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Donor sex and age influence the chondrogenic potential of human femoral bone marrow stem cells

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SUMMARY

Objective: Damaged articular cartilage does not heal well and can progress to osteoarthritis (OA). Human bone marrow stem cells (BMC) are promising cells for articular cartilage repair, yet age- and sex-related differences in their chondrogenesis have not been clearly identified. The purpose of this study is to test whether the chondrogenic potential of human femoral BMC varies based on the sex and/or age of the donor.

Design: BMC were isolated from 21 males (16–82 years old (y.o.)) and 20 females (20–77 y.o.) during orthopaedic procedures. Cumulative population doubling (CPD) was measured and chondrogenesis was evaluated by standard pellet culture assay in the presence or absence of transforming growth factor beta 1 (TGFβ1). Pellet area was measured, and chondrogenic differentiation was determined by Toluidine blue and Safranin O-Fast green histological grading using the Bern score and by glycosaminoglycan (GAG) content.

Results: No difference in CPD was observed due to donor sex or age. The increase in pellet area with addition of TGFβ1 and the Bern score significantly decreased with increasing donor age in male BMC, but not in female BMC. A significant reduction in GAG content per pellet was also observed with increasing donor age in male BMC. This was not observed in female BMC.

Conclusions: This study showed an age-related decline in chondroid differentiation with TGFβ1 stimulation in male BMC, but not in female BMC. Understanding the mechanisms for these differences will contribute to improved clinical use of autologous BMC for articular cartilage repair, and may lead to the development of customized age- or sex-based treatments to delay or prevent the onset of OA.

Introduction

Articular cartilage is an avascular tissue with limited intrinsic healing capacity. Due to its inability to heal efficiently, focal cartilage injuries of the knee have an increased risk of progressing to osteoarthritis (OA), a leading cause of disability[1]. Current treatment modalities for articular cartilage repair include debridement, osteochondral grafting, autologous chondrocyte implantation, and microfracture[2–4]. While good to excellent clinical outcome scores have been reported for each technique, the repaired cartilage is biomechanically dissimilar to the surrounding native cartilage. This can lead to degradation of the cartilage over time, which would set the stage for the progression of OA. Thus, there is an urgent need to improve articular cartilage repair, as it may delay, or even prevent the onset of debilitating OA.

Microfracture is minimally invasive and the simplest of the treatment techniques, as it involves penetrating the subchondral bone to access repair cells from the bone marrow to infiltrate the defect. Microfracture is, however, inconsistent[4–6]. A high degree of variability in the amount of repair cartilage that fills the defect has been reported, indicating that there may be a subpopulation of patients that do not produce sufficient repair tissue after microfracture, leading to early failure[4]. Age has also been shown to affect the clinical outcome of microfracture, with younger patients (<40 years old (y.o.)) showing better clinical outcome scores[6]. Since bone marrow cells are the main repair cells recruited to the defect during microfracture, it suggests an important and continued role for the autogenous application of bone marrow stem cells (BMC) in articular cartilage repair. The variability seen in clinical outcome measures suggests that BMC from different individuals could differ in their capacity for chondrogenic differentiation.

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While it has been well established that BMC are capable of chondrogenic differentiation *in vitro* and *in vivo*[7–15], few studies have focused on comparing the chondrogenesis of BMC obtained from a large number of individuals[10,11,13]. This comparison is important to determine whether there is inherent variability at the BMC level between individuals for chondrogenic repair, so that this can be taken into account to develop efficient BMC-based therapies for articular cartilage repair. Since OA and injuries to articular cartilage affect men and women of all ages, it is important to determine whether the chondrogenic capacity of BMC differs based on the sex and/or age of the donor. Sexual dimorphism in the prevalence of OA has been reported, with women being diagnosed more frequently than men, especially over the age of 50[1,16]. Recent studies have shown that cell sex can affect the differentiation potential of both mouse and human progenitor cells[17–20]. As well, aging has been shown to affect the osteogenic differentiation of BMC[21–23], although this was not conclusive in all studies[24–26]. Our understanding of age- and sex-related differences in the chondrogenic capacity of BMC has not been clearly defined.

Cells isolated from bone marrow procured from the distal femur are promising cells for articular cartilage repair, since this site could be accessed as an autologous cell source, and these cells are similar to those typically accessed during microfracture. Sex- and age-related differences in the chondrogenic potential of femoral BMC should be investigated to better develop BMC-based therapies for articular cartilage repair. Such studies may also provide information predictive of the outcome of microfracture, which may affect patient selection and treatment chosen. For this reason, this study was performed to test the hypothesis that the chondrogenic potential of femoral human BMC varies based on the sex and/or age of the donor. The findings obtained will be important to improving articular cartilage repair, a strategy for potentially delaying the onset of disabling OA.

Methods

Cell isolation and expansion

Femoral bone marrow reamings were obtained from males and females undergoing orthopaedic surgery for fracture stabilization or joint replacement according to an exempt IRB-approved protocol at the University of Pittsburgh for discarded tissue. A total of 41 femoral bone marrow reamings were obtained. The donors ranged in age from 16 to 82 y.o. and included 21 males (16–82, mean donor age 39 ± 22) and 20 females (20–77, mean donor age 53 ± 17). Freshly harvested bone marrow was minced, washed with phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) and vortexed. The cell suspension was then passed through a 70 μm cell strainer. Thirty-five ml of cell suspension was loaded onto 15 ml Histopaque®-1077 (Sigma–Aldrich, St. Louis, Mo., USA) and fractioned by centrifugation ($400\times g$) for 30 min. Mononucleated cells were recovered from the Histopaque-supernatant interface and cultured in Minimum Essential Medium Alpha Medium (Invitrogen) supplemented with 16.5% fetal bovine serum (FBS; lot selected for rapid growth of BMC, Atlanta Biologicals, Lawrenceville, GA, USA), 1% penicillin–streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen). Cells were allowed to adhere for 5–7 days at 37°C and 5% CO_2 before the first medium change, which would remove any non-adherent cells. Visible colonies were seen after 8–10 days of initial plating and were detached from the tissue-culture plastic with 0.25% trypsin (Invitrogen). BMC were replated at 100 cells/cm^2, trypsinized when they reached 70% confluence, and either used for experiments or replated at the same cell density for further expansion. Medium was refreshed every 3–4 days.

Cumulative population doublings (CPDs)

At passage 2, cells were placed in a T25-flask at a density of 100 cells/cm^2. After 7 days, cells were trypsinized, counted, and replated at 100 cells/cm^2. This was repeated until passage 8. Population doubling was calculated as the $\log_2 (N/N_0)$, where N_0 and N are the number of cells originally plated and the number of cells at the end of the expansion, respectively.

Chondrogenic differentiation

Chondrogenesis was tested on BMC at passage 3 or 4 using a previously described pellet culture assay[8]. A total of 2.5×10^5 cells were centrifuged at $500\times g$ for 5 min in 15-ml conical polypropylene tubes. The cell pellets were cultured in 0.5 ml chondrogenic medium (CM) that contained high-glucose Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 1% penicillin–streptomycin, 10^{-7} M dexamethasone (Sigma–Aldrich), 50 μg/ml L-ascorbic acid-2-phosphate (Sigma–Aldrich), 40 μg/ml proline (MP Biomedicals, Solon, OH, USA), and 1% BD^{TM} ITS + Premix (Becton-Dickinson, Franklin Lakes, NJ, USA), in the presence or absence of transforming growth factor beta 1 (TGFβ1) (10 ng/ml; R&D Systems, Minneapolis, MN, USA). All pellets were incubated at 37°C in 5% CO_2 for 21 days, and the CM was refreshed every 2–3 days.

Area measurement of pellets

After 21 days of culture, macroscopic images of the pellets were captured using a stereomicroscope (MVX-10 MacroView Systems, Olympus, Japan) equipped with a DP71 camera (Olympus), and the area was calculated using DP2-BSW software (Olympus). The change in pellet area was calculated by dividing the area measurement of pellets cultured in CM + TGFβ1 by that of pellets cultured in CM only.

Histological analysis

Pellets were fixed in 10% buffered formalin followed by paraffin embedding. Cross-sections (6 μm thick) were stained with Toluidine blue for sulfated polysaccharides and Safranin O-Fast green for sulfated glycosaminoglycans (GAG). Standard protocols for each of these stains were followed. Images were captured with DP2-BSW software (Olympus) using a Nikon TE-2000U Eclipse microscope equipped with a DP71 camera (Olympus). Safranin O-Fast green histology was graded by two blinded observers using the Bern score[27]. Table I indicates the criteria used to grade Safranin O-Fast green staining of pellets according to the Bern score.

Biochemical analysis

To measure total GAG content from BMC pellets, pellets cultured for 21 days were washed with PBS and each dry pellet was frozen at −80°C until all pellets were ready to be assayed. Each frozen pellet was placed in 0.2 ml papain buffer [50 mM phosphate buffer (pH 6.5), 5 mM EDTA, 5 mM cysteine HCl and 0.5 mg/ml papain] in a 60°C water bath overnight. The pellets were then vortexed and centrifuged for 5 min at $21{,}000\times g$ at room temperature. The supernatant was transferred to a new tube, followed by GAG quantification. GAG was quantified using the standard dimethylmethylene blue (DMMB) assay[28] with shark chondroitin-6-sulfate (Sigma–Aldrich) as the standard.