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Quantification of granulocyte-macrophage colony-stimulating factor hypersensitivity in juvenile myelomonocytic leukemia by ^{3}H-thymidine assay

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Abstract

Recent molecular studies have revealed that the GM-CSF/RAS signaling pathway plays a central role in the pathogenesis of juvenile myelomonocytic leukemia (JMML). CFU-GM colony assay is an important test for GM-CSF hypersensitivity in patients with JMML, but requires specific skills. We established a simple and easy quantification method to test GM-CSF hypersensitivity, using a ^{3}H-thymidine assay. With this quantification method, JMML patients with *RAS* mutations showed significantly higher GM-CSF sensitivity than JMML patients with PTPN11 mutations. This method will be useful not only in the diagnosis of JMML, but also to evaluate the difference of GM-CSF sensitivity among patients.
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1. Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare clonal disorder with both myelodysplatic and myeloproliferative features, representing only 2% of childhood leukemia and mainly affecting young children [1–3]. It is imperative to distinguish JMML from other diseases at the onset due to JMML's poor prognosis and requirement of aggressive treatment, including allogeneic stem cell transplantation [3–7]. Recent molecular studies have shown that granulocyte-macrophage colony-stimulating factor (GM-CSF)/RAS signaling pathway plays a central role in its pathogenesis [8–10]. Although these molecular studies have been of great importance for the diagnosis of JMML, analysis of GM-CSF hypersensitivity has still been important for disease diagnosis.

GM-CSF hypersensitivity has been evaluated by colony assay from JMML CD34^{+} cells in semi-solid medium. JMML CD34^{+} cells show an exaggerated growth response to low dose GM-CSF, which has no effect on control cells [11]. Conventional colony assay for GM-CSF hypersensitivity involves counting the number of colony-forming units granulocyte-macrophage (CFU-GM) from CD34^{+} cells of JMML patients on Days 10–14. In patients with JMML, the number of colonies is increased, and the size of each colony is larger than that in normal individuals [12]. We sometimes face difficulty because morphology of JMML CFU-GM is different from that of normal CFU-GM. A specific technique is required to evaluate the conventional colony assay for JMML. Thus, a

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simple and easy objective quantification method for GM-CSF hypersensitivity has to be investigated.

We established an objective quantification system for GM-CSF sensitivity using ^{3}H-thymidine assay and CD34^{+} cells obtained from JMML patients showed significantly higher proliferation with low dose GM-CSF than those from normal volunteers.

2. Materials and methods

2.1. Patient characteristics and samples

Eleven patients with JMML diagnosed between May 2005 and April 2007 were enrolled in this study. Clinical and laboratory data are shown in Table 1. This study was approved by the ethical committee of Nagoya University Graduate School of Medicine. Informed consent was obtained from the patients' guardians.

All eleven patients were diagnosed as JMML, according to the International JMML Working Group Diagnostic Criteria in 1998 [13]. All patients met laboratory criteria: lack of the Philadelphia chromosome, peripheral blood monocyte count $>1 \times 10^{9}\,l^{-1}$, and bone marrow blasts <20%. All except one patient showed splenomegaly. Additionally, all patients met at least two laboratory findings: myeloid precursors on peripheral blood smear and white blood cell count $>10 \times 10^{9}\,l^{-1}$.

Age at diagnosis ranged from 2 to 48 months (median 15 months). There were 5 boys and 6 girls. All patients showed peripheral blood blasts <20%. Patient no. 6 showed aortic and pulmonary stenosis with hypertrophic cardiomyopathy, all of which are considered concomitant with Noonan syndrome [14]. We diagnosed patient no. 6 with Noonan syndrome, as was later confirmed by the presence of a Noonan syndrome-specific mutation of *PTPN*11 [15]. There were no patients with clinical neurofibromatosis. Cytogenetic analysis revealed normal karyotype in 9 patients and monosomy 7 in 2 patients.

Fresh bone marrow (BM, $n = 8$) and peripheral blood (PB, $n = 3$) samples at diagnosis were obtained from the eleven JMML patients and were used for the following analyses. Normal samples of BM ($n = 6$) and granulocytic colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells (PBSC; $n = 3$) were also taken from 9 healthy, hematologically normal volunteers, after obtaining informed consent from all individuals.

2.2. Cell preparation

Mononuclear cells were isolated from each stem cell source by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation, washed twice, and resuspended in RPMI medium 1640 (Gibco, Peisley, UK) with 10% fetal bovine serum (JRH Bioscienses, Lenexa, KS). CD34^{+} cells were separated from each sample, using an autoMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec), according to manufacturers' instructions. Quality of CD34^{+} cells was evaluated using FACS Calibur (BD Biosciences, Heidelberg, Germany) with phycoerythrin(PE)-conjugated anti-human monoclonal antibodies specific to CD34 antigen (BD Pharmingen, San Diego, CA). Median purity was 98.9% (range: 92.8–99.7%).

2.3. Colony assay

CD34^{+} cells isolated from BM or PB were plated at concentrations of $1 \times 10^{3}\,ml^{-1}$ in 0.9% semi-solid methylcellulose medium (Methocult H4230; Stem Cell Technologies, Vancouver, BC, Canada), supplemented with or without 0.01–100 ng/ml of human recombinant GM-CSF (R&D, Minneapolis, MN). Final GM-CSF concentrations were set for 0, 0.01, 0.1, 1, 10 and 100 ng/ml. A volume of 1 ml of this mixture was placed in 35-mm Petri dishes in duplicate at 37°C in humidified air with 5% CO_2. Colonies were counted on Days 7 and 14 under an inverted microscope.

Table 1
Patient characteristics

Patient no.	Age (month)	Sex	WBC ($\times 10^{9}\,l^{-1}$)	Mono ($\times 10^{9}\,l^{-1}$)	Splenomegaly (cm)	HbF (%)	Cytogenetics/gene mutations
1	19	F	16.2	2.0	2	10.2	Monosomy 7
2	12	M	50.1	4.3	6	2.0	(−)
3	17	M	13.7	1.7	5	5.1	*NRAS* (G13D)
4	48	F	20.1	1.4	7	30.5	*PTPN*11 (A72D)
5	15	F	18.4	1.4	4	4.4	*NRAS* (G12S)
6[a]	3	M	24.8	3.2	0.5	57.2	*PTPN*11 (N308D)
7	2	M	79.3	11.1	6.5	25.1	*NRAS* (G12D)
8	39	M	22.6	1.1	5	19.0	*PTPN*11 (E76G)
9	29	F	56.2	4.4	3	55.0	*PTPN*11 (E76K), monosomy 7
10	15	F	56.2	4.5	4	20.8	*PTPN*11 (E76A)
11	15	F	20.8	5.0	3	3.5	(−)

WBC = white blood cell, mono = monocyte count.
[a] Patient with Noonan syndrome.

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