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Research paper

Leukemia cells impair normal hematopoiesis and induce functionally loss of hematopoietic stem cells through immune cells and inflammation

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ABSTRACT

Bone marrow (BM) failure is often seen in leukemia patients, indicating an abnormal hematopoietic process. However, hematopoiesis in leukemic milieus is largely unknown. In the present study, we utilized one of the most frequent leukemogenic translocations MLL-AF9 to induce leukemia and investigated the hematopoiesis and the activity of hematopoietic stem and progenitor cells (HSPCs) in a leukemic milieu. We found that the phenotypes of the non-leukemic population in leukemic BM were drastically different than normal BM, including blockage of differentiation and a drastically reduced Lin-/Sca+/c-kit+ (LSK) population that contains all HSPCs in leukemic BM. Further, transplantation assays demonstrated that stem cell function of HSPCs from leukemic BM was significantly compromised. Intriguingly, BM from a patient-derived xenograft leukemia model and from immunocompromised mice transplanted with murine MLL-AF9 cells, showed comparable percentage of hematopoietic stem cells (HSCs) to normal controls, indicating that an immunocompetent microenvironment is critical for leukemia-induced loss of HSPCs. Mechanistically, we found that the non-leukemic cells from leukemic BM possessed a more inflammatory profile than either leukemic cells or normal BM counterparts. Co-culturing or co-transplantation with non-leukemic cells from leukemic BM impaired the stem cell function of normal HSPCs in vitro and in vivo respectively, suggesting that the highly inflammatory non-leukemic population in leukemic BM not only is functionally abnormal but displayed a 'leukemia-like' characteristic. Finally, we tested the effect of the anti-inflammation drug diclofenac on leukemia mice. However, no phenotypic changes of HSPCs were observed upon diclofenac treatment due to only mild repression of inflammatory genes by diclofenac, further indicating that inflammation is a powerful negative regulator of HSPCs. Together, our results suggest that leukemia impairs normal hematopoiesis and inflammation as well as immune cells play a critical role in leukemia-induced BM failure.

1. Introduction

The function of hematopoietic stem and progenitor cells (HSPCs) are essential for a steady state of hematopoiesis. Abnormalities in HSPCs lead to bone marrow (BM) failure or malignant transformation. Therefore, the activity of HSPCs is tightly regulated and closely monitored. Biological disturbance to HSPCs such as dysregulation of cell cycle, which could be triggered by inflammation, results in exhaustion of HSPCs [1].

BM failure is often observed in leukemia patients and is one of the leading causes for leukemia-related death. Thus, there seems to be a causal relation between the malfunction of HSPCs and leukemia-induced BM failure. However, the biological activity of HSPCs and hematopoietic process in leukemic milieus remains largely unknown. Studies using immunocompromised mice transplanted with human leukemia samples indicate functional preservation of HSPCs in leukemic BM [2]. However, since the activity and function of HSPCs are highly regulated and maintained by immune cells and BM microenvironment [3,4], utilization of immunocompromised mice whose immune system and microenvironment are differing than immunocompetent mice, may not fully reflect the hematopoiesis in leukemic milieus. Therefore, using immunocompetent models is required for this line of investigation.

MLL translocations are found in leukemias across all ages especially in childhood leukemias [5,6]. Patients with MLL translocations are usually classified into an intermediate to poor risk groups [7,8]. In the present study, we explored the hematopoiesis in a MLL-AF9 translocation induced leukemia model. MLL-AF9 is one of the most frequent MLL rearrangements especially in childhood acute myeloid leukemia (AML) [5]. We found severely impaired hematopoiesis in leukemic BM. We

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further demonstrated stem cell function of non-leukemic compartment was significantly compromised. Additionally, we found that the non-leukemic compartment had a more inflammatory profile than leukemia cells and might result in defected HSPCs. Interestingly, we observed unaltered percentage of HSCs in immunocompromised mice transplanted with either human leukemia samples or murine MLL-AF9 leukemia cells, suggesting that the immune microenvironment was involved in leukemia-induced impairment of hematopoiesis.

2. Material and methods

2.1. Generation of leukemia models

MLL-AF9 leukemic model was generated by transplantation of Sca+/c-kit+/lin- (LSK) cells infected with GFP-MLL-AF9 retrovirus into normal recipients (C57BL/6J). In this study, a secondary leukemic transplantation was generated to study the hematopoiesis in a leukemic milieu. Briefly, LSK cells were isolated from normal BM by fluorescence-activated cell sorting (FACS) and cultured overnight in LSK medium (10% FBS IMDM containing 10 ng/ml IL3, 10 ng/ml IL6, 50 ng/ml SCF, 50 ng/ml Flt3-ligand). MLL-AF9 retrovirus was then introduced to LSK cells the following day. One day after infection, LSK cells were injected into recipient mice retro-orbitally. Primary leukemia cells from spleen and bone marrow (BM) were frozen for future usage. To generate secondary leukemia, GFP+ cells from primary leukemia were isolated by FACS and transplanted into unirradiated recipient mice (1 million cells/mouse).

To generate MLL-AF9 leukemia in NOD scid gamma (NSG) mice, GFP+ cells from primary leukemia were isolated and transplanted into unirradiated naïve NSG mice (1 million cells/mouse).

AML patient sample derived xenograft model was created by transplantation of AML patient samples to NOD scid gamma (NSG) mice (5 million cells/mouse). Recipients were sacrificed at 6 weeks post transplantation.

All animal experiments were approved by Cangzhou medical College.

2.2. In vivo BrdU labeling (Cell cycle analyses)

BrdU incorporation experiments were performed as described previously [9,10].

2.3. In vivo drug treatment

Starting at day 6 after leukemic transplantation, leukemia mice were treated with diclofenac (10 mg/kg/day, Sigma) intra-peritoneally. Treatment was stopped one day before sacrificing the mice.

2.4. Colony forming assay

Equal number of lin-/GFP- cells were sorted from leukemic and normal BM and resuspended in methylcellulose complete media (R&D system) (2000 cells/plate). Numbers of colonies were counted at day 10 after culturing. For 2nd plating, cells were recovered from methylcellulose media and equal number of cells (10000 cells/plate) were resuspended in methylcellulose complete media. Numbers of colonies were counted at day 10 after culturing.

2.5. HSPCs transplantation

Equal number of lin-/GFP- cells were sorted from leukemic and normal BM. Cells were then injected into lethally irradiated recipient mice (1 million cells/mouse). Survival of recipients was monitored.

2.6. Flow cytometry

All antibodies used for this study were purchased from BD biosciences. Analyses were performed using FACSCelesta (BD biosciences) and Fluorescence-activated cell sorting were done by FACSAria II sorter (BD biosciences).

2.7. Competitive transplantation and LSK cells coculturing

A CD45 chimerism system was utilized to generate leukemia for following competitive transplantation. Briefly, primary leukemia cells (GFP+ cells on a CD45.1 background) were transplanted into unirradiated recipient mice (CD45.2 background) to generate secondary leukemias. GFP- (CD45.2+) cells from leukemic or normal BM (CD45.2+) were isolated and mixed at 1:1 ratio with CD45.1+ cells isolated from normal BM respectively. Cell mixtures were injected into lethally irradiated recipient mice (1 million cells/mouse). Another group of recipients were transplanted with only CD45.1+ cells isolated from normal BM at a half cell dose (0.5 million cells/mouse).

For LSK cells coculturing experiment, GFP- (CD45.2+) cells from leukemic or normal BM (CD45.2+) were isolated and mixed at 10:1 ratio with CD45.1+ LSK cells isolated from normal BM respectively. Cell mixtures were cultured in LSK medium (10% FBS IMDM containing 10 ng/ml IL3, 10 ng/ml IL6, 50 ng/ml SCF, 50 ng/ml Flt3-ligand) for 10 days, and CD45.1 cells were isolated after coculturing and resuspended in methylcellulose complete media (50000 cells/plate). LSK cells cultured alone were served as control. Colony numbers were counted at 10 days post methylcellulose culturing.

2.8. ELISA analysis

Equal number of normal BM cells, GFP- (non-leukemia) cells and GFP+ (leukemia) cells were isolated and cultured in 10% FBS IMDM for 24 h (1 million cells/ml). Culturing medium was collected and spin down to get rid of cell debris. Supernatant was subjected to ELISA analysis per manufacturer's instruction (R & D system).

2.9. Statistical analysis

Two-tailed student's *t*-test with unpaired analysis was performed for relevance between biological factors. $P < 0.05$ was considered significant.

3. Results

3.1. Abnormal phenotypes of the non-leukemia population in the MLL-AF9 leukemia BM

To explore hematopoiesis in a leukemic milieu, we first examined the phenotype of the non-leukemic compartment in a MLL-AF9 translocation induced leukemia model. We transplanted primary MLL-AF9 leukemic cells into unirradiated mice to generate secondary leukemias and explored the hematopoiesis in these secondary leukemia mice. The expression of MLL-AF9 and thus the leukemia cells were monitored by green fluorescent protein (GFP) in this model [11]. We confirmed that MLL-AF9 was exclusively expressed in the GFP+ population not in the GFP- population (Supplemental Fig. 1).

We observed that the percentage of lineage- (lin-) cells in the non-leukemic (GFP-) compartment was significantly increased (Fig. 1A and B) when the leukemic burden was about 40% in BM (Supplemental Fig. 2A), indicating blockage of the normal hematopoietic differentiation. Cell cycle analyses showed that lin-/GFP- cells were more actively cycling than their counterparts in normal BM (Fig. 1C and D), which corroborated the expansion of lin-/GFP- cells in leukemic BM. Additionally, we analyzed the composition of lin+ population and found that the percentage of B cells was remarkably decreased while the

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