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Characterization of mesenchymal progenitor cells isolated from human bone marrow by negative selection☆,☆☆

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ABSTRACT

Studies on the pathogenesis of osteoporosis and other metabolic bone diseases would be greatly facilitated by the development of approaches to assess changes in gene expression in osteoblast/osteoprogenitor populations in vivo without the potentially confounding effects of in vitro culture and expansion of the cells. While positive selection to identify a progenitor population in human marrow can be used to select for cells capable of osteoblast differentiation, each of the markers that have been used to identify marrow mesenchymal populations (alkaline phosphatase [AP], Stro-1, CD29, CD49a, CD73, CD90, CD105, CD166, CD44, CD146 and CD271) may be expressed on distinct subsets of marrow mesenchymal cells. Thus, positive selection with one or more of these markers could exclude a possibly relevant cell population that may undergo important changes in various clinical conditions. In the present report, we describe the isolation and characterization of human osteoprogenitor cells obtained by depletion of bone marrow cells of all hematopoietic lineage/hematopoietic stem cells and endothelial/endothelial precursor cells (lin−/CD34/CD31−). The yield of lin−/CD34/CD31− cells from ~10 mL of bone marrow (~80 million mononuclear cells) was ~80,000 cells (0.1% of mononuclear cells). While not selected on the basis of expression for the mesenchymal marker, Stro-1, 68% of these cells were Stro-1+. Using linear whole transcriptome amplification followed by quantitative polymerase chain reaction (QPCR) analysis, we also demonstrated that, compared to lin− cells (which are already depleted of hematopoietic cells), lin−/CD34/31− cells expressed markedly lower mRNA levels for the endothelial/hematopoietic markers, CD34, CD31, CD45, and CD133. Lin−/CD34/31− cells were also enriched for the expression of mesenchymal/osteoblastic markers, with a further increase in runx2, osterix, and AP mRNA expression following in vitro culture under osteogenic conditions. Importantly, lin−/CD34/31− cells contained virtually all of the mineralizing cells in human marrow: while these cells displayed robust calcium deposition in vitro, lin−/CD34/31+ cells demonstrated little or no mineralization when cultured under identical osteogenic conditions. Lin−/CD34/31− cells thus represent a human bone marrow population highly enriched for mesenchymal/osteoblast progenitor cells that can be analyzed without in vitro culture in various metabolic bone disorders, including osteoporosis and aging.

Introduction

While considerable progress has been made in recent years in the clinical investigation of osteoporosis and other metabolic bone diseases using sophisticated imaging technologies [1,2] and serum bone biochemical markers [3], there remains an important need to better understand changes in gene expression in osteoblast/osteoblast progenitor populations in vivo in these various conditions. To this end, a number of approaches have been used, including assessment of mRNA expression in human bone biopsies [4] as well as analysis of bone marrow stromal cells following in vitro culture [5]. Each of these approaches has important strengths and limitations. For example, mRNA analysis of bone biopsy samples likely provides information on changes occurring in mature osteoblast/osteocyte populations but is confounded by the fact that the biopsy samples contain a heterogeneous population of cells, including not only osteoblasts and osteocytes, but also significant numbers of hematopoietic and endothelial cells. Bone marrow stromal cultures do represent a more homogeneous population, but the limitation of this approach is that even short term in vitro culture may alter the phenotype or gene expression profile of these cells.

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In recent studies, we have used an alternate approach that involves obtaining human bone marrow aspirates followed first by a depletion of hematopoietic lineage cells using a cocktail of antibodies (to CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a [glycophorin A]), thereby depleting the bone marrow cells of mature hematopoietic cells such as T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells [6,7]. Following this negative selection, the hematopoietic lineage negative (lin−) fraction was stained with an antibody to a mesenchymal marker, such as alkaline phosphatase (AP) [7] or Stro-1 [6]. The lin−/AP+ or lin−/Stro-1+ cells were then analyzed, without culturing, for expression of specific genes and pathways. Since the yields of RNA from these limited cell populations were relatively low for in-depth gene expression analyses, we coupled the cell isolation methods to a whole transcriptome linear amplification step that preserved the relative representation of each transcript species in the original sample during and after amplification [8,9].

While the above approach was useful and provided us insights into effects of estrogen on lin−/Stro-1+ cells [6] and PTH effects on lin−/AP+ cells [7], we recognize several limitations of these isolation methods. First, the hematopoietic cocktail did not include antibodies to CD34 or CD31. These are markers for hematopoietic stem cells or endothelial progenitor cells (CD34) [10,11] or for more mature endothelial populations (CD31) [12]. Moreover, neither AP nor Stro-1 expression is limited to mesenchymal cells [13–15]. Thus, the lin−/AP+ and the lin−/Stro-1 cells are likely still contaminated by immature hematopoietic and/or endothelial cells. Second, using positive selection to identify a progenitor population in human marrow has its own limitations, as each of the markers that have been used to identify marrow mesenchymal populations (AP, Stro-1, CD29, CD49a, CD73, CD90, CD105, CD166, CD44, CD146 and CD271 [for a review, see [16]) may be expressed on different (and sometimes mutually exclusive) subsets of marrow mesenchymal cells. Thus, positive selection with one or more of these markers may exclude a possibly relevant cell population that may undergo important changes in various clinical conditions.

Recognizing these concerns regarding positive selection for mesenchymal cells, Itoh et al. [17] recently described a method for purifying mesenchymal progenitor cells from mouse marrow using hematopoietic lineage depletion. These purified osteoprogenitors (HipOPs) were enriched for osteoblast differentiation markers and were able to differentiate into osteoblasts in vitro and in vivo. Importantly, however, ~17% of these cells expressed CD34, and the level of CD31 expression was not specified. Consistent with this, the HipOPs contained not only mesenchymal but also hematopoietic precursor cells, and it is possible that a sub-population of endothelial progenitor cells was also present in this population.

In the present report, we describe the isolation and characterization of human bone marrow lin−, CD34 and CD31 negative (lin−/CD34/31−) cells. These lin−/CD34/31− cells represent a population almost completely depleted of all hematopoietic/endothelial lineages and highly enriched for a mesenchymal progenitor population. Our results demonstrate that these cells can be isolated and analyzed without in vitro culture and may thus represent a useful population to employ in future clinical-investigative studies of osteoporosis, age-related bone loss, and other metabolic bone diseases.

Materials and methods

Experimental subjects

Bone marrow aspirates and small bone biopsies were obtained from healthy female volunteers between the ages of 29 and 83 years. For the aspirates, we used a 15 gauge needle, being careful to rotate the needle after every 2 mL of marrow aspiration in order to minimize contamination by peripheral blood. For the biopsies, we used an 8 gauge needle, attempting to obtain as much cortical bone as possible with associated trabecular elements. Exclusion criteria were: 1) use of bisphosphonates, estrogen (oral or transdermal), raloxifene, or PTH (or other bone-active drugs) in the past 3 years; 2) history of Paget's disease, other metabolic bone disease, diabetes, or significant cardiac, renal, or liver disease; 3) history of any fracture within the past 5 years; 4) hysterectomy; 5) abnormalities in the screening laboratory studies. The study was approved by the Mayo Institutional Review Board and all subjects provided written, informed consent prior to the study.

Hematopoietic lineage depletion and isolation of lin−/CD34/31− cells

Bone marrow cells were initially subjected to Ficoll gradient centrifugation for mononuclear (MNC) cell enrichment. Depletion of mature hematopoietic cells such as T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, erythroid cells, and their committed precursors was accomplished by MACS (Miltenyi) using a lineage negative selection cocktail (Miltenyi) containing biotin-conjugated antibodies to CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a (glycophorin A). The MACS sorted lin− cells were subsequently incubated with PE conjugated CD34 and CD31 antibodies (BD Biosciences) in the dark at 4 C for 30 min. The two cell populations lin−/CD34/CD31− and lin−/CD34/CD31+ were isolated by fluorochrome activated cell sorting (FACS). The fluorescence threshold was set based on excluding the highly fluorescent CD34/CD31 population, and the same fluorescence threshold for CD34/CD31 was used in all of the analyses.

Costaining for CD45 and stro-1

To determine the coexpression level of the hematopoietic cell marker CD45 in the different cell populations, lin−/CD34/CD31+ and lin−/CD34/CD31− cells were isolated as described above. In addition, MACS sorted lin+ cells were used as a comparison. The cells were incubated with a FITC conjugated monoclonal CD45 antibody for 30 min in the dark at 4 C. Coexpression for the osteoblastic marker, Stro-1, was determined by using a specific Stro-1-IgM antibody (R & D Systems) which was subsequently incubated with a FITC conjugated anti-IgM antibody (R & D Systems). The samples were run on BD FACScan and analyzed using CellQuest Pro. For CD45, the fluorescence threshold was set based on the lin+ cells, excluding the highly fluorescent CD45+ cells. Since Stro-1 did not identify a clearly distinct population of cells as positive, an IgM isotype control antibody (AbD Serotec) was used to set the fluorescence threshold.

Processing of bone biopsies

The bone biopsies were immediately placed in lysis buffer (QIAzol, Qiagen) and subjected to homogenization using a variable speed homogenizer (Tissue Tearor, Cole-Parmer).

Gene expression analysis

Total RNA from the various sorted cell populations and bone biopsies was isolated using microfuge columns (MicroColumns, Qiagen). DNase treatment to digest all genomic DNA that could lead to false positive gene expression results was done following RNA isolation using Turbo DNA-free DNase (Ambion). RNA quality and purity were confirmed with a Nanodrop spectrophomtometer (Thermo Scientific). Since the overall number of the various cell populations was limited (generally<100,000 cells) for the performance of in-depth gene expression analyses, we used the WT-OvationTM Pico RNA whole transcriptome amplification system (NuGen Technologies, Inc) to synthesize μg quantities of amplified cDNA starting with total RNA input of ~50 ng. In this linear amplification system, the relative representation of each transcript species in the original sample is maintained during and after amplification [8,9].