Bone 94 (2017) 1–9

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Hematopoietic derived cells do not contribute to osteogenesis as osteoblasts

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ARTICLE INFO

Article history:
Received 25 May 2016
Revised 26 September 2016
Accepted 6 October 2016
Available online 07 October 2016

Keywords:
Heterotopic ossification
Bone lining cells
Osteoblasts
CD45

ABSTRACT

Despite years of extensive investigation, the cellular origin of heterotopic ossification (HO) has not been fully elucidated. We have previously shown that circulating bone marrow-derived osteoblast progenitor cells, characterized by the immunophenotype CD45−/CD44+/CXCR4+, contributed to the formation of heterotopic bone induced by bone morphogenetic protein (BMP)-2. In contrast, other reports have demonstrated the contribution of CD45+ hematopoietic derived cells to HO. Therefore, in this study, we developed a novel triple transgenic mouse strain that allows us to visualize CD45+ cells with red fluorescence and mature osteoblasts with green fluorescence. These mice were generated by crossing CD45-Cre mice with Z/RED mice that express DsRed, a variant of red fluorescent protein, after Cre-mediated recombination, and then crossing with Col2.3GFP mice that express green fluorescent protein (GFP) in mature osteoblasts. Utilizing this model, we were able to investigate if hematopoietic derived cells have the potential to give rise to mature osteoblasts. Analyses of this triple transgenic mouse model demonstrated that DsRed and GFP did not co-localize in either normal skeletogenesis, bone regeneration after fracture, or HO. This indicates that in these conditions hematopoietic derived cells do not differentiate into mature osteoblasts. Interestingly, we observed the presence of previously unidentified DsRed positive bone lining cells (red BLCs) which are derived from hematopoietic cells but lack CD45 expression. These red BLCs fail to produce GFP even under in vitro osteogenic conditions. These findings indicate that, even though both osteoblasts and hematopoietic cells are developmentally derived from mesoderm, hematopoietic derived cells do not contribute to osteogenesis in fracture healing or HO.

1. Introduction

Heterotopic ossification (HO), the process of bone formation outside of the skeleton, is known to be induced following traumatic injury or surgery, as well as in genetic disorders such as fibrodysplasia ossificans progressiva (FOP). The process of HO is initiated by an inflammatory phase similar to bone regeneration in fracture healing while normal skeletogenesis during development does not require inflammation, suggesting that a distinct cellular mechanism is involved in HO. It has recently been demonstrated that cells playing an important role in HO differ from those in normal skeletogenesis [1]. The extensive research on the type and source of cells which give rise to osteoblasts in HO has provided a variety of candidates including circulating mesenchymal progenitor cells [2–5], Tie2-expressing cells [6] and GLAST-expressing cells [1]. Previously, we have reported that circulating bone marrow-derived osteoblast progenitor cells are recruited to bone-forming sites and give rise to osteoblasts [3]. These circulating osteoblast progenitor cells are positive for CD44 and CXCR4 and negative for CD45 [4], suggesting that they were derived from non-hematopoietic bone marrow cells. However, others have reported that circulating CD45+/Collagen I+ osteogenic precursor cells contribute to HO in patients with FOP [5], and that hematopoietic derived fibroblast-like cells, so-called fibrocytes, also have the potential to become osteoblasts [2,7,8]. Moreover, it has been reported that hematopoietic stem cells can generate functional osteoblasts [9,10]. Considering these findings and given that osteoblasts and blood cells are both embryonically derived from mesoderm, CD45+ hematopoietic derived cells might lose their CD45 expression

Abbreviations: HO, heterotopic ossification; GFP, green fluorescent protein; BLC, bone lining cell; BMP, bone morphogenetic protein.

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http://dx.doi.org/10.1016/j.bone.2016.10.003
8756-3282/

postnatally and contribute to HO directly by differentiating into osteoblasts. To address whether hematopoietic derived cells give rise to osteoblasts in HO, it is necessary to trace their cell fate in vivo.

The Cre/loxP system makes it possible to trace cell lineages in vivo [11] by crossing tissue-specific Cre recombinase-expressing mouse strains with floxed fluorescent reporter mice. Several different Cre lines have been utilized in the study of HO [1,6,12] including Lyz-Cre (monocyte/macrophage lineage), CD19-Cre (B cell lineage), and LCK-Cre (T cell lineage) mice to study the hematopoietic lineages in HO models [12]; however, none of these lineages directly contribute to bone formation. Since these Cre lines label differentiated lineage cells instead of hematopoietic stem/progenitor cells, alternative Cre lines which cover a majority of hematopoietic cells including primitive stem cells are needed.

All blood cells in the adult hematopoietic system originate developmentally from definitive hematopoietic stem cells (dHSCs) in the embryonic hematopoietic system. In the mouse embryo, dHSCs, characterized by a VE-cadherin +/CD45 + immunophenotype, emerge in the aorta-gonad-mesonephros (AGM) by embryonic day 11 [13,14]. Since VE-cadherin is also expressed by endothelial cells, a VE-cadherin-Cre line is not ideal to specifically label hematopoietic cells. Conversely, CD45 is recognized as a pan-leukocyte marker and its expression is limited to hematopoietic cells including dHSCs but excluding mature erythrocytes, indicating that a CD45-Cre line would specifically label hematopoietic cells. Therefore, in this study, we used CD45-Cre mice with Z/RED reporter mice in conjunction with Col2.3GFP mice, which express GFP in mature osteoblasts, and tracked hematopoietic cells to examine whether they differentiate into functional mature osteoblasts in regular skeletogenesis, a bone morphogenetic protein-2 (BMP-2) induced HO model, or a tibia fracture model.

2. Materials and methods

2.1. Mice

CD45-Cre mice were kindly gifted by Dr. Eva Mezey and were produced as previously described [15]. Briefly, Cre recombinase cDNA was inserted into the 3′ end of exon 33 of the complete murine *CD45* gene with an internal ribosomal entry site (IRES) using a bacteria artificial chromosome (BAC). C57BL/6 wild type, Z/RED [16] and Col2.3GFP [17] mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Double transgenic F1 mice were developed by breeding CD45-Cre mice with Z/RED mice. Triple transgenic F2 mice were developed by crossing CD45-Cre/Z/RED F1 mice with Col2.3GFP mice. Colonies of these strains were maintained in the vivarium in The Research Institute at Nationwide Children's Hospital. All animal protocols were approved by the Institutional Animals Care and Use Committees of The Research Institute at Nationwide Children's Hospital.

2.2. Bone-associated cell isolation and culture

Femora and tibiae were harvested from mice. Muscle and soft tissues were removed from bones carefully under a stereomicroscope. After both ends of bone were removed, bone marrow cells were flushed with phosphate-buffered saline (PBS) using a 5-mL syringe and a 27G needle. Empty bones containing the diaphysis and a part of the metaphysis were placed in conical glass vials (REACTI VIALS; Thermo Fisher Scientific, Waltham, MA) and minced into small fragments in Dulbecco's modified Eagle medium-Ham's F12 mixture (DMEM/F12; Mediatech Inc., Manassas, VA, 3.151 g of glucose per liter). During this step, bone fragments were washed with DMEM/F12 several times and the supernatants were pooled for further cell isolation. Bone granules were digested on a shaker for 120 min at 37 °C with collagenase P (0.2 mg/mL; Roche Diagnostics, Mannheim, Germany) in DMEM/F12 supplemented with 100 U/mL penicillin/streptomycin (Mediatech Inc.) and washed with DMEM/F12. Following digestion, the collagenase P solution and washing media were combined with the above pooled supernatants, and were filtered through a 40 μm cell strainer (Corning, Corning, NY). Bone-associated cells were collected by centrifugation (500*g*, 5 min). The digested bone granules were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 50 μM L-ascorbic acid (Sigma-Aldrich, St. Louis, MO), and 100 U/mL penicillin/streptomycin as previously described [18]. Bone-associated cells, especially DsRed positive cells, grew significantly better in DMEM/F12 than in Minimum Essential Medium Eagle Alpha (αMEM) as shown in Supplemental Fig. 1.

2.3. In vitro differentiation

Osteoblastic differentiation was induced by switching the culture medium to DMEM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerol phosphate (Sigma-Aldrich), and 50 μM L-ascorbic acid. After 4 weeks of osteoblastic differentiation, cells were imaged for fluorescence on an AxioObserver A1 (Carl Zeiss, Thornwood, NY) using AxioVision 4.5SP1 software (Carl Zeiss). To isolate cells from mineralized extracellular matrix, Cells were then treated with 0.25% trypsin solution (Mediatech Inc.) and 173 U/mL collagenase type I solution (Worthington Biochemical Corporation, Lakewood, NJ) at 37 °C for 60 min. Cells were collected with a cell scraper followed by centrifugation (500*g*, 5 min). After fixation with 4% paraformaldehyde in PBS (4% PFA) at 4 °C for 45 min, cells were decalcified with 15% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) in PBS at 4 °C overnight. The cells were further dissociated by collagenase type I treatment at 37 °C overnight. Cells were filtered with a 70 μm cell strainer (Corning) prior to flow cytometric analysis. The cell recovery efficiency after the filtration was 88.2 ± 11.0% of initial cell population as assessed by measuring the quantity of DNA in the cells using a QIAamp DNA Mini Kit (Qiagen, Germantown, MD; Supplemental Fig. 2).

2.4. Flow cytometry

Peripheral blood was collected from the retro-orbital sinus. Following red blood cell (RBC) lysis, blood, bone marrow cells, and bone-associated cells were analyzed on a BD FACSCalibur and a BD LSRII (BD Biosciences, San Jose, CA) using allophycocyanin (APC) conjugated anti-mouse CD45 (30-F11; BD Biosciences). CD45 −/DsRed +/GFP − cells and CD45 −/DsRed −/GFP + cells were sorted from bone-associated cells isolated from the triple transgenic mice using a BD FACSAria. The DsRed positivity in CD45-positive cells was calculated with the following formula: DsRed positivity = the percentage of CD45 +/DsRed + cells / the percentage of CD45 + cells × 100. Data were analyzed using FlowJo software version 7.6 (Tree Star, Inc., Ashland, OR).

2.5. Immunofluorescent staining and analysis

Bones were fixed in 4% PFA at 4 °C for 24 h following the removal of soft tissues. After decalcification with 15% EDTA solution for 3 weeks, bones were embedded in paraffin and cut into 6 μm sections. After deparaffinization, sections were treated with 0.25% trypsin solution for 13 min at room temperature to retrieve antigens before blocking with normal serum. These sections were incubated at 4 °C overnight with polyclonal goat anti-GFP antibody (1:200, Novus Biologicals LLC., Littleton, CO), polyclonal rabbit anti-RFP antibody (1:200, Abcam, Cambridge, MA), monoclonal rat anti-mouse CD45 antibody (30-F11, 1:100, BD Biosciences), polyclonal rabbit anti-mouse osteocalcin antibody (1:1000, Takara Bio, Japan), monoclonal rat anti-mouse F4/80 antibody (A3-1, 1: 200, AbD Serotec, Raleigh, NC), polyclonal rabbit anti-rat Cathepsin K (1:100, Abcam), monoclonal rat anti-mouse Ter119 antibody (TER-119, 1:100, BD Biosciences), monoclonal rat anti-mouse CD3 (RM0027-3B19, 1:200, Abcam), monoclonal rat anti-mouse CD45R antibody (RA3-6B2, 1:200, BD Biosciences), monoclonal rat anti-mouse Gr-1 (RB6-8C5, 1:200, AbD Serotec) and/or monoclonal rat anti-mouse CD11b (M1/70, 1:50, Abcam). Subsequently, sections were stained