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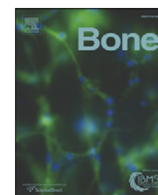

Original Full Length Article

Mesenchymal progenitors residing close to the bone surface are functionally distinct from those in the central bone marrow

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A B S T R A C T

Long bone is an anatomically complicated tissue with trabecular-rich metaphyses at two ends and cortical-rich diaphysis at the center. The traditional flushing method isolates only mesenchymal progenitor cells from the central region of long bones and these cells are distant from the bone surface. We propose that mesenchymal progenitors residing in endosteal bone marrow that is close to the sites of bone formation, such as trabecular bone and endosteum, behave differently from those in the central bone marrow. In this report, we separately isolated endosteal bone marrow using a unique enzymatic digestion approach and demonstrated that it contained a much higher frequency of mesenchymal progenitors than the central bone marrow. Endosteal mesenchymal progenitors express common mesenchymal stem cell markers and are capable of multi-lineage differentiation. However, we found that mesenchymal progenitors isolated from different anatomical regions of the marrow did exhibit important functional differences. Compared with their central marrow counterparts, endosteal mesenchymal progenitors have superior proliferative ability with reduced expression of cell cycle inhibitors. They showed greater immunosuppressive activity in culture and in a mouse model of inflammatory bowel disease. Aging is a major contributing factor for trabecular bone loss. We found that old mice have a dramatically decreased number of endosteal mesenchymal progenitors compared with young mice. Parathyroid hormone (PTH) treatment potently stimulates bone formation. A single PTH injection greatly increased the number of endosteal mesenchymal progenitors, particularly those located at the metaphyseal bone, but had no effect on their central counterparts. In summary, endosteal mesenchymal progenitors are more metabolically active and relevant to physiological bone formation than central mesenchymal progenitors. Hence, they represent a biologically important target for future mesenchymal stem cell studies.

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Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells capable of differentiating into multiple cell lineages including osteoblasts, chondrocytes, adipocytes, smooth muscle cells, myocytes, endothelial cells, and neurons. Together with their homing ability to sites of injury and immunoregulatory effects, they are a promising therapeutic tool for tissue engineering and regeneration [1]. MSCs reside in bone marrow and many other tissues and organs, such as placenta, peripheral blood, cord blood, cord Wharton's jelly, adipose tissue, amniotic fluid, periosteum, synovial membrane, synovial fluid, articular cartilage, and muscle. Due to a lack of specific cell surface markers to unequivocally identify these cells, currently there is no commonly accepted method to isolate true MSCs. The standard approach to study MSCs is to isolate and culture bone marrow cells from rodent long bones to obtain plastic-adherent and clonogenic fibroblastoid mesenchymal progenitors, which is a heterogeneous population containing not only true MSCs but also their proliferative progeny. Bone marrow from the central part of the rodent diaphyseal bone can be easily separated from the surrounding cortical shell by flushing and this source of bone marrow is commonly used to make mesenchymal progenitor cultures. On

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the other hand, bone marrow in the metaphyseal region is evenly distributed within honeycombed or spongy-shaped trabecular bone and therefore, is not easily isolated.

The bone marrow is also the home of another important stem cell, the hematopoietic stem cell (HSC). Interestingly, recent studies demonstrate that HSCs, particularly the long-term HSCs, residing in the endosteal region have a superior proliferative capacity and homing efficiency compared with their counterparts in the diaphyseal bone [2,3], indicating that the microanatomical location of stem cells might have a great influence on their behavior. Previous publications [4–6] described the endosteal cells as those cells within a 12 cell distance from the bone surface and proposed that all bone marrow cells within mouse trabecular bone are in the endosteal region [4]. These data suggest the importance of isolating the mesenchymal progenitors within the endosteal region and comparing their stem cell properties and biological functions with their counterparts derived from the central region of diaphyseal bone.

Aging is one of the most important risk factors for osteoporosis. A number of studies using the conventional colony forming unit-fibroblast (CFU-F) assay to access mesenchymal progenitor numbers and cultured mesenchymal progenitors for functional studies have indicated that aging may decrease the number and activity of mesenchymal progenitors in the central bone marrow of rodents [7–11] and humans [12–16], suggesting that mesenchymal progenitors play an important role in age-related osteoporosis. However, there are also reports demonstrating no effect or a marginal effect of aging on mesenchymal progenitor numbers or differentiation potential in rodents [17,18] and humans [19,20]. Parathyroid hormone (PTH) is the only approved anabolic therapy for the treatment of postmenopausal women and men with osteoporosis who are at a high risk of fractures. Intermittent injection of PTH1–34 (teriparatide) induces an anabolic effect on bone, increasing trabecular bone volume, connectivity, plate-like microarchitecture [21,22], periosteal bone mass [23], cortical cross-sectional area [24], and reducing fractures [25]. PTH injection greatly stimulates bone formation and increases osteoblast numbers, which involves activating bone lining cells, inhibiting osteoblast apoptosis, and enhancing osteoblast maturation through suppressing the expression of sclerostin from osteocytes [26,27]. Mendez-Ferrer et al. recently reported that a 5-week injection of PTH doubles the number of nestin$^+$ MSCs in bone marrow [6]. However, CFU-F assays yield conflicting results, with an increase [28–30], decrease [31], or no change [32,33] in the number of mesenchymal progenitors after PTH injections. All these experiments were performed with a flushed bone marrow population and therefore, the effects of aging and PTH on the mesenchymal progenitors within the endosteal region of bone has not yet been investigated.

Several enzymatic digestion approaches, together with chopping and crushing bones into small pieces, have been reported to obtain mesenchymal progenitors either from rodent whole bone [34,35], pre-flushed long bone [36,37], or cortical bone [38,39]. While several of these reports [34–36] showed a dramatic increase in the number of CFU-F colonies from the cells isolated by collagenase digestion methods, the anatomical location and biological functions of the mesenchymal progenitors isolated by these approaches have not been analyzed. In this report, we used enzymatic digestion following bone marrow flushing to separately isolate mesenchymal progenitors from the endosteal and central regions of bones. We show that, while they are phenotypically identical to their central counterparts in terms of stem cell surface marker expression and multi-differentiation ability, endosteal mesenchymal progenitors exist at a higher frequency and display important functional differences, such as an elevated proliferative rate and greater immunomodulatory ability. Most interestingly, we demonstrate that endosteal mesenchymal progenitors, but not central mesenchymal progenitors, are highly responsive to aging and PTH treatment, suggesting that they are a major target of the anabolic PTH therapy.

Material and methods

Isolation and culture of rodent endosteal and central mesenchymal progenitors

All animal experiments in this study were performed under the institutionally approved protocols for the use of animal research either at the University of Pennsylvania or at the University of South California.

Sprague-Dawley rats and C57Bl/6 mice (Charles River) were euthanized by CO_2 inhalation and the bilateral femora and tibiae were dissected free of surrounding tissues under sterile conditions and washed in αMEM. Both ends of the long bones were removed at growth plate sites and the bone marrow was flushed out of the bone with αMEM using a 25-gauge needle (Fig. 1A step 1). Flushed cells (the central bone marrow) were passed through a 70-micron cell strainer and then either used directly for flow cytometry, or plated at 3×10^6/T25 flask for CFU-F assays and 30-50$\times 10^6$/100 mm dish for expanding. To obtain endosteal mesenchymal progenitors, the outer surface of flushed long bones was scraped several times and then digested with a protease solution (2 mg/ml collagenase A and 2.5 mg/ml trypsin in PBS) for 20 min to remove the periosteum and periosteal progenitors. Next, bones were longitudinally cut into two halves (step 2), gently washed to remove loosely attached bone marrow (step 3), and then digested in the protease solution for 1 h (step 4). Cells within the supernatant (endosteal bone marrow) were collected, washed one time with culture medium, passed through a cell strainer, and then either used directly for flow cytometry, or plated at 1×10^6/T25 flask for CFU-F assays and 3-5$\times 10^6$/100 mm dish for expanding. For rat mesenchymal progenitors, the growth medium was αMEM with 15% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. For mouse mesenchymal progenitors, the growth medium was αMEM with 20% FBS, 55 μm β-mercaptoethanol, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After reaching 80–90% confluence, adherent mesenchymal progenitors were detached from flasks by trypsin and EDTA and subcultured at a dilution of 1:3 to 1:5. Only passages less than five were used in the experiments.

For PTH treatment, 1-month-old Sprague Dawley rats or C57Bl/6 mice were injected subcutaneously with various PTH peptides [1–34 (human), 1–31 (human), and 3–34 (bovine), Bachem] at 80 μg/kg or vehicle (saline). Twenty-four hours later, central and endosteal bone marrow cells were isolated as described above and plated for CFU-F assays. In addition, rats were injected with PTH(1–34) 80 μg/kg or vehicle daily for 12 days. Both central and endosteal bone marrow cells were isolated 1 day after the last injection for CFU-F assay.

CFU-F Assays

Endosteal and central bone marrow cells in growth medium were allowed to form colonies for 5 and 7 days, respectively. Cells were fixed and stained for alkaline phosphatase (ALP) activity using a leukocyte ALP kit (Sigma-Aldrich). The number and diameter of ALP-positive colonies were counted and measured under a microscope. The flasks were then stained with 3% crystal violet in methanol and total CFU-F numbers and diameters were quantified. Only the colonies composed of more than 50 cells were counted.

Cell sorting

To sort for GFP-positive and negative cells, endosteal bone marrow cells were isolated from 1-month-old transgenic mice expressing GFP under the control of the 2.3-kb fragment rat a1 (I) collagen gene promoter [40] as described above. Cells were washed, re-suspended in PBS containing 0.5% BSA, and sorted into GFP$^+$ and GFP$^-$ populations using a FACSDiva Cell Sorter (BD Biosciences). GFP$^-$

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