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Circulating osteogenic cells: Characterization and relationship to rates of bone loss in postmenopausal women ☆

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ABSTRACT

There is increasing evidence that osteogenic cells are present not only in bone marrow (BM) but also in peripheral blood (PB). Since staining for alkaline phosphatase (AP) identifies osteoprogenitor cells in BM, we sought to further characterize BM versus PB hematopoietic lineage negative (lin−)/AP+ cells and to compare gene expression in PB lin−/AP+ cells from postmenopausal women undergoing rapid versus slow bone loss. PB lin−/AP+ cells were smaller than their BM counterparts, and both were negative for the pan-hematopoietic marker, CD45. BM and PB lin−/AP+ cells were capable of mineralization *in vitro*. Using whole genome linear amplification followed by quantitative polymerase chain reaction (QPCR) analysis, we found that relative to the BM cells, PB lin−/AP+ cells expressed similar levels of a number of key osteoblast marker genes (runx2, osterix, osteopontin, OPG, periostin), consistent with the PB cells being in the osteoblastic lineage. Importantly, however, compared to the BM cells, PB lin−/AP+ cells expressed lower levels of mRNAs for AP, type I collagen, and for a panel of proliferation markers, but higher levels of osteocalcin, osteonectin, and PTHR1 mRNAs, as well as those for RANKL and ICAM-1, both of which are important in supporting osteoclastogenesis. Using microarray followed by QPCR analysis, we further demonstrated that, compared to postmenopausal women undergoing slow bone loss, PB lin−/AP+ cells from women undergoing rapid bone loss expressed lower levels of mRNAs for hydroxyprostaglandin dehydrogenase, interferon regulator factor 3, Wnt1-induced secreted protein 1, and TGFβ2, but higher levels of the Smad3 interacting protein, zinc finger DHHC-type containing 4 and col1α2. These data thus demonstrate that while PB lin−/AP+ cells express a number of osteoblastic genes and are capable of mineralization, they are a relatively quiescent cell population, both in terms of cell proliferation and matrix synthesis. However, their higher expression of RANKL and ICAM-1 mRNAs as compared to BM lin−/AP+ cells suggests a role for the PB lin−/AP+ cells in regulating osteoclastogenesis that warrants further investigation. Our study also provides "proof-of-concept" for the use of PB lin−/AP+ cells in clinical-investigative studies, and identifies several pathways that could potentially regulate rates of bone loss in postmenopausal women.

Introduction

A number of potential markers for the identification of multipotent bone marrow stromal cells have been developed in recent years [1]. While alkaline phosphatase (AP) is expressed by mature osteoblasts, recent studies have demonstrated that the AP+ fraction of human bone marrow (BM) contains virtually all of the osteoprogenitor population in the marrow [2], indicating that AP expression is associated not only with mature osteoblasts, but likely also with osteoblastic cells at varying stages of differentiation, including relatively early mesenchymal cells.

Although the presence of early mesenchymal and osteoprogenitor cells in BM is well established, the issue of whether there are similar, or at least related, populations in peripheral blood (PB) remains controversial. Based largely on work by Long et al. [3–6] who used cell sorting with antibodies to osteocalcin (OCN) and AP to identify non-adherent BM cells with osteogenic potential, we used flow cytometry following staining with antibodies to these markers to identify OCN+ and AP+ cells in human PB [7,8]. Subsequent studies by others have provided further support for the existence of circulating osteoprogenitor cells, although the precise relationship of these circulating cells to their BM counterparts remains to be fully defined. Thus, Otsuru et al. [9] showed that BM cells were recruited via the circulation into BMP-2 containing collagen pellets and contributed to ectopic bone formation. Subsequently, this group demonstrated

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that the PB cells mobilized in response to BMP-2 were negative for the pan-hematopoietic marker, CD45, and that a major chemokine for recruitment of these cells was stromal cell-derived factor-1 (SDF-1), which was induced locally by BMP-2 [10]. Interestingly, the ability to form bone may not strictly be limited to mesenchymal cells. For example, Suda et al. [11] have recently demonstrated that a population of circulating CD45+ cells that express type I collagen can also form bone both *in vitro* and *in vivo*. These cells are present in the circulation of normal subjects, but increased in PB of subjects with fibrodysplasia ossificans progressiva.

Recent studies have also demonstrated that BM cells expressing the smooth muscle cell marker, α-smooth muscle actin (αSMA) [12], and pericytes surrounding marrow vessels [13] may represent true mesenchymal cells with osteoblastic potential. Thus, Kalajzic et al. [12] found that in a mouse model of osteoblast ablation, repopulation of bone surfaces by osteoblasts was preceded by expansion of an αSMA+ population of cells that subsequently differentiated into osteoblasts. In addition, Sacchetti et al. [13] showed that subendothelial (pericytic) cells expressing MCAM/CD146 were capable of forming bone and providing a niche for hematopoiesis in heterotopic sites.

In the present study, we focused on hematopoietic lineage negative (lin−) cells (which should be enriched for mesenchymal cells) that also expressed AP and sought to identify possible similarities or differences between BM versus PB lin−/AP+ populations. We first characterized these cells for their ability to mineralize, followed by a systematic comparison of expression of key osteoblastic, proliferation, smooth muscle cell, pericytic, and other genes. Finally, to begin to apply these methods in clinical investigation for bone diseases, we performed a study to test whether postmenopausal women undergoing rapid trabecular bone loss had differences in the expression of specific genes, including candidate genes related to bone metabolism, in PB lin−/AP+ cells as compared to cells from similar aged women undergoing slow bone loss.

Methods

Study subjects

All studies were approved by the Mayo Clinic's Institutional Review Board, and the study subjects provided written, informed consent. BM and PB samples were initially obtained from normal healthy female volunteers for flow cytometry analysis and mineralization assays ($n = 7$, mean age, 61 years). Following local anesthesia, 30 ml of BM aspirate was collected in an anticoagulant (EDTA) from the iliac crest using a standard procedure, and 250 ml of blood from each subject was also collected in EDTA. For the clinical study, we selected 20 postmenopausal subjects between 50 and 80 years of age from an ongoing longitudinal study [14]. The study subjects were identified based on the rate of bone loss determined by quantitative computed tomography (QCT) of the lumbar spine done at baseline, 3, and 6 years. Details regarding this cohort and methods for the QCT analysis have previously been published [14,15]. None of the subjects were on bisphosphonates, estrogen, calcitonin, teriparatide, glucocorticoids, or other drugs known to affect bone metabolism. None had any significant medical comorbidities (e.g., underlying malignancy, congestive heart failure, renal failure, etc.). Subjects were grouped into either the rapid or slow cohort by selecting individuals meeting the above criteria and at the two extremes for rates of vertebral trabecular volumetric bone mineral density (vBMD) bone loss. The subjects underwent a fasting blood draw of 250 ml in the outpatient Clinical Research Unit, following which the blood samples were processed as described below for obtaining the relevant cell populations and serum assays.

For the comparison of gene expression in PB versus BM lin−/AP+ cells, we selected six subjects (three from the rapid loss group with the lowest rates of vertebral bone loss and three from the slow loss with the highest rates of vertebral bone loss, so as to avoid the extremes of bone loss for this comparison of PB versus BM lin−/AP+ cells) and performed the detailed quantitative polymerase chain reaction (QPCR) assays described below. Since we did not obtain BM samples in these subjects, we used BM samples from untreated control subjects obtained as part of an ongoing study examining effects of PTH 1–34 on gene expression in BM lin−/AP+ cells. From this untreated control group, we selected six subjects in a similar age range to those on whom we had processed the PB lin−/AP+ cells.

Mononuclear cell (MNC) isolation and flow cytometry analysis

Following Ficoll extraction, MNCs from BM and PB were labeled with a biotinylated anti-human AP monoclonal antibody (Ab) (B4-78) [16] and an isotype matched control, both from R&D Systems (Minneapolis, MN). Subsequently, the cells were stained with a PE-conjugated Streptavidin antibody (eBioscience, San Diego, CA) and a FITC-conjugated lineage (lin) cocktail of monoclonal Abs (BD Biosciences, San Jose, CA). The labeled samples were immediately analyzed on the Becton Dickinson FACScan flow cytometer (San Jose, CA). On flow analysis, the gate was determined on a forward versus side scatter dot plot on the live cell population in the lymphocyte/monocyte-enriched area. Using this specific gate and based on the coexpression of AP and lin markers, the lin−/AP+ population was defined. The percentage of lin−/AP+ cells was compared against the isotype control, and the percent positive lin−/AP+ fraction was defined by subtraction. In some analyses, we also costained for AP and the pan-hematopoietic marker, CD45, labeled with FITC (BD Biosciences).

Magnetic Activated Cell Sorting (MACS)

For cell separation using MACS, post-Ficoll BM and PB cells were magnetically labeled with a human hematopoietic lineage cell depletion kit (Miltenyi Biotec GmbH, Germany) containing Abs to CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and glycophorin A, according to the manufacturer's instructions. After one wash, the cell suspension was loaded onto an autoMACS cell sorter (Miltenyi Biotec GmbH). Subsequently, lin− BM and PB cells were MACS-sorted using a biotinylated anti-human AP monoclonal Ab (R&D Systems) and anti-biotin beads (Miltenyi Biotec GmbH). MACS sorted lin−/AP+ cells from BM and PB were then stored in RLT buffer at −80 °C for later extraction of RNA, as described below.

Osteogenic cultures

MACS-sorted lin−/AP+ BM cells were cultured in plastic plates at a plating density of 2.5×10^5 per cm^2 in medium containing phenol red-free αMEM, 10% FBS and 1% penicillin–streptomycin mixture, all obtained from Invitrogen (Carlsbad, CA). After 1 week, the medium was replaced with osteogenic differentiation medium containing phenol red-free αMEM, 10% FBS, 50 μM ascorbic acid, 10^{-8} dexamethasone and 10 mM β-glycerolphosphate. The cultures were maintained for 21 days, fixed in 10% formalin, and then stained for calcium deposits using 2% alizarin red. MACS-sorted lin−/AP+ PB cells were suspended in growth medium (MesenCult Basal Medium, Stem Cell Technologies, Vancouver, BC, Canada) containing 10% FBS and 1% penicillin–streptomycin mixture and plated in fibronectin-coated plates (Becton Dickinson) at a plating density of 3.5×10^5 per cm^2. On day 21 the medium was changed to differentiation medium containing MesenCult Basal Medium with 15% osteogenic stimulatory supplements, 3.5 mM β-glycerophosphate, 10^{-8} M dexamethasone, and 50 μg/ml ascorbic acid, all purchased from Stem Cell Technologies. After 3 weeks of differentiation, the cells were stained for alizarin red as described above. Throughout the culture of the PB cells, the total media with the non-adherent cell fraction from each well was