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Analyzing the cellular contribution of bone marrow to fracture healing using bone marrow transplantation in mice

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Abstract

The bone marrow is believed to play important roles during fracture healing such as providing progenitor cells for inflammation, matrix remodeling, and cartilage and bone formation. Given the complex nature of bone repair, it remains difficult to distinguish the contributions of various cell types. Here we describe a mouse model based on bone marrow transplantation and genetic labeling to track cells originating from bone marrow during fracture healing. Following lethal irradiation and engraftment of bone marrow expressing the *LacZ* transgene constitutively, wild type mice underwent tibial fracture. Donor bone marrow-derived cells, which originated from the hematopoietic compartment, did not participate in the chondrogenic and osteogenic lineages during fracture healing. Instead, the donor bone marrow contributed to inflammatory and bone resorbing cells. This model can be exploited in the future to investigate the role of inflammation and matrix remodeling during bone repair, independent from osteogenesis and chondrogenesis.

Keywords: Bone marrow transplantation; β-Galactosidase; Fracture repair; Mouse; Inflammation; Osteoblast; Osteocyte; Chondrocyte; Osteoclast

The essential role of the bone marrow during fracture healing is well recognized. Mechanical disruption of the bone marrow, by aspirating cells in the marrow cavity and/or reaming the endosteum, delays healing. This delay may be achieved by removing the mesenchymal stem cells that may participate in osteogenesis and chondrogenesis, and by impinging on the formation of the hematoma, which supplies cytokines and growth factors in the early stages of repair [1–4]. Indeed, the bone marrow provides niches for both hematopoietic and mesenchymal stem cells [5,6].

Hematopoietic stem cells give rise to inflammatory cells, which invade the fracture site during the early stages of healing. Several studies have demonstrated the importance of inflammation during bone repair, however, the role of the inflammatory response in regulating cartilage and bone differentiation remains underestimated [7–10]. Osteoclasts are also derived from the hematopoietic lineage and are recruited during the early stages of healing [11–13]. Previous studies have focused on their role in cartilage and bone remodeling in the callus [12,14–16]. Yet, the interactions between matrix forming and matrix degrading cells during fracture repair remain to be further investigated.

Mesenchymal stem cells, including skeletal stem cells, appear to reside in the stromal compartment of the bone marrow [17,18]. Mesenchymal stem cells placed in culture can give rise to various cell types, including chondrocytes and osteoblasts [19,20]. When combined with grafting materials, mesenchymal stem cells transplanted in critical size defects in animal models can improve healing [21,22]. This osteogenic capacity of the stromal compartment of the bone marrow is largely utilized in humans. Iliac crest autologous bone grafts in combination with bone allografts are currently performed to treat fractures with a decreased chance of healing [23]. These results indicate that the bone marrow has the potential to provide osteoblasts and

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chondrocytes during bone healing. In vivo studies have also shown that transplanted mesenchymal stem cells can participate in the formation of the fracture callus and particularly in osteogenesis, however, the role of the stromal compartment of the bone marrow compared to the hematopoietic compartment remains to be further elucidated [24,25]. Here, we have developed an in vivo model using bone marrow transplantation in mice, where we can specifically assess the cellular contribution of the hematopoietic compartment of the bone marrow during fracture healing. After transplanting genetically labeled bone marrow cells into lethally irradiated wild type mice, we carefully analyzed the localization of labeled bone marrow-derived cells in the fracture callus. Our results reinforce the primary role of the hematopoietic compartment of the bone marrow in regulating inflammation and matrix remodeling during fracture healing.

Materials and methods

Irradiation and bone marrow transplantation. All procedures followed protocols approved by the UCSF IACUC. Host C57B6 wild type mice (8–10-week-old males; $n = 25$ mice) were given Neomycin sulfate (2 mg/ml) in sterile drinking water 2 days before, and for 14 days following, irradiation. Neomycin water was changed daily. Host mice received two lethal irradiations in a 3 h interval (300 rad from an X-ray source) and bone marrow transplantation from donor mice, 3 h following the second dose of radiation.

Bone marrow was isolated from wild type mice carrying a *LacZ* transgene that resulted in the constitutive expression of β-galactosidase (β-gal, Rosa26 mice). Six to eight-week-old male Rosa26 donor mice from the same genetic background as host mice (C57B6) ($n = 13$ Rosa26 mice) were anesthetized and euthanized by cervical dislocation. Tibias, femurs, and humeri were collected sterily and flushed in RPMI 1640 medium. Cells were centrifuged, incubated in red blood cell lysing buffer (Sigma, R7757), washed in RPMI 1640 medium, and re-suspended in Hanks' BSS (HBSS) (10^7 cells/200 μl). Bone marrow cells (200 μl, 10^7 cells) were injected in the tail vein of host-irradiated wild type mice using a 27 g needle. To accomplish this, mice were placed in a restraining device and the tail placed in warm water (37–40 °C) to allow dilation of the tail vein. Mice were monitored daily for weight loss and infection. Mice recovered for a minimum of 6–8 weeks before undergoing tibial fracture. We refer to these hosts as BMT mice, to distinguish them from positive control (Rosa26; $n = 12$ mice) or negative control (wild type; $n = 11$ mice) mice that have not undergone either X-irradiation or bone marrow transplantation.

Creating non-stabilized fractures. Following anesthesia, a closed, standardized non-stable fracture was produced in the mid-diaphysis [12,26]. Mice were sacrificed at days 0, 5, 7, 10, 14, 21, and 28 post-surgery ($n = 25$ BMT mice; $n = 11$ wild type negative control mice and $n = 12$ Rosa26 positive control mice).

Tissue processing, histological, and immunohistological analyses. Mice were euthanized under anesthesia and tibias were collected free of skin at various time points following surgery. Tissues were fixed for 24 h at 4 °C in a 0.2% glutaraldehyde solution containing 2 mM $MgCl_2$, 5 mM EDTA, and 0.02% NP40 in PBS, decalcified at 4 °C in 19% EDTA, pH 7.4, for 10–14 days, equilibrated in 30% sucrose in PBS for 24 h at 4 °C, and cryo-embedded. The calluses were cryo-sectioned longitudinally (10 μm thick). To detect the distribution of bone marrow-derived cells expressing β-galactosidase, one tissue section every tenth was post-fixed in the 0.2% glutaraldehyde solution, washed three times for 15 min in a solution containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP40 in PBS, stained overnight in wash solution containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-D-β-galactoside), 2.1 mg/ml potassium ferrocyanide, 1.64 mg/ml potassium ferricyanide, and 20 mM Tris–HCl, pH 7.3, and counterstained with eosin. Blue X-gal staining indicated which of the cells were derived from the donor bone marrow. Adjacent sections were stained with Safranin-O/Fast Green to detect the formation of cartilage and trichrome to detect the formation of bone in the callus [12]. Double staining for β-galactosidase activity and Tartrate resistant acid phosphatase (TRAP) activity was performed using X-gal staining and a leukocyte acid phosphatase kit (Sigma, St. Louis, MO) to identify matrix-resorbing osteoclasts derived from the donor bone marrow [12]. Sections were lightly counterstained with eosin. To detect bone marrow-derived inflammatory cells, sections were double stained with X-gal and inflammatory cell specific antibodies: a polyclonal rat anti-mouse F4/80 antibody (eBioscience, San Diego, CA) was used to visualize cells from the monocyte lineage, including macrophages and osteoclast precursors and a polyclonal rat anti-mouse neutrophil antibody (Serotec, Raleigh, NC) to localize neutrophils. Following X-gal staining, sections were immersed in 5% H_2O_2/PBS for 5 min, washed in PBS, and then incubated in 5% goat serum for 30 min. Sections were incubated overnight at 4 °C in primary antibody, washed in PBS, blocked in 5% sheep serum for 30 min, and incubated for 1 h at room temperature in biotinylated anti-rat IgG (BD PharMingen, San Diego, CA). Slides were washed in PBS and incubated in horseradish peroxidase-conjugated streptavidin (Amersham, Cleveland, OH). Bound antibody was revealed by incubation in a diaminobenzidine solution. Sections were lightly counterstained with eosin.

Results

Engraftment of donor Rosa26 bone marrow after bone marrow transplantation

Although an intact bone marrow is important for normal bone healing [1,20,27], its contribution to fracture healing has not been entirely defined. To distinguish marrow-derived cells from cells of the surrounding soft tissue or the periosteum during healing, we performed lineage analysis by isolating bone marrow from wild type mice carrying a ubiquitously expressed *LacZ* transgene (Rosa26 mice). Rosa26 marrow was transplanted into lethally irradiated wild type mice. Following engraftment and reconstitution of the marrow (6–8 weeks), a non-stabilized tibial fracture was created and analyzed at various time points post-fracture. While all tissues exhibited positive-X-gal staining in Rosa26 control mice (Fig. 1A, D, and G), no donor bone marrow-derived cells were detected in the growth plate (Fig. 1B), cortical bone, periosteum or muscles in BMT mice (irradiated and bone marrow transplanted; Fig. 1E). Conversely, the majority of marrow cells in BMT mice stained with X-gal indicating successful bone marrow engraftment (Fig. 1B and H).

Absence of bone marrow-derived cells in chondrogenic and osteogenic lineages during fracture healing

By day 10 post-fracture, no X-gal-positive chondrocytes were detected in the BMT, and wild type fracture calluses (Fig. 2B, C, E, and F). Chondrocytes were X-gal-positive in Rosa26 callus demonstrating that chondrocytes were not derived from the donor bone marrow in BMT mice. We did not observe the presence of X-gal-positive osteoblasts and osteocytes in BMT calluses at day 10 and up to day 28 post-fracture (Fig. 2G–L) indicating that the