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Decreased Osteogenic Activity of Mesenchymal Stem Cells in Patients With Corticosteroid-Induced Osteonecrosis of the Femoral Head

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A B S T R A C T

Background: Osteonecrosis (ON) of the femoral head occurs when cells of trabecular bone spontaneously die. Mesenchymal stem cells (MSCs) have been introduced into the femoral head in an attempt to halt progression of the disease. The purpose of this study was to functionally compare MSCs in patients with ON of the femoral head with patients without.
Methods: Mesenchymal stem cells were isolated from 20 patients with corticosteroid-induced ON and 10 controls without. Colony-forming unit and proliferation assays were used to assess MSC proliferation. Mesenchymal stem cells were differentiated into bone, fat, and cartilage. Functional assays were used to quantify the differentiation capacity.
Results: Control MSCs demonstrated greater cellular growth potential and improved ability to differentiate into bone.
Conclusion: The decreased ability to differentiate into bone may be a reason why patients treated with autologous MSC infusion fail regenerative treatment strategies and progress to collapse.

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Osteonecrosis (ON) of the femoral head occurs when cells of trabecular bone in the femoral head spontaneously die, leading to fracture and collapse of the femoral articular surface [1,2]. Once collapse of the articular surface occurs, the disease course rarely regresses, leading to severe pain and functional disability [3-6]. Currently, most patients with ON of the femoral head require total hip arthroplasty (THA) at a younger age than most patients undergoing THA for osteoarthritis [7-9].

If the disease course is recognized early before collapse of the femoral head, patients can be treated with core decompression [10-12]. Although this technique has been used for decades, randomized trials of core decompression alone have not shown any ability to halt progression of the disease and prevent collapse [13]. Several studies have used human mesenchymal stem cells (MSCs) obtained from iliac crest bone marrow concentrate (BMC) to augment the femoral head site at the time of decompression [14-17]. Although this is a current treatment option, previous reports have shown that a large percentage of patients eventually progress to collapse [14-17]. Currently, risk factors that have been described for disease progression and femoral head collapse include stage of the disease at the time of treatment, concentration of MSCs augmenting the decompression site, and the location and size of the necrotic lesion [14-18]. The purpose of this study was to determine if human MSCs isolated from the iliac crest harbor deficiencies in the setting of ON, which may account for failure, with a focus on (1) cellular viability and function, and (2) ability of MSCs to perform multilineage differentiation.

Materials and Methods

After institutional review board approval, bone marrow was obtained from the iliac crest of 20 patients undergoing minimally invasive core decompression and autologous bone marrow transplantation for ON. Iliac crest bone marrow was also isolated from 10 patients undergoing revision hip arthroplasty without a previous diagnosis of ON or corticosteroid exposure, where the revision site was supplemented with concentrated bone marrow. Eligibility criteria included patients

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with Steinberg Grade I or II ON [19], age older than 18 years, and normal bone marrow function defined as an absolute neutrophil count greater than 1500 cells/μL. Patients with an active infection, current alcohol use greater than 2 drinks per day, immunodeficiency, poorly controlled diabetes (hemoglobin A1C >8%), and those receiving hematopoietic growth factors or chemotherapy were excluded. Bone marrow was aspirated from the iliac crest of all patients and concentrated in the operating room using the BioCUE system (Biomet Biologics, Warsaw, IN).

Isolation of MSCs From Concentrated Bone Marrow

The ON group consisted of 11 females and 9 males with a mean age of 43 years (range, 20-66 years) at the time of surgery. Five patients were current tobacco users. All patients had a history of chronic corticosteroid exposure. At the time of the surgical procedure, 12 patients were not actively taking prescribed corticosteroids, 4 were taking oral corticosteroids daily, and 4 were intermittently taking oral corticosteroids for asthma exacerbations. Based on the Steinberg system [19], patients were classified as having stage I (n = 3) or stage II (n = 17) ON. The control group consisted of 4 females and 6 males with a mean age 63 years (range, 33-95 years) at the time of surgery. Two patients were current tobacco users (Table).

Determination of Cell Recovery and Viability

Bone marrow concentrate was digested as previously described [20]. Bone marrow cell viability was determined using a Countess (Invitrogen, Grant Island, NY) automated cell counter coupled with 0.4% trypan blue (Invitrogen) staining.

Mesenchymal Stem Cell Culture

Mesenchymal stem cell cultures were obtained from the native BMC by plating the resuspended cell pellets obtained after digestion as previously described [20]. Mesenchymal stem cells were identified as cells with the ability to proliferate in culture with an adherent, spindle-shaped morphology. When the cells reached approximately 80% confluence, they were detached with 0.05% Trypsin (Invitrogen) and quantified using the Countess machine. Thirty-thousand cells were then replated on new 100-mm plates with expansion media and 10% fetal bovine serum (FBS). The process was repeated for a total of 2 cell passages before the start of differentiation or growth experiments.

Immunophenotypic Characterization of MSCs

Isolated cells were collected at the time of initial plating after digestion as well as after 2 cell passages in culture. The isolated cells were immunostained for cell surface markers using an Aria SE flow cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA). Mesenchymal stem cells were identified as a population of cells expressing CD90/105, with an absence of CD14/34/45 [20]. Cells were incubated with either CD34-allophycocyanin, CD14–fluorescein isothiocyanate, CD45 phycoerythrin cyanine 7 (all from BD Biosciences), CD90 peridinin chlorophyll-cyanine-5.5, or CD105 phycoerythrin (both from eBioscience, San Diego, CA).

Table
Comparison of Patients With and Without Osteonecrosis of the Femoral Head.

Demographics	Osteonecrosis (n = 20)	No Osteonecrosis (n = 10)	*P*
Males	9	6	.69
Females	11	4	
Mean age (y)	43	63	**.0008**
Tobacco use	5	2	1.0

Proliferation Assay

Bone marrow–derived MSCs were initially plated at 5000 cells/cm^2 in 10% FBS-supplemented media and grown for a total of 5 passages, representing 40 to 60 cell division events depending on the conditions. Cumulative population doubling time was calculated using the formula $[\log_{10}(N_H)-\log_{10}(N_1)]/\log_{10}2$ where N_H is the harvested cell number and N_1 is the plated cell number [21]. Each passage was calculated and added to the population doubling time as previously described [21]. Briefly, the generation time (time between 2 cell doubling events) was calculated as $[\log_{10}2 \times \Delta t]/[\log_{10}(N_H)-\log_{10}(N_1)]$, where Δt is the time between passages. Shorter generation time represents faster cell growth [21].

Fibroblast Colony-Forming Unit Assay and Quantification of Cell Function

Fibroblast colony-forming unit (CFU) assays were used to assess MSC yield as previously described [22] in addition to a functional assay (below). Mesenchymal stem cell precursors were quantified after 2 weeks by washing cells with phosphate-buffered saline (PBS) and staining with 0.5% crystal violet (Sigma Aldrich, St Louis, MO) in methanol for 5 minutes at room temperature. The plates were then washed, and visible colonies were counted. All experiments were performed in triplicate and reported as a mean of the 3 trials.

Cells were trypsinized once they reached 80% confluence (Invitrogen). Ten thousand cells per well were then plated on 12-well culture dishes. After 14 days of growth, the cellular viability and proliferation rates were measured using the MTS Cell Proliferation Assay (Promega Corporation, Madison, WI) per the manufacturer protocol.

In Vitro Differentiation

Osteogenic, chondrogenic, and adipogenic differentiation was achieved using StemPro Osteogenesis, Chondrogenesis and Adipogenesis differentiation media (all from Invitrogen) with 10% FBS, per the manufacturer protocol. Cells were cultured for a total of 14 days with media changed every 48 hours. After day 14, differentiation assays were performed as described below.

Quantification of Differentiation

To quantify adipocyte differentiation, staining was performed using 0.2% oil red O in 100% isopropanol (Sigma-Aldrich). Briefly, samples were fixed in 10% formaldehyde, stained with oil red O for 10 minutes, and rinsed with 60% isopropanol (Sigma-Aldrich). The oil red O was eluted by adding 100% isopropanol for 10 minutes. The optical density (OD) of the solution was measured at 490 nm, for 0.5 seconds.

Sulfated glycosaminoglycan content was determined using a Blyscan Kit (Biocolor, Carrickfergus, United Kingdom) per the manufacturer protocol. Likewise, alkaline phosphatase activity was measured using an Alkaline Phosphatase Assay Kit (Abcam, Cambridge, MA) per the manufacturer protocol.

Statistical Analysis

Results were expressed as mean ± SEM for each experiment. Analysis was performed through an unpaired Student *t* test using JMP 10 software (SAS, Cary, NC) with statistical significance set at a $P < .05$.

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