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Research paper

RUNX1-EVI1 induces dysplastic hematopoiesis and acute leukemia of the megakaryocytic lineage in mice

Yuka Nakamura[a], Motoshi Ichikawa[a], Hideaki Oda[b], Ieharu Yamazaki[d,c], Ko Sasaki[a], Kinuko Mitani[a,*]

[a] Department of Hematology and Oncology, Dokkyo Medical University School of Medicine, Tochigi, Japan
[b] Department of Pathology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan
[c] Department of Molecular Pathology, Tokyo Medical University, School of Medicine, Tokyo, Japan
[d] Department of Electron Microscope, BML Research Institute Inc., Saitama, Japan

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ABSTRACT

The *RUNX1-EVI1* gene generated by the t(3;21) translocation encodes a chimeric transcription factor and is a causative gene in the development of *de novo* acute megakaryoblastic leukemia and leukemic transformation of hematopoietic stem cell tumors. Heterozygous *RUNX1-EVI1* knock-in mice die *in utero* due to hemorrhage in the central nervous system and spinal cord and complete abolishment of definitive hematopoiesis in the fetal liver. On the other hand, the chimeric knock-in mouse develops acute megakaryoblastic leukemia. We created another mouse model of *RUNX1-EVI1* using transplantation of retrovirus-infected bone marrow cells. Some mice transplanted with *RUNX1-EVI1*-expressing bone marrow cells developed acute megakaryoblastic leukemia within eight months, and the other non-leukemic mice showed thrombocytosis at around a year. In the non-leukemic mice, dysplastic megakaryocytes proliferated in the bone marrow and frequently infiltrated into the spleen, which was not associated with marrow fibrosis. In the leukemic mice, their tumor cells were positive for c-kit and CD41, and negative for TER119. Although they were negative for platelet peroxidase in the electron microscopic analysis, they had multiple centrioles in the cytoplasm, which are characteristic of megakaryocytes that undergo endomitosis. The leukemic cells were serially transplantable, and gene-expression analyses using quantitative RT-PCR arrays revealed that they showed significantly elevated expression of stem cell, primitive hematopoietic cell and endothelial cell-related genes compared with normal bone marrow cells. All these data suggested that *RUNX1-EVI1* caused dysplastic hematopoiesis or leukemia of the megakaryocytic lineage and endowed gene expression profiles distinctive of immature hematopoietic cells.

1. Introduction

The t(3;21)(q26;q22) translocation is a recurrent chromosomal abnormality found in chronic myelogenous leukemia in blastic crisis, myelodysplastic syndromes (MDS)-derived leukemia and *de novo* acute megakaryoblastic leukemia (AMKL) [1]. The translocation creates the *RUNX1-EVI1* fusion gene whose resultant molecules contain the N-terminus of RUNX1 and almost the entire region of EVI1 [2]. RUNX1-EVI1 exhibits two main molecular functions [3]. One is a dominant-negative function over normal RUNX1 function, and the other is an aberrant EVI1 expression leading to repression of TGFβ signaling [4], inhibition of CEBPA [5] and stimulation of AP-1 activity [6]. We previously reported phenotypes of *RUNX1-EVI1* knock-in mice [7,8]. Heterozygous *RUNX1-EVI1* embryos died *in utero* as a result of hemorrhage in the central nervous system and lack of definitive hematopoiesis in the fetal liver [7], which macroscopically mimicked phenotypes of *RUNX1* knock-out embryos [9,10], while a chimeric knock-in mouse developed AMKL [8]. To further understand the nature of hematopoietic diseases caused by *RUNX1-EVI1*, we generated another mouse model by retroviral transduction and transplantation.

2. Methods

RUNX1-EVI1 cDNA [2] was subcloned into MSCV-IRES-GFP retroviral vector [11]. To produce *RUNX1-EVI1* virus, Plat-E packaging cells [12] were transfected with retroviral constructs using FuGENE6

* Corresponding author at: Department of Hematology and Oncology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi 321-0293, Japan.
E-mail address: kinukom-tky@umin.ac.jp (K. Mitani).

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Table 1
Phenotypes of the mice transplanted with mock or RUNX1-EVI1-positive retrovirus-transduced bone marrow cells.

ID	Donor Ly5 isotype	Latency, months	Body weight, g		Spleen weight, mg		WBC count, /μl		Hemoglobin, g/dL		Platelet count, x10^4/μl		% BM donor chimerism		% GFP+/ donor derived	
			mock	R/E	mock	R/E	mock	R/E	mock	R/E	mock	R/E	mock	R/E	mock	R/E
B	Ly5.2	8	ND	ND	43.5	188.0	7900	2,200	15.1	8.1	ND	ND	0	46.1	0	2.9
D	Ly5.1	11	24.5	25.0	70.3	103.5	7600	1,100	12.3	14.2	62.1	74.2	10.5	6.6	2.21	2.2
G	Ly5.1	12	21.7	19.6	46.2	66.4	3000	10,200	14.2	13.6	85.1	144.2	1.98	10.4	9.22	0.7
J2	Ly5.1	11	19.4	21.3	48.6	52.3	15500	9,900	14.9	14.8	40	95.1	8.75	21.3	4.71	1.9
S1	Ly5.2	6	20.2	16.4	56	177.0	8100	4,900	14.1	6.3	33.8	9.4	1.77	82.9	53.7	66.3
S2	Ly5.2	7	21.6	12.6	54.8	61.1	11100	2,500	14.1	2.1	14.6	10.4	12.48	71.9	19	83.5

Mice (C57BL/6 (Ly5.2) and B6.SJL (Ly5.1)) were transplanted with bone marrow cells from mice with different Ly5 antigens (B6.SJL and C57BL/6, respectively and designated as "Donor Ly5 isotype") along with supporting bone marrow cells with the same Ly5 antigens of the recipient mice, and were killed for analysis at specified latencies. Bone marrow donor chimerisms were analyzed by flow cytometry using antibodies against the Ly5 antigens of the transduced cells, and cells expressing the gene on the retrovirus were detected as GFP- and donor Ly5-antigen-positive cells. Mock: retrovirus expressing GFP only, R/E: retrovirus expressing RUNX1-EVI1 fusion protein and GFP. ND: not determined.

transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) as described previously [13].

Mouse bone marrow (BM) transplantation was performed using C57BL/6-Ly-5.1/Ly-5.2 congenic mouse system, using Ly-5.2 mice as donors and Ly-5.1 mice as recipients, or vice versa. Cell isolation from BM and infection with *RUNX1-EVI1*-expressing retrovirus were performed as described previously [13]. Briefly, BM mononuclear cells were isolated from the femurs and tibias of donor mice 5 days after intraperitoneal administration of 150 mg/kg 5-fluorouracil (Sigma-Aldrich, St Louis, MO) and lineage-negative (Lin^-) BM cells were obtained by depletion with the use of Mouse Lineage Cell Depletion kit (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions. After 18 h of pre-stimulation culture in the medium [Iscove's modification of Dulbecco's medium (IMDM) supplemented with 10% FBS, 100 ng/ml mouse stem cell factor (PeproTech, Rocky Hill, NJ), 6 ng/ml mouse interleukin-3 (PeproTech) and 10 ng/ml mouse interleukin-6 (PeproTech)], Lin^- BM cells were transduced with retrovirus constructs using Retronectin (Takara Bio, Japan) according to the manufacturer's instructions. Then, after 48 h of transduction in the same medium, 0.5 to 2.0 × 10^6 of infected BM cells were injected through tail vein into recipient mice which had been administered a lethal dose of 9.5 Gy total-body X-ray irradiation. Infection efficiency (frequency of the GFP + cells using flow cytometry) was 19.1 ± 17.8 (s.d., n = 9) % for mock virus (transplanted GFP + cells: 9.6 to 38.2 ± 35.6 × 10^4 cells) and 5.3 ± 7.4 (s.d., n = 9) % for *RUNX1-EVI1*- expressing virus (transplanted GFP + cells: 2.7 to 10.6 ± 14.4 × 10^4 cells). For the lethally irradiated mice, a radioprotective dose of 2 × 10^5 of BM cells from mice with the Ly5 isotype identical with the recipients was simultaneously injected. Serial transplantation was performed that secondary and tertiary recipient mice received 0.5 to 2.0 × 10^5 BM cells from primary and secondary diseased mouse with 2.0 × 10^5 competitor cells.

Blood counts were monitored intermittently, and moribund mice were killed and morphologically examined using standard protocols. Peripheral blood (PB) was obtained from the saphenous vein and blood cells were counted by Automatic Blood Cell Counter (ERMA INC, Tokyo, Japan). PB, BM, and spleen smears were stained with Wright-Giemsa stain solution (Wako, Japan) for evaluation of cell morphology. Tissues were fixed in 20% buffered formalin, embedded in paraffin, sectioned, and followed by staining with hematoxylin and eosin (H&E) or silver impregnation. For ultrastructural studies, BM and spleen cells were treated for the platelet peroxidase reaction and examined with a JEOL 1200CX electron microscope. For flow cytometry, red blood cells were lysed by using Ammonium Chloride Lysing Reagent (BD Biosciences, San Jose, CA) in PB or single-cell suspensions of BM and spleen. Cells were then stained with fluorescent-dye-conjugated monoclonal antibodies against mouse antigens from BioLegend : PerCP/Cy5.5-conjugated Mac-1 (M1/70), Gr-1 (RB6–8C5), B220 (RA3–6B2), CD3 (145–2C11), Ter119, CD31 (MEC13.3); PE/Cy7-conjugated CD41 (MWReg30); APC-conjugated c-Kit (2B8); PE-conjugated CD45.2 (104), and analyzed using FACSAria (BD Biosciences). For analyses on gene expression profiles, mouse BM cells from the serially transplanted leukemic mice and control C57BL/6 mice were collected, and total RNA was extracted using the RNeasy Mini RNA extraction kit (QIAGEN). Extracted RNA was then analyzed using the RT^2 Profiler PCR array system (PAMM-054ZA, PAMM-405ZA, and PAMM-013ZA, QIAGEN) in the BM cells according to the manufacturer's instructions [13]. All animal studies were approved by the Animal Care Committee of Dokkyo Medical University School of Medicine.

3. Results

The transplanted mice exhibited two distinct phenotypes. Some transplanted mice (#B, D, G and J2) developed dysplastic megakaryopoiesis with a tendency to increases in platelet counts ($P = 0.299$, paired *t*-test) in the PB at around a year and the others (#S1 and S2) died of AMKL within 8 months.

Three of the non-leukemic mice (#B, #G and #J2) showed platelet counts around or over 100 × 10^4/μl, while all of them revealed platelet aggregation in the blood smear (Table 1 and Fig. 1A, B). One of them (#B) was associated with severe anemia and two (#B and #D) with leukocytopenia. The #B mouse indicated polychromatophilic red blood cells in the blood smear (Fig. 1B), indicating dysbiosynthesis of hemoglobin. In contrast, dysplasia in mature granulocytes was unclear in either of #B or #D. In the BM, an increase in dysplastic megakaryocytes was observed in all the mice (Fig. 1C, D). The fraction of CD41-positive cells was larger in the RUNX1-EVI1-expressing BM of #G than control marrow (Fig. 1E, F). Their megakaryocytes were markedly enlarged and had more immature nuclei with abnormal chromatin clumping and lobulation (Fig. 1D, G, H). Marked dyserythropoiesis including uneven mitosis, karryorrhexis and nuclear fragmentation was also detected in #B (Fig. 1I). Despite less prominently, all the other mice also had dysplastic erythroblasts in the BM. Reticulin staining revealed no fibrosis in the BM of either mouse (Fig. 1J, K). The spleen was enlarged in #B and #D, but not in #G and J2, compared to normal spleen. Structure of the white pulp became dispersed in the enlarged spleens (#B and #D), while maintained in the normal-sized ones (#G and #J2) (Fig. 1L, N and data not shown). Interestingly, irrespective of the size, dysplastic megakaryocytes and erythroblasts were infiltrated into the white pulp of the spleen in all the diseased mice (Fig. 1O). However, the infiltration of erythroblasts was more massive in #B and #D than #G and #J2, relating to the disappearance of the white pulp (Fig. 1N).

In contrast to the mice without apparent leukemic phenotype, both of the leukemic mice (#S1 and #S2) had pale liver and marked splenomegaly (Fig. 2B). They revealed pancytopenia in the PB including severe anemia (Table 1). The BM in the *RUNX1-EVI1*-expressing mice