to AD-MSCs in the treatment of OA. In this study, we compared the therapeutic efficacy between PB-MSC and AD-MSC for alleviation of traumatic-induced OA development.

Methods

Isolation of mouse adipose-derived mesenchymal stem cells

To isolate murine AD-MSCs, adipose tissues were isolated from 8 ~ 10 weeks old male C57BL/6J mice. Adipose tissues were digested at 37°C for 30 min with 0.075% type I collagenase after washed with sterilized PBS. Enzyme activity was neutralized with α -MEM, containing 10% FBS and centrifuged at 200 g for 10 min to obtain a pellet. The pellet was incubated overnight at 37°C/5% CO₂ in control medium (α -MEM, 10% FBS, 100 units/ml of penicillin, 100 μ g/ml of streptomycin). Following incubation, the tissue culture plates were washed to remove residual nonadherent cells and maintained at 37°C /5% CO₂ in control medium. When the monolayer of adherent cells has reached confluence (P0), cells were trypsinized (0.25% trypsin; Sigma), resuspended in α -MEM containing 10% FBS, and sub-cultured at a concentration of 2,000 cells/cm². Cells of passage 3 ~ 5 were used in the following study. To collect peripheral blood, C57BL/6J mice were anesthetized with isoflurane. Peripheral blood was collected into a microcentrifuge tube containing heparin. To lyse red blood cells, harvested blood was treated with red blood cell lysis buffer (Bio Legend, San Diego, CA, USA). Collected cells were resuspended in complete culture medium containing α -minimum essential medium (α -MEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 17% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin-streptomycin-neomycin. Then, cells were seeded in 6-well plates at a density of 0.5×10^6 cells/cm². Culture medium was half changed every 3 d. Once cells reached a confluence of over 50%, cells were

passaged regularly and cultured in complete culture medium supplemented with 10% FBS. Cells of passage 3 ~ 5 were used in the following assays.

Immunophenotypic analysis

Cells of passage 3 were enzymatically digested with 0.25% trypsin-EDTA (Thermo Fisher Scientific) for 30 s at room temperature, respectively. Cells were resuspended in resuspension buffer containing 1% BSA (Millipore Sigma) at 10^6 cells/ml. We transferred a volume of 300 μ l cells into a 5-ml flow cytometry tube. Cell aliquots were incubated with PE-conjugated CD29 (SC018; R&D Systems, Minneapolis, MN, USA), APC-conjugated Sca-1 (stem cell antigen 1; SC018; R&D Systems, USA), peridinin chlorophyll protein (PerCP)-conjugated CD45 (SC018; R&D Systems), or each corresponding isotype control antibody (SC018; R&D Systems), respectively, for 30 min at room temperature in the dark after washing with PBS. Cells were resuspended in 1000 μ l resuspension buffer after washing with PBS. The cell suspension was analyzed by flow cytometric analysis using CellQuest software (BD Biosciences). BD FACS Canto II was used for data acquisition by adjusting voltage and compensation using appropriate excitation and detection channels. Data analysis was performed using Flow Jo (BD Biosciences) software.

Safranin O-fast green staining and Alcian Blue staining

Samples were fixed in 4% PFA and embedded in paraffin. Safranin O staining [0.1% (wt/vol) Safranin O in 0.1% (vol/vol) in acetic acid] or Alcian blue (Sigma-Aldrich) were applied. The staining results were viewed under a phase contrast microscope (Leica Microsystems, Wetzlar, Germany).

Destabilization of the medial meniscus surgery-induced Osteoarthritis Model

The surgical procedures were approved by Animal Experimental Ethical Committee of the Chinese University of Hong Kong and performed following the well-established protocol [24]. Twenty-eight C57BL/6J mice (8–10 weeks old male) were anesthetized with peritoneal injection of ketamine (50 mg/kg), and destabilization of the medial meniscus (DMM) surgery was performed with a microsurgical scalpel on the right knee by sectioning of the medial meniscotibial ligament (MMTL) that anchored the medial meniscus (MM) to the tibial plateau. The lateral meniscotibial ligament was identified and protected during the surgery. Contralateral knee joints received sham surgery in which the MMTL was exposed without sectioning. Mice were allowed completely free movement following DMM surgery. Eight weeks later, the mice were terminated and the knee joints were harvested for histological analysis and other examinations.

Intra-articular injections of MSCs

After DMM surgeries, mice were randomly allocated into three groups ($n = 7$ per group), which were treated with saline (10 μ l, vehicle control), mouse PB-MSCs (PB-MSCs, 5×10^5 cells/mouse), mouse AD-MSCs (AD-MSCs, 5×10^5 cells/mouse) three times per week after surgery through 32-gauge micro-needles (Hamilton, Sigma-Aldrich). The mice underwent sham surgeries without injection were served as healthy controls ($n = 7$). To verify the accurate intra-articular injection, Alcian blue dye was injected in a pilot study. To avoid any leakage, the injections were performed by gently inserting the needle into the joint at a depth around 3 mm, waiting for 5 seconds and then slowly withdrawing the needle. The procedures were performed under isoflurane anesthesia.

3D microstructure of subchondral bone

Subchondral bone microstructure under medial side of tibial plateaus was determined by high-resolution micro-CT 40 system (Scanco Medical, Wangen-Brüttisellen, Switzerland). Besides the total subchondral bone, trabecular bone compartment was also segregated by selecting the region of interest at the cancellous bone and excluding the subchondral plate and the calcified growth plate. A total of one hundred 3D sagittal images of the tibiae medial subchondral bone ($n = 7$) were evaluated at global threshold (158 mg hydroxyapatite/ cm^3) and a Gaussian filter was used (sigma: 0.8, support: 2) to suppress image noise. Parameters including bone mineral density (BMD) and bone volume/ total tissue volume (BV/TV) were determined with a built-in program (Image Processing Language v4.29d, Scanco Medical) according to well-established protocols [25].

Histological assays for joint tissue sectioning

Joint tissue samples were fixed in 4% (wt/vol) paraformaldehyde (PFA), then subjected to 0.5 M EDTA at 4°C with constant shaking for decalcification. Decalcified tissue samples were paraffin embedded, sectioned at 5 μ m, dewaxed, and rehydrated by standard procedures. The slides were stained with Hematoxylin Eosin (H & E) and Safranin O-fast green staining. The Osteoarthritis Research Society International (OARSI) OA scoring system was adopted to analyze the pathological changes in the joint as previously described [26]. Briefly, the depth of the cartilage damage as well as the extent of the damaged surface was scored in a blinded manner at two different locations in the rat knee joint, i.e., the lateral and medial tibial articular cartilage. The OA score was defined as the product of the multiplication of these two scores. The mean OA score was calculated using the scores for the two individual locations examined by three researchers.

Immunohistochemistry assays

To detect the expression of matrix metalloproteinase13 (MMP13) in articular cartilage, immunohistochemistry was performed. Tissue samples were fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. Paraffin sections were deparaffinized, blocked in 0.3% hydrogen peroxide in methanol for 20 min and rehydrated through graded alcohol. Antigen retrieval was performed by incubating in a 10 mM warm citrate buffer (pH 6.0) for 20 min at 60°C. The sections were incubated

with primary antibodies (in blocking solution): anti-MMP13 (1:300; ab219620, Abcam, USA) overnight at 4°C after blocked with blocking solution (5% animal serum in PBS /1% bovine serum albumin). 5 random microscopic fields were captured from each sample and images were captured under a phase-contrast microscope (Leica Microsystems, Wetzlar, Germany).

Enzyme-linked immunosorbent assays (ELISAs)

Peripheral sera were harvested through centrifugation of peripheral blood mixed with heparin. The secretion of serum inflammatory cytokines of Interleukin-6 (IL-6) in peripheral sera was measured using Mouse IL-6 Quantikine ELISA kits (SM6000B; R&D Systems, Minneapolis, MN, USA) following the kit instructions.

Statistical analysis

All statistical analysis was performed by the statistical software SPSS 15.0 (SPSS, Chicago, Illinois, USA). Data were presented as means $\pm$ SEM among groups. OA score in animal study was compared using Kruskal-Wallis test with Dunn's post-test. Data from animal study except for OA score were analyzed by one-way analysis of variance (one-way ANOVA) followed by post hoc multiple comparison tests (Tukey's test when equal variance was assumed, or Games-Howell test when equal variance was not assumed). A p-value < 0.05 was considered significant.

Results

Isolation and characterization of PB-MSCs and AD-MSCs

Murine peripheral blood-derived mesenchymal stem cells (PB-MSCs) and adipose-derived mesenchymal stem cells (AD-MSCs) were isolated and expanded *ex vivo* respectively. (Fig. 1A). PB-MSCs and AD-MSCs both expressed cell surface antigens of MSCs (CD29, Sca-1), while lacked the expression of hematopoietic marker CD45 (Fig. 1B). Tri-lineage differentiation induction assays confirmed that both PB-MSCs and AD-MSCs possessed multi-differentiation capacity into osteoblasts, adipocytes, and chondrocytes (Fig. 1C) under appropriate culture conditions.

Amelioration of articular cartilage degeneration and subchondral bone hardening through MSCs transplantation

The results of 3D reconstruction for the right tibial subchondral bones through μ CT analysis (Fig. 2B-2D) suggested that DMM-induced osteoarthritis (OA) of saline control group exhibited remarkably increased bone density of subchondral bones, confirming traumatic-induced OA model was well-established. While PB-MSCs treatment ameliorated abnormal bone formation in subchondral bones in mice after surgery, which is comparable to the therapeutic efficacy of AD-MSCs treatment. Also, results of histological assays (Fig. 3) suggested that PB-MSCs or AD-MSCs treatment significantly attenuated the degradation of joint articular cartilage respectively. In the meanwhile, the protein expression of MMP13 on joint

cartilage was significantly suppressed in the PB-MSCs and AD-MSCs-treated group comparing to saline controls (Figure-5).

Observation of BrdU/Sox9-expressing cells and reduction of peripheral sera IL-6 through MSC treatment. In addition, BrdU-Sox9-coexpressing proliferating chondrocytes were observed in mPB-MSCs or mAD-MSCs treatment group (Fig. 4). Simultaneously, MSCs treatment significantly reduced expression of MMP13 (Fig. 5) on articular cartilage surface of right knee joints comparing to saline controls. And the concentrations of IL-6 within peripheral sera of saline control mice were significantly higher than sham surgery and MSCs-treated groups (Fig. 6).

Discussion

The management of osteoarthritis (OA) remains a major challenge in the orthopaedic surgery despite extensive preclinical research has been performed through diverse therapeutic approaches [27–29]. Cell source-specific features exist among different tissue-derived adult stem cells [30, 31]. Inter- and intra-source heterogeneity with differential gene expression profiling and functionality has been reported between different source derived MSCs [7]. A recent study has demonstrated that adipose-derived stem cells exerted as a preferred source for OA treatment when compared with bone marrow-derived MSCs through meta-analysis of clinical therapeutic outcomes, as well as different biologic characteristics between AD-MSCs and BM-MSCs indicated by single-cell RNA sequencing analysis [32]. To further identify a specific subpopulation of MSCs with potent chondrogenic differentiation and proliferation capacity may be conducive to enhanced survival rate of transplanted stem cells and optimization of the therapeutic outcome.

In this study, 3 times of repetitive injections of allogeneic MSCs were applied for the treatment of DMM surgery-induced mice OA model, as previous study suggested repeat injections of allogeneic MSCs caused the lowest signs of synovitis [33]. However, adverse immune response after repeated injection of allogeneic MSCs was also reported [34]. This animal model usually quickly exhibited OA phenotype around 4 weeks post-surgery, and we expected to evaluate the relative long-term therapeutic outcome (8 weeks after surgery), which may better mimic the clinical settings for the treatments of OA patients. And recent studies from our group have demonstrated allogeneic MSCs transplantation possessed similar therapeutic efficacy as autologous MSCs on bone formation of distraction osteogenesis rat model, indicating lower immune response associated to allogeneic MSCs therapy [35].

Peripheral blood has emerged as a novel ideal cell source of adult stem cells due to its availability of acquisition with minimal invasiveness and ease of cell biobank, which is conducive for further autologous cell transplantation for personalized cellular therapy [20, 36, 37]. We found that PB-MSCs displayed comparably effective efficacy in attenuation of traumatic-induced OA as AD-MSCs. Increasing evidence has suggested that adult stem cells may facilitate tissue regeneration through various molecular mechanisms, such as immunomodulation of local microenvironment, differentiation into functional cells, cell “empowerment” via paracrine secretion, immunoregulation, and intercellular

mitochondrial transfer [38]. Notably, stem cell-derived exosomes or extracellular vesicles have recently been demonstrated effective for cartilage repair and OA attenuation [39–41]. Also, mitochondrial transfer from transplanted MSCs has recently been demonstrated as a crucial approach of cell therapy to protect against cartilage degeneration through improving mitochondrial function, cell proliferation, and inhibiting apoptosis in chondrocytes [42]. Thus, injected MSCs may function via paracrine secretion of exosomes or extracellular vesicles, and various growth factors, as well as mitochondrial transfer for ‘cell empowerment’ to the resident chondrocytes besides direct chondrogenic differentiation.

However, there are still limitations in this present study. For instance, how many subsets PB-MSCs consist of, and which subpopulation of PB-MSCs mainly functions during the processes of cartilage protection and regeneration during OA therapy, still remain unclear. Further investigations are required for the identification of a specific PB-MSCs subpopulation as a preferred cell source for cartilage repair and OA attenuation. Also, further improved treatments of cell therapy for therapeutic optimization, such as MSCs encapsulated with injectable hydrogels or micro-carriers, may be necessary for optimized therapeutic efficacy [43–45].

Conclusions

In summary, the present study demonstrated that repeated intra-articular injections of PB-MSCs ameliorated trauma-induced osteoarthritis progression through stimulation of regeneration and proliferation of de novo chondrocytes, and inhibition of hypertrophic marker and inflammatory cytokines release via regulation of local microenvironments. PB-MSCs may become an ideal and alternative cell source for osteoarthritis therapy.

Abbreviations

OA: Osteoarthritis

PB-MSCs: Peripheral blood derived mesenchymal stem cells

DMM: Destabilization of the medial meniscus surgeries

MSCs: Mesenchymal stem cells

AD-MSCs: Adipose tissue derived MSCs

FBS: Fetal bovine serum

MMTL: Medial meniscotibial ligament

H & E: Hematoxylin Eosin

BMD: Bone mineral density

BV/TV: Bone volume/ total tissue volume

OARSI: Osteoarthritis Research Society International

MMP13: Matrix metalloproteinase13

ELISAs: Enzyme-linked immunosorbent assays

One-way ANOVA: one-way analysis of variance

Declarations

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Acknowledgements

Not applicable

Contributions

GL and SL designed the study. SL, WL, HW, and QP conducted the animal experiments. ZY, XZ and WL analyzed the data. All authors contributed to the article and approved the submitted version.

Ethics declarations

Ethics approval and consent to participate

All the animal care and experimental procedures were performed with the ethical approval from the Department of Health in the Hong Kong Special Administrative Region and The Chinese University of Hong Kong.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

A & B, Morphology observation of murine peripheral blood-derived mesenchymal stem cells (mPB-MSCs) and adipose-derived mesenchymal stem cells (mAD-MSCs). C & D, Alizarin red staining of mPB-MSCs and mAD-MSCs after 2-week-osteogenic induction. E & F, red oil O staining of mPB-MSCs and mAD-MSCs after 2-week-adipogenic induction. G & H, Alcian blue staining of mPB-MSCs and mAD-MSCs after 2-week-chondrogenic induction. Scale bar, 100 μ m.

Figure 2

A, Schematic summary of workflow of in vivo assays. B, 3D μ CT reconstruction images of the tibia subchondral bone medial compartment of right joints of mice. Scale bar, 0.5 mm. C, bone volume/total tissue volume (BV/TV), and D, apparent bone mineral density (BMD) were analyzed for tibia subchondral bones by μ CT analysis. Data are presented as mean $\pm$ SEM. (*: $p < 0.05$; $n=3$ /group).

Figure 3

A, Results of histological assays (Safranin O-fast green and H&E stainings) for joint samples. Scale bar, 100 μ m. B, Quantification of the histologic results through the Osteoarthritis Research Society International Cartilage Histopathology Assessment System (OARSI score) indicated articular cartilage damage for all groups. Data are presented as mean $\pm$ SEM. (*: $p < 0.05$; $n=7$ /group).

Figure 4

Immuno-fluorescence staining of sox9 and BrdU. A, Immunofluorescent staining results of BrdU and Sox9 for knee joint samples of 8-week-postsurgery mice. Scale bar, 100 μ m. B, the ratio of Sox9 / BrdU-expressing cell numbers for the four groups samples. Data are presented as mean $\pm$ SD. (#:p > 0.05 compared to Saline Control; n=3/group).

Figure 5

Immuno-histochemical staining results of MMP13 for the knee joint samples. The expression of MMP13 on articular cartilage of PBMSCs- and ADMSCs-treated groups was reduced than saline controls. Scale bar, 100 μ m.

Figure 6

Concentrations of IL-6 within peripheral sera through ELISA assays. The concentration of IL-6 within peripheral sera increased remarkably than sham surgery group, whilst peripheral sera IL-6 concentrations of PB-MSCs and AD-MSCs-treated groups mice were much lower than saline controls. (*: $p < 0.05$; $n=4$ /group).