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Impaired function of bone marrow stromal cells in systemic mastocytosis

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Abstract
Patients with systemic mastocytosis (SM) have a wide variety of problems, including skeletal abnormalities. The disease results from a mutation of the stem cell receptor (c-kit) in mast cells and we wondered if the function of bone marrow stromal cells (BMSCs; also known as MSCs or mesenchymal stem cells) might be affected by the invasion of bone marrow by mutant mast cells. As expected, BMSCs from SM patients do not have a mutation in c-kit, but they proliferate poorly. In addition, while osteogenic differentiation of the BMSCs seems to be deficient, their adipogenic potential appears to be increased. Since the hematopoietic supportive abilities of BMSCs are also important, we also studied the engraftment in NSG mice of human $CD34^+$ hematopoietic progenitors, after being co-cultured with BMSCs of healthy volunteers vs. BMSCs derived from patients with SM. BMSCs derived from the bone marrow of patients with SM could not support hematopoiesis to the extent that healthy BMSCs do. Finally, we performed an expression analysis and found significant differences between healthy and SM derived BMSCs in the expression of genes with a variety of functions, including the WNT signaling, ossification, and bone remodeling. We suggest that some of the symptoms associated with SM might be driven by epigenetic changes in BMSCs caused by dysfunctional mast cells in the bone marrow of the patients.

Introduction

Mast cells express c-kit (CD117), which is the stem cell factor (SCF) receptor. When this receptor is constitutively activated due to genetic mutation(s), an increase in mast cell (MC) proliferation occurs and leads to the disease systemic

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mastocytosis (SM) (Carter et al., 2014). More than 90% of patients with SM carry the somatic D816V activating mutation in the *KIT* gene (Nagata et al., 1997). The over proliferation of MCs in the bone marrow (BM) results in an alteration in the expression of additional genes (D'Ambrosio et al., 2003) and mediators including histamine within the bone marrow and thus a change in the immediate environment of bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells or MSCs) that are responsible for generating cartilage, bone and marrow adipocytes in BM during bone turnover and repair, as well as supporting hematopoiesis.

A number of pathologic features of SM suggest a possible link between specific disease findings and a role for BMSCs. These include the well-documented skeletal disease in patients with SM (Delsignore et al., 1996; Rossini et al., 2014), and abnormalities in the cellular composition of the BM including anemia (George and Horny, 2011). As we have previously established that the human BMSCs bear all four histamine receptors (Nemeth et al., 2012), we wanted to examine if the increase in MCs might be associated with abnormalities of the BMSC compartment which might in turn impact disease pathology.

Methods

Patients

Patients with SM were evaluated at the National Institutes of Health (NIH; Bethesda, MD) as part of Institutional Review Board (IRB)-approved research protocol designed to study the pathogenesis of SM (NCT00044122) and were diagnosed according to the World Health Organization (WHO) criteria. KIT *D816V* mutation analysis was performed described (Maric et al., 2007). Healthy controls participated under the IRB approved protocol NCT01071577. The biopsy and aspirates were obtained from the same affected site of the patients. Neoplastic mast cell "free" aspirates were not obtained. Although of scientific interest, obtaining these samples would have been technically challenging and would have subjected the patients to repeated bone marrow aspirations.

Culturing of BMSCs

Bone marrow aspirates were diluted according to cell counts and we plated 0.25–0.5 × 10^6 nucleated cells/cm^2 in 75-cm^2 culture flasks (patient samples did not always have this number of cells available) and incubated the cells in complete medium for 24 h in a 37 °C, 5% CO_2 incubator. The medium was changed every 3 days and cells were passaged before reaching confluency. To isolate normal and abnormal colonies the patients' BMSCs were grown in a flask and monitored daily. When the colonies were large enough to determine their nature, the top of the flask was removed and the normal and abnormal looking colonies were isolated and seeded again in separate culture plates.

Since the abnormal colonies grew very slowly and stopped growing very early, we had to pool the abnormal colonies to have enough cells for experiments to perform. For more details see Nemeth et al. (2013).

Proliferation studies of BMSCs

BMSCs were serum starved in a basal medium for 72 h. 5000 human serum starved BMSCs were plated in 96-well plates/200 μL each well. After an overnight incubation the serum free medium was changed to either 20% serum medium or basal medium containing 10 ng/mL FGF-2. Twenty-four hours later BrdU was added at a final cc of 10 μM. After another 12 h cells were fixed with fix-perm buffer and the next steps were done according to the Roche BrdU ELISA protocol.

Beta-galactosidase (β-gal) staining of expanded BMSC

BMSCs were stained using a senescence associated beta-gal staining kit (Sigma Aldrich, St. Louis, MO) following the manufacturer's protocol. Briefly, BMSCs were washed with PBS, fixed with a fixation buffer for 7 min at room temperature, washed with PBS, and then incubated with a staining mixture at 37 °C overnight. The senescent cells under 10 randomly selected high-magnitude microscope fields (100×) were counted on the following day.

Primary colony-forming efficiency (CFE) and measurement of $CD34^+$ cells of bone marrow aspirate

Primary CFE of bone marrow aspirates was analyzed by methods as reported previously (Sabatino et al., 2012). Briefly, 1 × 10^5 nucleated cells were plated in a T25 flask (Corning, Corning, NY) in duplicate and cultured for 13 days without changing the culture medium. The colonies were fixed with methanol for 30 min and stained with saturated methyl-violet water solution for 20 min. Colonies were observed under a low magnitude light microscope field (25×). Colonies containing 50 or more cells of fibroblastic morphology were counted (Sabatino et al., 2012).

The percentage of $CD34^+$ hematopoietic progenitor cell (HPC) was determined by following standardized protocols in the Cell Processing Section (CPS), Department of Transfusion Medicine, Clinical Center of NIH. Briefly, 1 × 10^6 nucleated cells were incubated with CD34 APC, CD3 PE-Cy7, 7AAD and CD45 APC-Cy7 (BD Biosciences, San Jose, CA) for 20 min at 4° and then lysed in a lysis washing assistant equipment (BD Biosciences). The $CD34^+$ percentage was measured on a FACSCanto cytometer (BD Biosciences) (Sabatino et al., 2012; Ren et al., 2013).

Population doubling (PD) curve of expanded BMSC

PD was calculated to analyze the proliferation of BMSC. The number of BMSCs at each passage was manually counted and the PD for each passage was calculated using equation: N = log2 (NH/N1) where N = population doublings, N H = cell harvest number, N I = plating cell number, and the cumulative PDs were calculated in relation to the number of cells at the first passage (Sabatino et al., 2012; Ren et al., 2013).