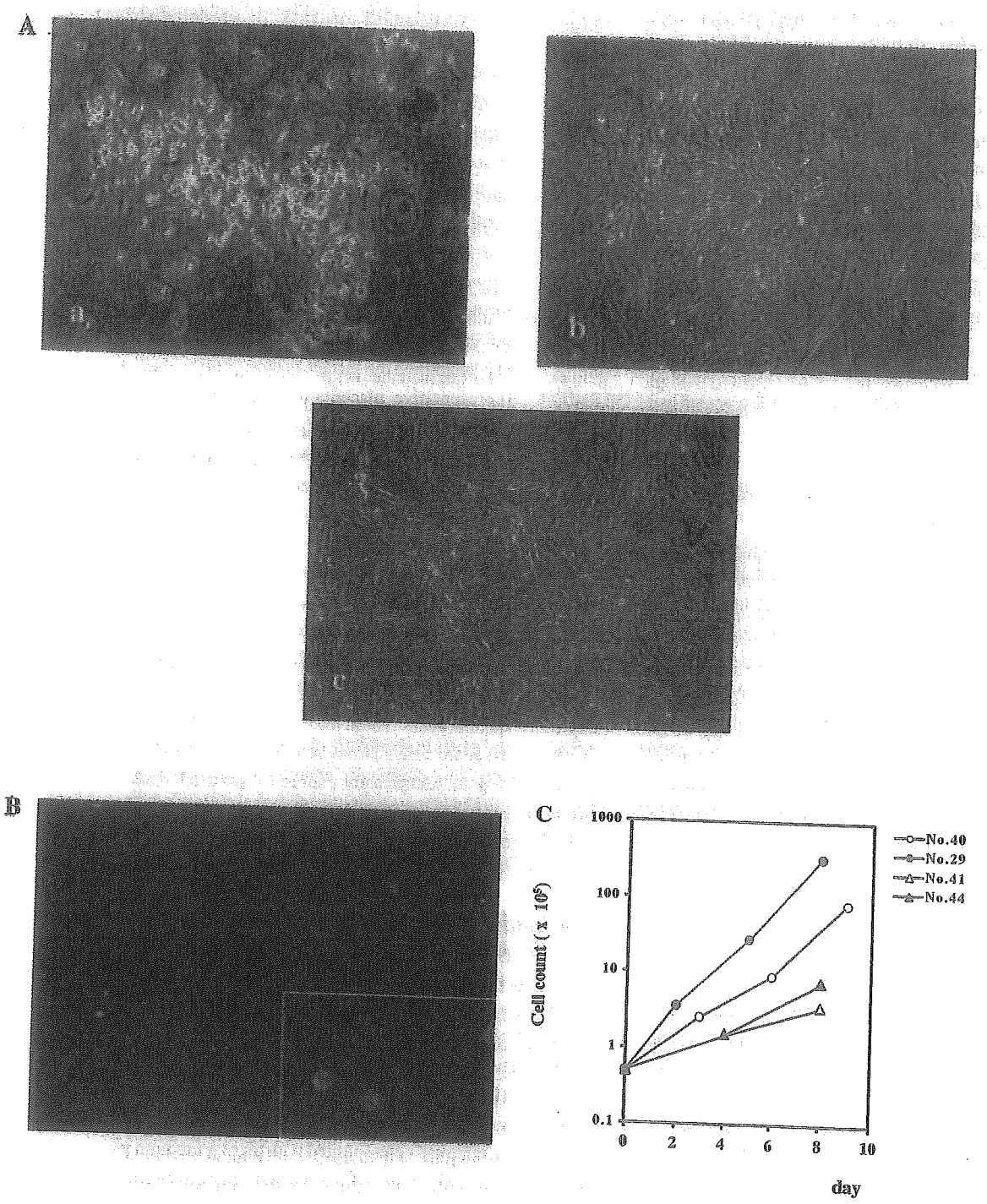
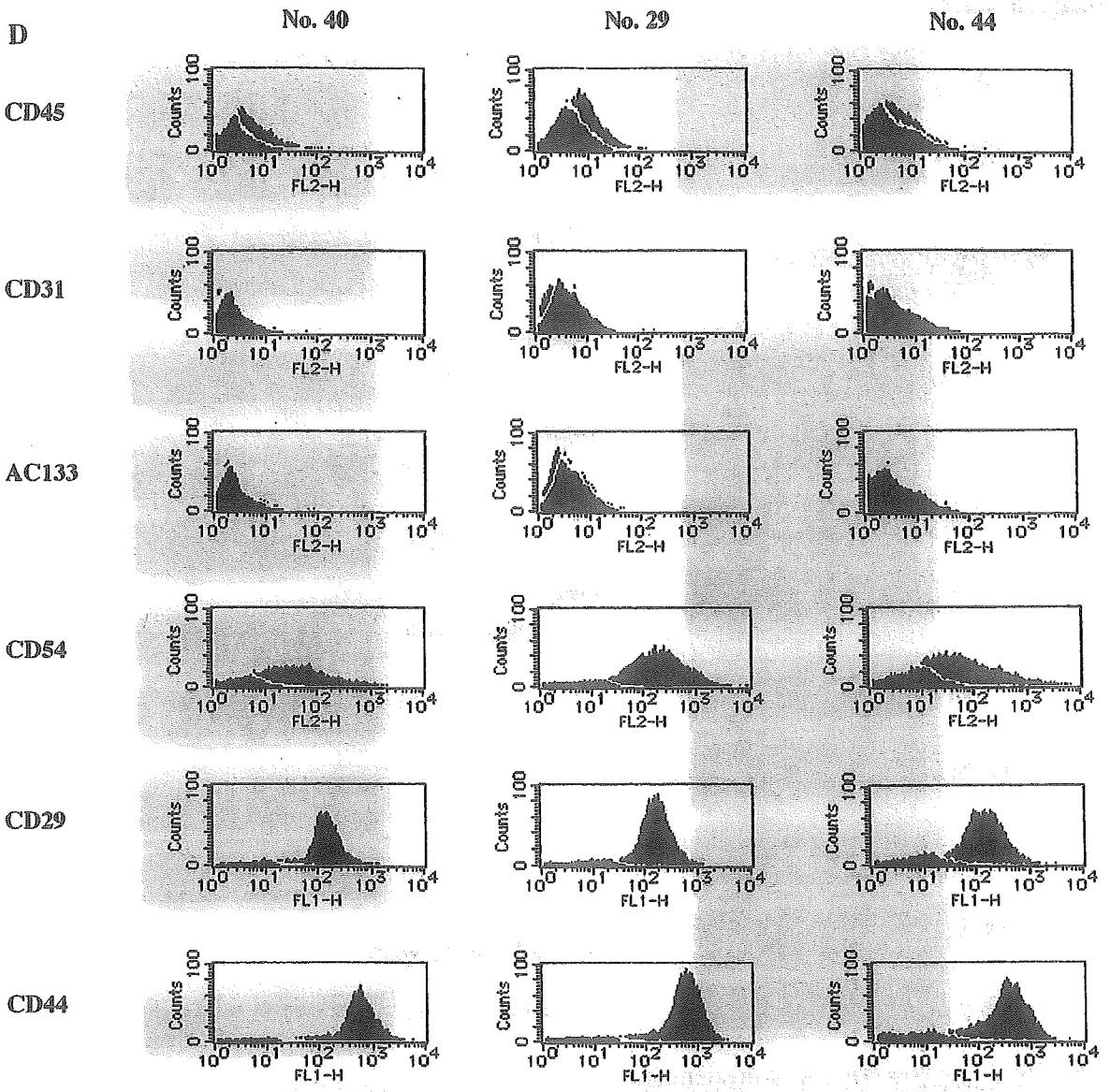


Figure 1. Isolation and characterization of placenta-derived cells. **(A):** Morphology of placenta-derived cells. Cells from a single-cell suspension easily expanded through the formation of colony-forming unit fibroblast–like colonies. a: 10 days after isolation (× 100 magnification); b: 3 weeks after isolation (× 40 magnification); c: 6 weeks after isolation (passage 3; × 40 magnification). **(B):** Fluorescence in situ hybridization analysis for human X/Y chromosomes. Cells from male placenta have Y-positive (green) and X-positive (orange) signals. **(C):** Growth curve of placenta-derived cells. Frozen and thawed cells ($n = 4$, started at passage six or seven) were seeded at 0.5×10^5 cells per well and cultured until 90% confluence was reached. These cells were resuspended, enumerated, and reseeded at the same density for 10 days. **(D):** Immunophenotype of placenta-derived cells. Cells were stained with phycoerythrin-conjugated or fluorescein isothiocyanate–conjugated antibodies against CD45, CD31, AC133, CD54, CD29, CD44, or immunoglobulin isotype control antibodies. Cells were analyzed using fluorescence-activated cell sorter Calibur. Individual placenta-derived cells were given serial numbers of placenta isolation. Representative samples were used for these figures. **(Figure 1 D continued on next page.)**



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Discussion

In this study, we successfully isolated placenta-derived cells from human term placentas ($n = 57$) and then characterized morphology, cell-surface antigens, gene expression patterns, and differentiation capacity of these cells. Results of RT-PCR analysis of 15 individual placenta-derived cells showed that the expression patterns of seven genes (HOXB4, CD34, AC133, flk-1, Tie-2, GATA-4, and myogenin) varied but expressions of 14 other genes were quite similar (Fig. 2

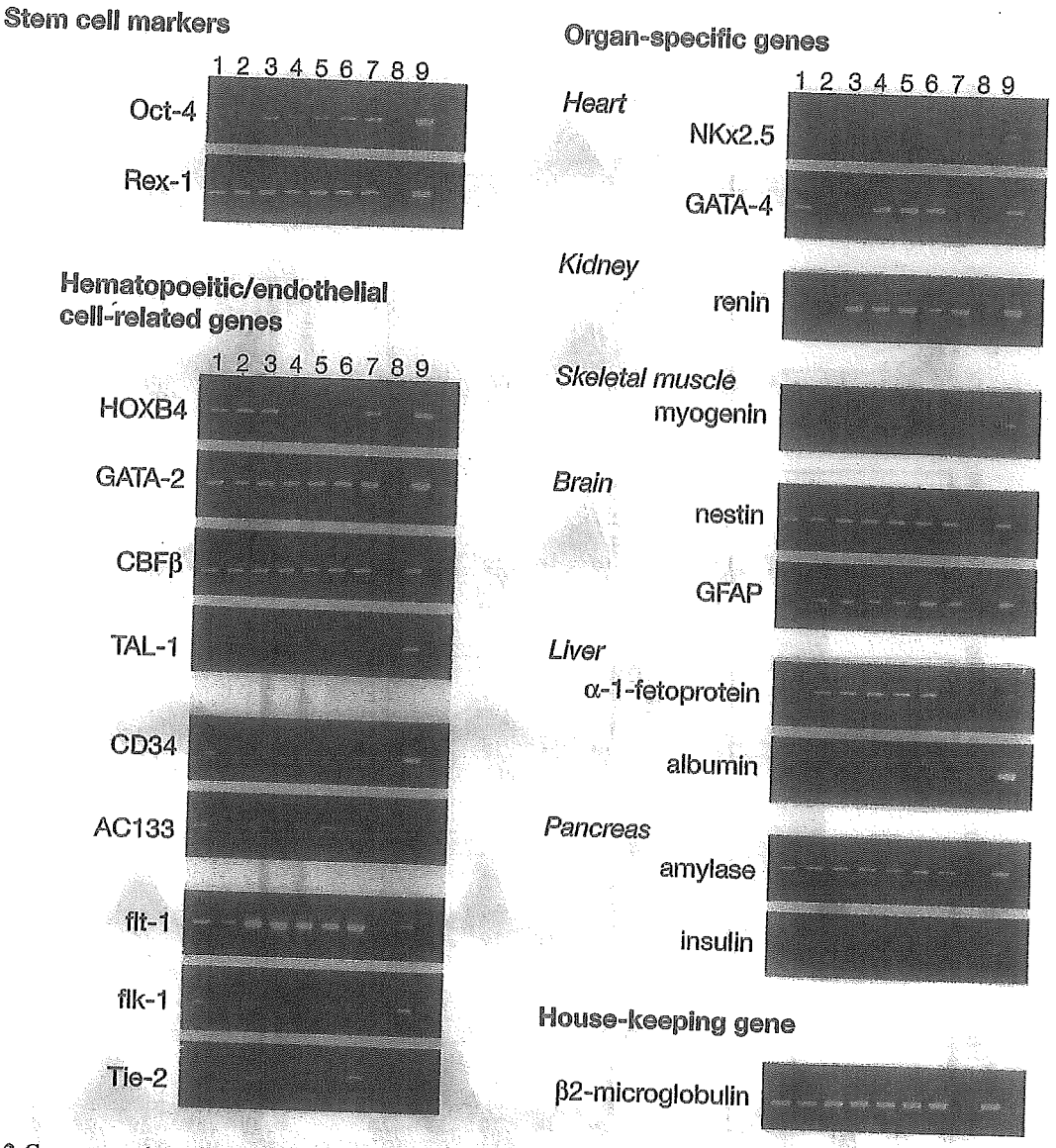
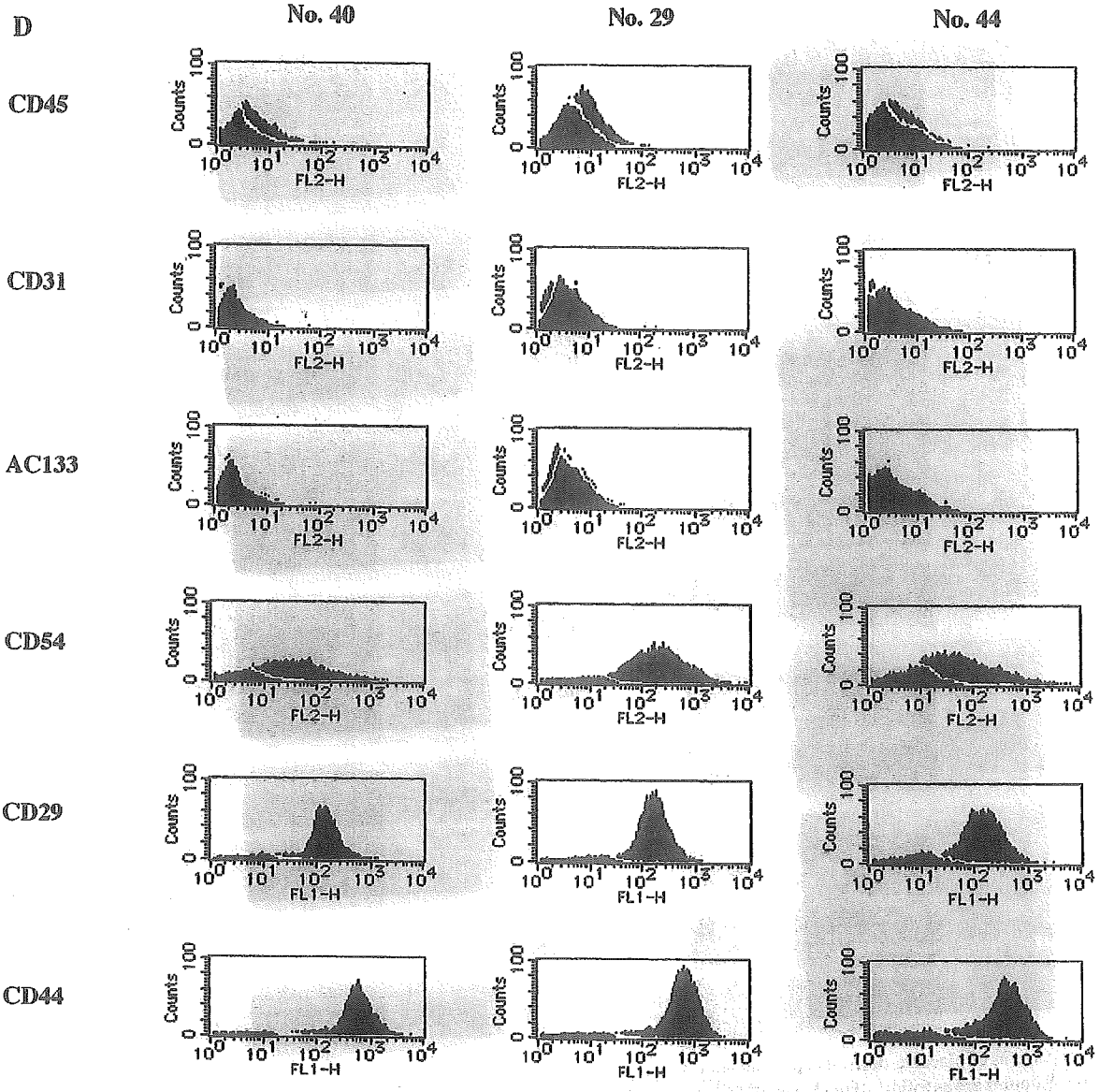


Figure 2. Gene expression patterns of placenta-derived cells. Placenta-derived cells were characterized using reverse transcription–polymerase chain reaction. Samples are as follows: lane 1, noncultured placenta (trypsin-digested residue); lane 2, human bone marrow–derived mesenchymal stem/progenitor cells; lanes 3–7, placenta (Nos. 29, 41, 42, 44, and 40)-derived cells; lane 8, reagent control; lane 9, positive control (i.e., Oct-4, Rex-1, HOXB4, and β2-microglobulin were used for EoL-3. GATA-2, CBFβ, TAL-1, CD34, AC133, flk-1, and flt-1 were used for TF-1. NKx2.5 and GATA-4 were used for human heart RNA. Renin and myogenin were used for human kidney RNA and skeletal muscle RNA, respectively. Nestin and GFAP were used for human brain RNA. α-1-fetoprotein and albumin were used for human liver RNA. Amylase and insulin were used for human pancreas RNA). In this figure, we took up the data from the five representative placenta-derived cells, and each of placenta-derived cells was shown by serial numbers of placenta isolation.

shows evidence of six placenta-derived cells; nine are not shown). These expression patterns resemble those of hBM-derived MSCs, except for renin and flt-1 (Fig. 2, lane 2). Comparison of the two types of placenta-derived cells with distinct growth characteristics (one that propagates more than 20 passages [Fig. 1C, Nos. 40 and 29] and the other with growth limitation [Nos. 41, 42, and 44]) showed that the expressions of HOXB4, CD34, Tie-2, and GATA-4 were different among these groups. The former more resembled the hBM-derived MSCs for gene expression patterns (Fig. 2). Collectively, these results indicate that the placenta-derived cells have MSC-like gene expression patterns. In addition, they showed a differentiation capacity toward both osteoblasts and adipocytes (Fig. 3), suggesting that these cells have MSC-like differentiation potential.

Because the original culture of 57 placenta-derived cell lines should be a mixture of a variety of cell types, we attempted to subclone these cells to do a detailed analysis.



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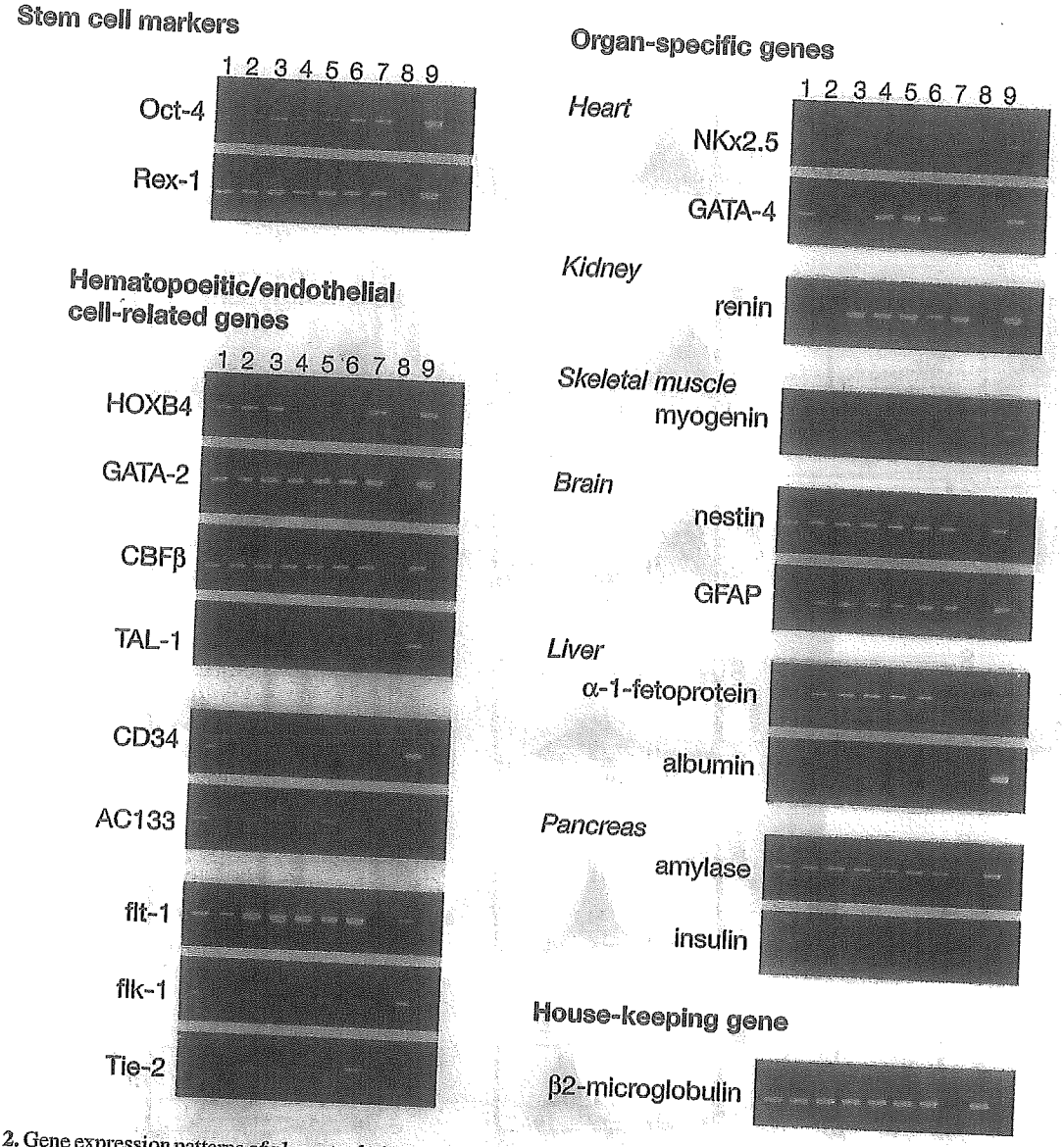

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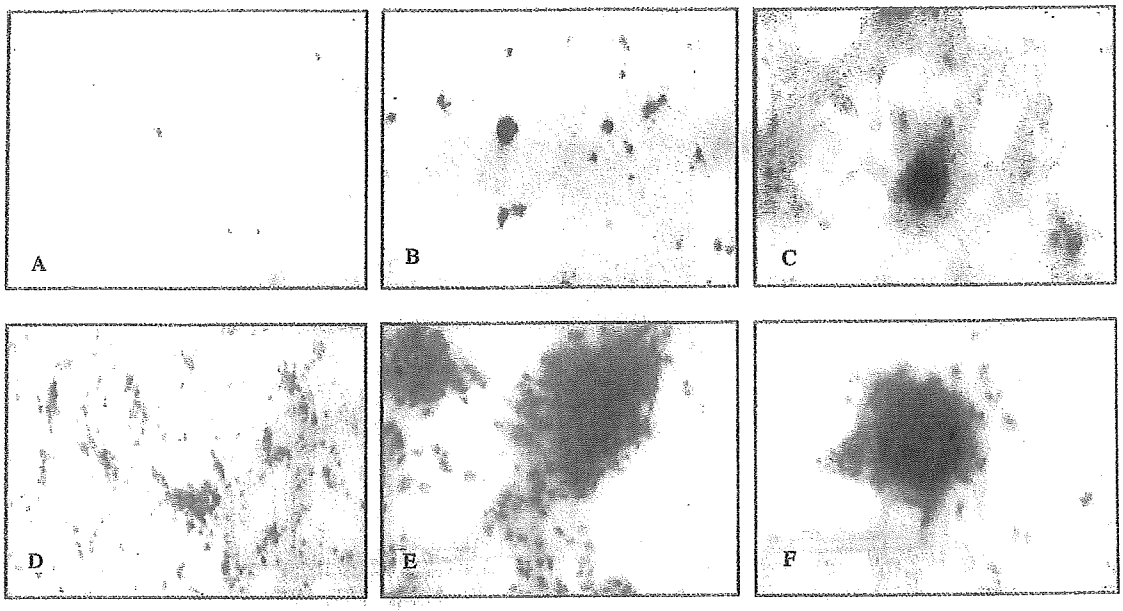

Figure 3. Differentiation potential of placenta-derived cells. After a 2-week culture in osteogenic (B, C) or adipogenic (E, F) medium or regular medium (A, D), each of the placenta-derived cells was evaluated for osteogenic or adipogenic differentiation using specific staining and hematoxylin counterstaining. Magnification: A, B, D, E, ×40; C, F, ×100. A representative sample was used for this figure.

Two established clones retained almost all of the phenotypes of parental No. 40 placenta-derived cells, including morphology, cell-surface populations, gene expression patterns, and differentiation capacity. However, these clones also had some differences in mRNA expression, such as CD34 and α-1-fetoprotein. These genes were upregulated compared with the parent mixture cells (Figs. 2, 4C). In some reports, small proportions of hMSCs expressed low levels of CD34 [6, 25]. Further experiments are required to determine the meaning of expressions of these genes.

Rex-1 is known to be important for maintaining undifferentiated embryonic stem cells [26, 27]. However, the role of this gene in MSCs is not clear. The result of RT-PCR analysis showed that Rex-1 is expressed in both BM-derived MSCs and placenta-derived cells (Fig. 2) but not in the two clones (Fig. 4C). Analysis of parental placenta-derived cells at various time points during passages (passages 3, 5, 9, and 18 for original cells; passages 13 and 26 for GFP-labeled mixture cells) using RT-PCR showed that only Rex-1 expression switched from positive (before passage 5, Fig. 2) to negative (after passage 9; data not shown). Additional analysis is required to know the role of Rex-1 in placenta-derived cells.

Interestingly, cell-surface markers analyzed using FACS revealed that the placenta-derived mixture cells and clones had the $CD45^{low}CD31^{-}AC133^{-}CD54^{+}CD29^{+}CD44^{+}$ phenotype (Figs. 1D, 4B), and the expression of CD45 and AC133 antigens differed from MSCs derived from other sources [2, 7, 10, 24]. As for the expression of AC133, the results were negative with FACS yet positive with RT-PCR analysis. This contradictory finding may be due to a damaged AC133 epitope by trypsin treatment of the cells. As for the expression of CD45, some reports showed that unprocessed or fresh MSCs were $CD45^{med,low}$, whereas cultured MSCs and more mature cells were $CD45^{-}$ [24, 28]. However, as our results showed, the expression of CD45 was low during passages. The $CD45^{low}$ phenotype might be one of the specific characteristics of the placenta-derived cells.

This study showed that the placenta-derived MSC-like cells could be easily isolated and expanded without morphological and characteristic changes in medium supplemented only with FBS. Therefore, the placenta may prove to be an attractive and rich source of MSCs. Further studies are required to better understand the precise nature of placenta-derived cells and to explore their potential clinical applications.

Acknowledgments

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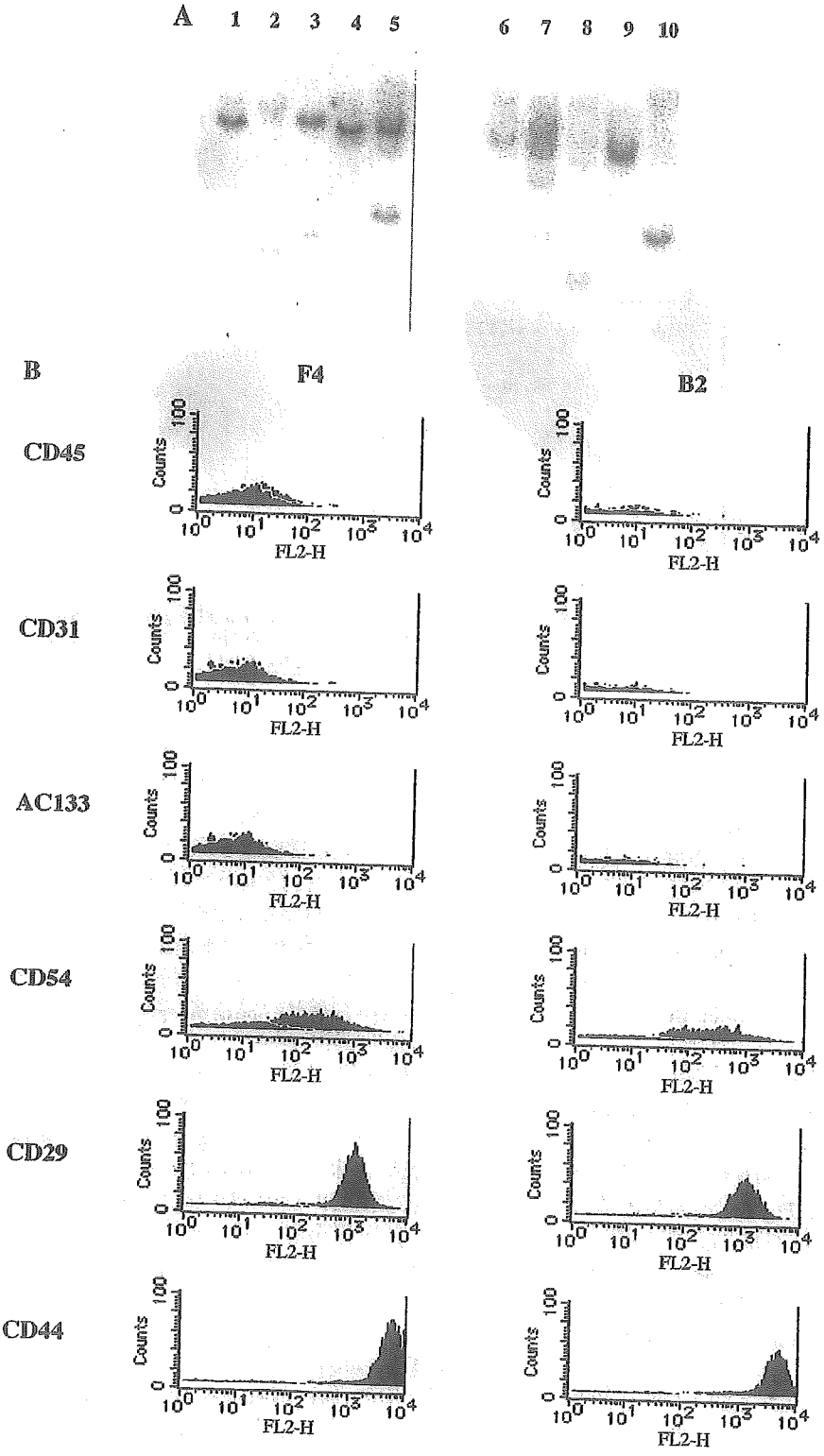
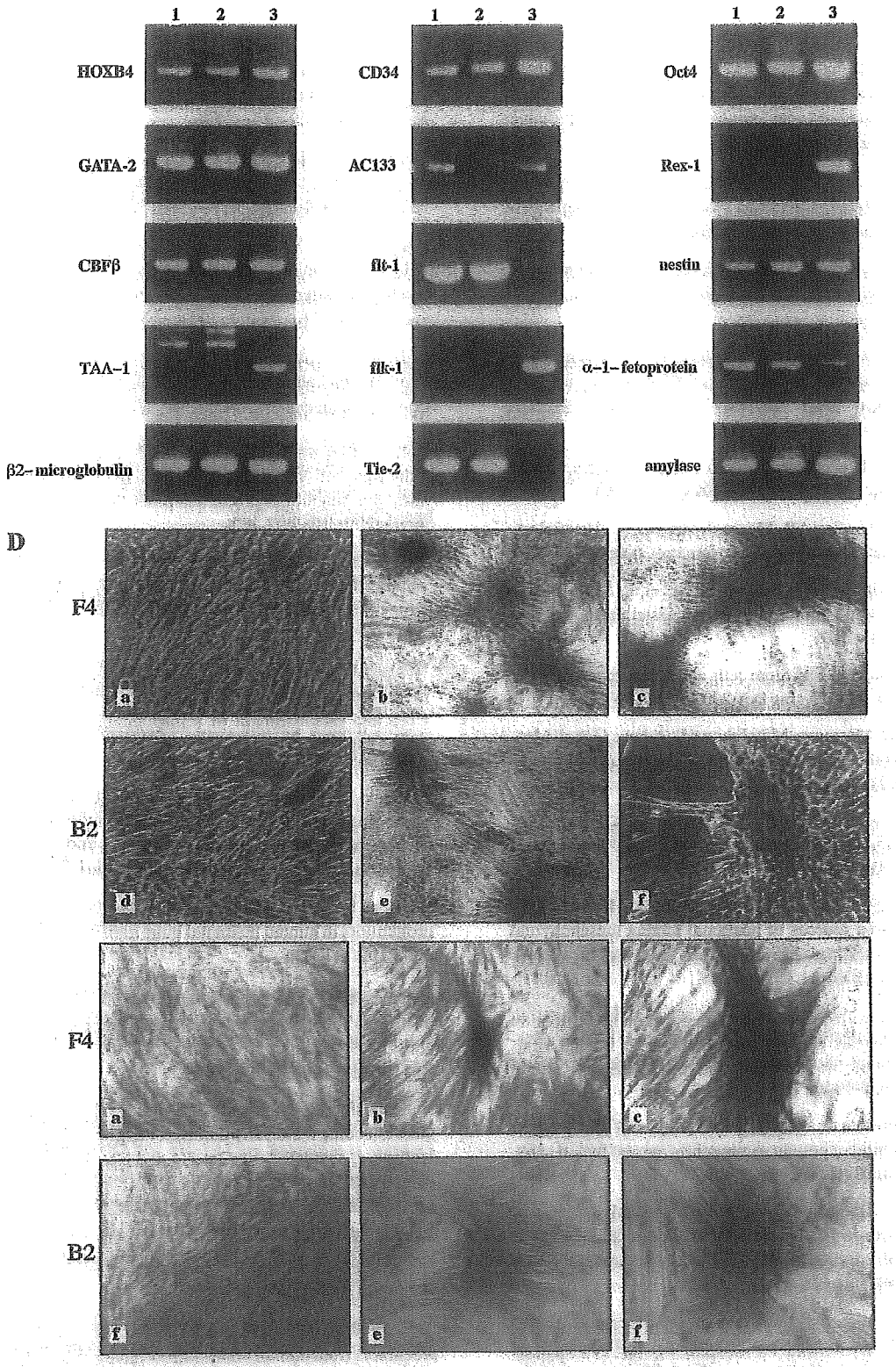


Figure 4. Establishment and characterization of two clones from No. 40 placenta-derived cells. **(A):** Establishment of placenta-derived clones. No. 40 placenta-derived cells were transduced with MSCV-IRES-GFP retrovirus, and green fluorescent protein (GFP)-positive population was sorted by fluorescence-activated cell sorting, then replated onto a 96-well dish at 5 or 10 cells per well and expanded. DNAs from these GFP-positive No. 40 placenta-derived subclones were digested overnight with BamHI (cut only once in the MSCV-IRES-GFP plasmid), and fragments were separated by electrophoresis and probed with a 32P-labeled GFP cDNA probe. Samples are as follows for lanes 1-6, 8, and 9: subclones B2, B4, D2, D3, E4, G3, F1, and F4, respectively. These subclones were obtained from sub-cloning of five cells per ell. Lanes 7 and 10, subclones E4 and G4. (Figure 4C and D continued on next page.) **(Figure 4 C and D continued on next page.)**



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Viral Infections in Juvenile Myelomonocytic Leukemia: Prevalence and Clinical Implications

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Objectives: Viral infections may complicate the diagnosis of juvenile myelomonocytic leukemia (JMML) in a substantial proportion of patients, but this possibility has not been tested in a prospective study. The authors therefore measured the cellular expression of the MxA protein, a reliable marker of viral infection, at diagnosis in children with JMML to estimate the prevalence of such infections.

Methods: Eighteen children, aged 1 to 69 months, who met the diagnostic criteria of the International JMML Working Group were prospectively studied. MxA expression was assessed by flow cytometric analysis of peripheral blood mononuclear cells stained with an anti-human MxA antibody. All data were obtained through the MDS Committee of the Japanese Society of Pediatric Hematology.

Results: Twelve patients (67%) had elevated levels of the MxA protein, with rotavirus, RS virus, or CMV infection documented in three of these patients. Although none of the patients had primary Epstein-Barr virus (EBV) infection, reactivation of the virus was strongly suspected in four children, including two with monosomy 7, each having increased levels of MxA. Southern blot analysis revealed monoclonal integration of the EBV genome into bone marrow mononuclear cells from one of these patients. There was no discernible correlation between increases in the marker protein and the presenting features or course of the disease.

Conclusions: Viral infection may be present in two thirds of children with newly diagnosed JMML, but it does not constitute a basis for revising clinical management. The possibility that EBV or other viruses contribute to JMML pathogenesis by stimulating pre-exiting malignant clones warrants further investigation.

Key Words: juvenile myelomonocytic leukemia, viral infections, monosomy 7, Epstein-Barr virus, MxA

(*J Pediatr Hematol Oncol* 2004;26:636–641)

Juvenile myelomonocytic leukemia (JMML), previously described as juvenile chronic myelocytic leukemia or chronic myelomonocytic leukemia,[1,2] is a rare disease with a generally ominous prognosis. Niemeyer et al[3] reported a 10-year survival rate of 39% for patients who underwent hematopoietic stem cell transplantation (HSCT), compared with only 6% for patients who did not receive this treatment. Thus, allogeneic HSCT is recommended for most children with JMML.[4–8]

A consensus on the diagnosis of JMML was reached only recently. The World Health Organization collaborative study group classified JMML as a disease having characteristics of both myelodysplastic syndrome (MDS) and myeloproliferative disorders,[9] while the International JMML Working Group introduced a new set of diagnostic criteria that combine suggestive clinical observations with major and minor laboratory findings (Table 1).[10] Although this revised approach to the diagnosis of JMML has gained wide acceptance, cytomegalovirus (CMV), Epstein-Barr virus (EBV), or human herpesvirus-6 infection can produce signs and symptoms closely resembling those seen in patients with JMML, leading some investigators to suggest that documentation of a viral infection in a child with suspected JMML should raise questions regarding the diagnosis of a malignant disease.[11–13]

It was previously shown that MxA protein is specifically expressed in circulating mononuclear cells in response to stimulation by type I interferons in the presence of viral infections.[14–22] We therefore prospectively studied the peripheral blood mononuclear cells of children with newly diagnosed JMML for evidence of viral infection, using cytoplasmic expression of the MxA protein as a surrogate marker, and then

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TABLE 1. Diagnostic Criteria for JMML

Suggestive clinical features
1. Hepatosplenomegaly
2. Lymphadenopathy
3. Pallor
4. Fever
5. Skin rash

Major laboratory criteria (all three required)
1. No Philadelphia chromosome; or no BCR-ABL rearrangement
2. Peripheral blood monocyte count >1 × 10^9/L
3. Bone marrow blasts >20%

Minor laboratory criteria (minimum of two required)
1. Increased fetal hemoglobin, corrected for age
2. Myeloid precursors evident on peripheral blood smear
3. White blood cell count >10 × 10^9/L
4. Clonal abnormalities, including monosomy 7
5. GM-CSF hypersensitivity of myeloid progenitors in vitro

Adapted from Niemeyer et al.[10]

related the findings to the clinical course and presenting features of the disease.

METHODS

Patients

Eighteen children with an established diagnosis of JMML were enrolled in the study. Clinical data and samples for laboratory analysis were obtained through the MDS Committee of the Japanese Society of Pediatric Hematology.[23] Informed consent was given by the guardians of the patients. Age at diagnosis ranged from 1 to 69 months (median 15 months); follow-up for the surviving patients ranged from 4 to 33 months (median 12 months). There were 12 boys and 6 girls. One patient had neurofibromatosis 1 (NF-1) and another had Noonan syndrome. This study was approved by the MDS Committee of the Japanese Society of Pediatric Hematology.

MxA Protein Expression in Lymphocytes

The expression of MxA was tested in blood samples (1 mL heparinized venous blood) collected at diagnosis. After separation on a Ficoll-Metrizamide density gradient (Lymphoprep; Nyegaad, Oslo, Norway), the mononuclear cells were washed and resuspended in alpha-MEM (Gibco, NY) supple-

ABLE 2. Clinical and Laboratory Characteristics of the Patients

atient No.*	Age (mo)	Sex	Clinical Features†	Minor Criteria†	Karyotype	Spontaneous Colony Formation	CRP (mg/dL)	Other Conditions
1	1	F	4	1,2,3,5	Normal	+	4.1	
2	1	M	1	2,3,5	Normal	+	1.6	
3	3	M	1,2,3,4	1,2,3	Normal	+	2.8	
4	6	M	1,3,4	2,3	Normal	+	0.2	
5	8	M	1,2,3	2,3,5	Normal	−	1.7	
6	16	M	1,3,4	2,3,4,5	−7	+	0.9	
7	24	M	1,2,5	1,2,3,5	Normal	+	0.0	
8	24	F	1,2	1,2,3,5	Normal	+	0.2	
9	28	M	1,2,5	1,3	Normal	+	1.6	
10	37	M	1,2,3,4	1,2,3,4	−7	−	1.7	
11	37	F	1,2,4,5	1,2,3	Normal	+	3.0	
12	69	M	1,4	3,4,5	−7	+	3.5	NF-1
13	1	F	1,2,3	1,2,3,5	Normal	−	3.3	
14	2	M	1	1,2,3	Normal	+	4.8	NS
15	4	F	1,2,4	1,2,3,4,5	t(2;19)	+	0.3	
16	13	M	1,2	1,2,3	Normal	+	1.6	
17	32	M	1,2,3,4,5	1,2,3,4,5	8+, −Y	+	3.1	
18	56	F	1,3,4	1,2,3	Normal	−	0.6	

All patients met major criteria: lack of the Philadelphia chromosome (or no BCR-ABL rearrangement), peripheral blood monocyte count >1 × 10^9/L, and bone arrow blasts <20%.
CRP, C-reactive protein (normal value <1 mg/dL); NF-1, neurofibromatosis-1; NS, Noonan syndrome.
*Patients with MxA protein level greater than 2.0 FI are indicated by boldface type.
†Numbers correspond to diagnostic guidelines listed in Table 1.

mented with 10% FCS (HyClone, Logan, UT). The cytoplasmic staining technique has been described.[24] Briefly, the cells were fixed and their membranes permeabilized with Fix and Perm (An Der Grub, Austria), followed by staining with antihuman MxA antibody (KM1135, a mouse monoclonal antibody, IgG1 isotype, donated by Kyowa Co., Tokyo, Japan). Mouse IgG1 (DAKO) served as a control, and PE-labeled goat antimouse IgG (DAKO) was used as a secondary antibody. The expression of MxA protein in mononuclear cells in a lymphoid gate was analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

As a measure of the intensity of staining, we used a fluorescence index (FI), representing the ratio of the fluorescence intensity of cells stained with the antihuman MxA antibody versus that of cells stained with the isotype-matched control. In some experiments, the mononuclear cells were incubated in complete medium with 1,000 U/mL of interferon-alpha (IFN-α; INTRON A, Schering Corp.) at 37°C for 4 hours before cytoplasmic staining was performed. In a previous study,[14] we showed that the FI of MxA in virus-infected patients ranged from 2.6 to 38.9 (median 6.7), contrasted with 0.9 to 2.0 (median 1.2) in control subjects without fever ($P < 0.05$, Mann-Whitney test). Hence, an FI of 2.0 was selected as the threshold value for the diagnosis of viral infections.

Southern Blot Hybridization

The clonality of EBV infection was determined by Southern blotting with a terminal repeat probe, as previously described.[25] Genomic DNA extracted from bone marrow was digested with *Eco*RI, subjected to gel electrophoresis, transferred to a nylon membrane, hybridized with a ^{32}P-labeled *Bam*HI-W fragment from the terminal region of EBV, and visualized by autoradiography.

RESULTS

All 18 patients fulfilled the diagnostic criteria of the International JMML Working Group, although the constellation of features varied widely (Table 2). Seventeen patients had hepatosplenomegaly, 11 had lymphadenopathy, 8 had pallor, 10 had a temperature of more than 38°C, and 4 had skin rash. Among the minor laboratory criteria (a minimum of two required), an elevated hemoglobin F level was noted in 13 patients, myeloid precursors on a peripheral blood smear in 16, a white blood cell count above 10×10^9/L in 18, a clonal chromosomal abnormality in 5, and hypersensitivity of myeloid progenitor cells to granulocyte/macrophage colony-stimulating factor (GM-CSF) in vitro in 10 of 16 evaluable patients. Myeloid precursors from 14 patients showed spontaneous colony-forming ability in vitro.

TABLE 3. Results of MxA Expression Analysis and Serologic Tests

Patient No.*	MxA (no IFN-α) (FI)	MxA (with IFN-α) (FI)	CMV Titer	EBV Titer	Other Virus
1	5.2	NT	IgM−, IgG−	EBNA 20, VCA-IgG 80	
2	2.8	NT	IgM+, IgG+	EBNA <10, VCA-IgG <10	
3	2.3	2.4	IgM−, IgG+	EBNA <10, VCA-IgG <10	Rota
4	2.1	4.5	IgM−, IgG−	EBNA <10, VCA-IgG <10	
5	2.1	NT	IgM−, IgG+	EBNA <10, VCA-IgG <10	
6	4.0	2.7	IgM−, IgG+	EBNA 40, VCA-IgG 2560	
7	8.1	3.8	IgM−, IgG+	EBNA+, VCA-IgG+	
8	8.5	NT	IgM−, IgG−	EBNA <10, VCA-IgG <10	RS
9	3.3	6.3	IgM−, IgG−	EBNA 10, VCA-IgG 640	
10	4.4	NT	IgM−, IgG+	EBNA+, VCA-IgG+	
11	2.2	4.4	IgM−, IgG+	EBNA 40, VCA-IgG 320	
12	14.0	NT	IgM−, IgG−	EBNA 40, VCA-IgG 320	
13	1.7	4.0	IgM−, IgG−	EBNA <10, VCA-IgG <10	
14	1.0	1.8	IgM−, IgG−	EBNA <10, VCA-IgG <10	
15	1.0	1.2	IgM−, IgG−	EBNA <10, VCA-IgG <10	
16	1.9	4.5	IgM−, IgG+	NT	
17	1.2	NT	IgM−, IgG−	EBNA <10, VCA-IgG <10	
18	1.2	3.4	IgM−, IgG+	EBNA 40, VCA-IgG+	

FI, fluorescence index; NT, not tested; VCA, viral capsid antigen; EBNA, Epstein-Barr virus nuclear antigen; RS, respiratory syncytial.

*Patients with increased MxA expression (FI > 2.0) are denoted by boldface type.

VCA-IgM was negative in all patients.

Karyotypic abnormalities included monosomy 7 in three patients, the chromosomal translocation t(2;19) in one, and trisomy 8 with loss of the Y chromosome in one. Three patients had clinically apparent viral infections (rotavirus, respiratory syncytial virus, and CMV). Serologic studies for CMV and EBV were performed in all but one patient (Table 3). The results were not diagnostic of primary EBV infection but indicated reactivation of EBV in four patients (patients 6, 9, 11, and 12), including two with monosomy 7.

Analysis of MxA Protein Expression and EBV Clonality

The expression of MxA in peripheral blood mononuclear cells was increased (>2.0 FI) in 12 patients (see Table 3). This finding extended to each of the three patients with monosomy 7, including the patient with NF-1, but not to those with other karyotypic abnormalities or to the child with Noonan syndrome. MxA protein levels greater than 2.0 FI did not correlate with age at diagnosis, the presence of fever, the serum level of C-reactive protein, or any of the other presenting features determined in this study. As expected, each of the three patients with clinically apparent viral infection had an increased FI value.

The MxA level was also elevated in the four patients with apparent EBV reactivation, prompting us to analyze the clonality of these cases, using a probe derived from EBV terminal repeats. Southern blotting showed monoclonal integration of the EBV genome into the bone marrow mononuclear cells of patient 6 (Fig. 1) but not of patients 11 and 12 (not shown).

Since MxA expression is specifically induced by IFN-α, we considered that lymphocytes from some patients with JMML might lack the ability to respond to the cytokine. Table 3 shows the FI values for 11 patients whose blood mononuclear cells were stained with antihuman MxA antibody after IFN-α stimulation in culture. In nine of these patients, the values were higher in stimulated mononuclear cells than in unstimulated cells. One of the two exceptions was patient 14, who had Noonan syndrome; the other was patient 15, whose myeloid progenitor cells carried the t(2;19). These results suggest that low expression of MxA (<2.0 FI) in unstimulated cells simply reflects the lack of interferon production in vivo rather than any intrinsic defect of mononuclear cells that might block responsiveness to IFN-α.

Clinical Outcome

All patients were managed clinically according to a single protocol developed by the authors under the auspices of the MDS Committee of the Japanese Society of Pediatric Hematology. Two patients, one with documented CMV infection (patient 2) and the other with an increased FI but no specific virus (patient 4), had spontaneous regression of all disease (Table 4). In patient 2, the CMV genome was detected in the blood and the signs of JMML disappeared without specific treatment. In patient 4, the clinical characteristics of the disease resolved within 3 months while the child was awaiting transplantation. Of the remaining 16 patients, 10 underwent allogeneic HSCT, 3 received chemotherapy only, and 3 were not treated because of early complications, including leukemic cell infiltration to the lungs and congestive heart failure associated with Noonan syndrome. Seven of the 10 patients who received a transplant remain in complete hematologic remission for 8+ to 26+ months (median 17+ months). Three patients are awaiting allogeneic HSCT.

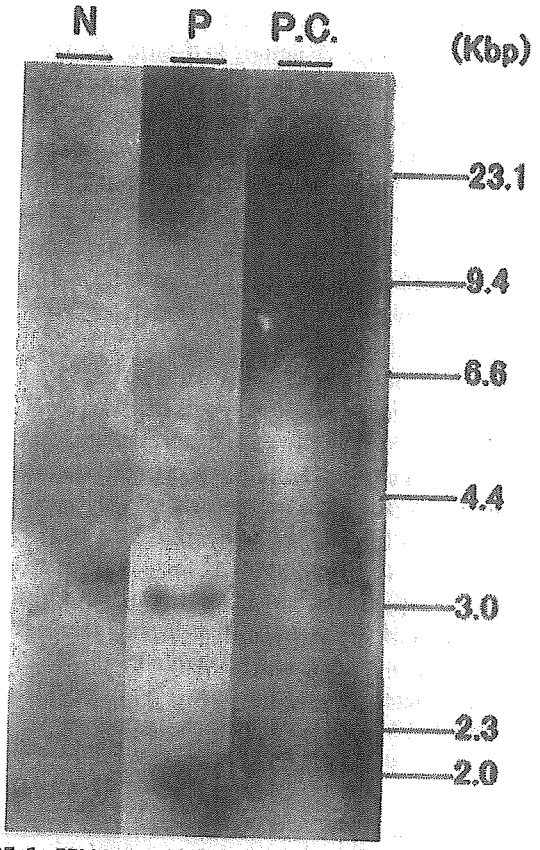

FIGURE 1. EBV-DNA analysis. A DNA sample from patient 6 was digested with *EcoRI* and hybridized with a probe recognizing the termini of the EBV genome. N, normal control; P, patient sample; PC, positive control (Raji cell line). A single 3.0-kb band indicates monoclonal integration of EBV into the patient's DNA.

DISCUSSION

MxA protein is specifically expressed in circulating mononuclear cells in response to stimulation by type I interferons.[15,16] Since the biologic half-life of IFN-α is very short (1–2 hours) but that of MxA is rather long (2.3–2.5 days),[17,26] detection of an elevated level of MxA soon after the onset of viral infection should be diagnostic, a prediction supported by

TABLE 4. Treatment and Clinical Outcome

Patient No.*	Treatment	Disease Progression	Survival (mo)
1	Related BMT	Extramedullary tumor	8 (VOD)
2	No treatment	Spontaneous regression	8+
3	Related PBSCT		12+
4	No treatment	Spontaneous regression	33+
5	6MP		5+
6	Related PBSCT		20+
7	U-CBT		26+
8	6MP		4 (GI bleeding)
9	No treatment	Infiltration to lungs	1 (with disease)
10	Related CBT & BMT		11+
11	U-BMT		17+
12	No treatment		4+
13	U-CBT		8+
14	No treatment		4 (heart failure)
15	U-BMT	Extramedullary tumor	6 (with disease)
16	U-CBT		22 (GVHD, pneumonia)
17	6MP		7+
18	U-BMT	AML-M6	24+

U-CBT, unrelated cord blood transplantation; U-BMT, unrelated bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; AML, acute myeloid leukemia; VOD, veno-occlusive disease of liver; 6MP, 6-mercaptopurine; GVHD, graft-versus-host disease.

*Patients with MxA protein levels greater than 2.0 FI are indicated by boldface type.

correlative studies of cellular MxA protein concentrations in patients with suspected or proven viral infections.[14,17–22] We therefore sought to exploit MxA expression in blood mononuclear cells as a surrogate marker of viral infection in patients meeting the international diagnostic criteria for JMML.[10]

Our results showed a concomitant viral infection in 12 (67%) of the 18 children studied. CMV, rotavirus, or respiratory syncytial virus infection was documented in three children with elevated MxA protein levels, while in four others there was apparent reactivation of EBV, although the early antigen (D) or the amount of the EBV genome was not tested. Of the five patients with a chromosomal abnormality, indicative of malignant disease arising from a single transformed hematopoietic progenitor, three had increased MxA levels. The remaining nine cases with marker evidence of viral infection occurred in patients with "normal" karyotypes; however, we cannot rule out the possibility of monoclonal JMML associated with point mutations in the *NRAS* or *KRAS* proto-oncogene, recently identified PTPN11 mutations, or other molecular genetic alterations.[1,2,27] These observations demonstrate that concomitant viral infection is not restricted to JMML patients with a normal karyotype but may extend to cases of clonal disease, perhaps contributing to their pathogenesis (as discussed below). There were no reports describing the expression of the MxA protein in patients with malignancies including monocytic predominant disorders other than JMML in the literature, and the sensitivity and the specificity of the measurement of MxA for viral infections versus the other processes are unknown. Accordingly, we cannot rule out the possibility that MxA is apparently expressed in the absence of viral infections in a subset of patients with JMML.

The association of reactivated EBV infection with JMML (in patient 6) is a provocative finding and has not been reported by others, although Stollmann et al[28] described a child with persistent EBV infection and a t(3;5)-positive myeloproliferative disease resembling JMML. EBV causes a variety of malignant diseases, including nasopharyngeal carcinoma, Burkitt lymphoma, Hodgkin disease, and lymphoproliferative disease.[29] Moreover, in patients with chronic active EBV infection, which can evolve to virus-associated hemophagocytic syndrome,[30] the peripheral blood T cells and natural killer cells showed monoclonal integration of the EBV genome,[31] a hallmark of natural killer cell-associated leukemia/lymphoma, Hodgkin disease, and gastric cancer, among other malignancies. We speculate that some cases of JMML may be exacerbated by chronic active EBV infection, either by viral stimulation of a pre-existing (e.g., monosomy 7) malignant clone or by direct induction of chronic myelomonocytic leukemia. This

hypothesis requires testing in larger numbers of patients with an established diagnosis of JMML.

There has been a longstanding debate over the significance of a viral infection in children with JMML, in particular whether this finding should supersede the primary diagnosis.[11–13,32] Although we demonstrated a substantial rate of MxA protein positivity (67%), elevated levels of this surrogate maker of viral infection did not correlate with diagnostic criteria of the International JMML Working Group or with other clinical and laboratory features, suggesting that the presenting profiles of most of our patients represented leukemia and not a virally induced reactive process. We conclude that evidence of viral infection in JMML patients diagnosed with criteria similar to ours is not sufficient to motivate a revision of clinical management plans.

In this small series, the results of allogeneic HSCT were quite good (7 of 10 patients alive in complete hematologic remission for 8+ to 26+ months), confirming the efficacy of this treatment modality in JMML.[4–8] We cannot rule out that a minor subset of these patients suffered from infection rather than leukemia and would have fared equally well on less aggressive therapy. This issue is difficult to address given the current understanding of JMML pathogenesis; however, continued refinement and evaluation of diagnostic guidelines with emphasis on newer molecular genetic techniques should greatly reduce the likelihood of mistaken interventions.

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RAS-blocking bisphosphonate zoledronic acid inhibits the abnormal proliferation and differentiation of juvenile myelomonocytic leukemia cells in vitro

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Juvenile myelomonocytic leukemia (JMML) is a clonal myeloproliferative/myelodysplastic disorder of early childhood with a poor prognosis. JMML cells are characterized by hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) caused by a continuously activated GM-CSF receptor–retrovirus-associated sequence (RAS) signal transduction pathway through various molecular mechanisms, resulting in spontaneous GM colony formation in vitro. Bisphosphonate zoledronic acid (ZOL), a RAS-blocking compound, suppressed colony formation from bone marrow (BM) cells of 8 patients with JMML and 5 healthy control subjects without and with GM-CSF (10 ng/mL), respectively, in a dose-dependent manner in clonal culture. At 10 μM ZOL, however, spontaneous GM colony formation from JMML BM cells decreased to 3%, but the formation of G colonies containing granulocytes, but no macrophages, was enhanced, whereas 40% of GM colonies were retained and G colony formation was not affected in culture of normal BM cells with GM-CSF. In suspension culture, cytochemical and flow cytometric analyses showed that 10 μM ZOL also inhibited spontaneous proliferation and differentiation along monocyte/macrophage lineage of JMML BM cells but not the development of normal BM cells by GM-CSF. The inhibitory effect of ZOL on JMML cells was confirmed at a single-clone level and observed even at 3 μM. The current result offers a novel approach to therapy in JMML. (Blood. 2005; 106:3134-3141)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare clonal myeloproliferative/myelodysplastic disorder of infancy and early childhood.[1-3] Despite various approaches to therapy, the mortality rate in patients with JMML is still high. Intensive chemotherapeutic regimens have largely proved futile in inducing durable remissions.[4] Low-dose chemotherapy with 6-mercaptopurine, for example, has been temporarily effective in some patients, but generally it has not been shown to result in long-term disease control.[5,6] Allogeneic hematopoietic stem cell transplantation (SCT) is presently the only therapy capable of producing durable remissions, but the 4- or 5-year probability of event-free survival in patients with JMML treated by SCT is approximately 50%.[5,7] Therefore, the development of a new treatment for patients with JMML is awaited.

JMML cells are characterized by the ability to spontaneously proliferate in the absence of hematopoietic growth factors in vitro,[8] giving rise to granulocyte-macrophage (GM) colonies caused by hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) released from monocytes/macrophages.[9] Several investigators have shown that deregulated GM-CSF signal transduction through the retrovirus-associated sequence (RAS) pathway plays a key role in the characteristic property of JMML cells. Activating mutations of the *RAS* gene were found in 15% to 30% of patients with JMML.[10-14] It was also reported that 30% of patients with JMML had mutations of the gene *NF1* encoding neurofibromin, a guanosine triphosphatase acting protein that inactivates RAS, leading to a continuously activated GM-CSF receptor (GM-CSFR)–RAS pathway.[15] Furthermore, somatic mutations of *PTPN11* encoding a cytoplasmic Src homology-2 domain containing protein that controls RAS functions were found in 34% of patients with JMML without Noonan syndrome.[16] Given these reports, linking the pathogenesis of JMML to the continuously activated GM-CSFR–RAS signal transduction pathway, it is reasonable to explore molecular mechanism-based therapy for JMML.

A GM-CSF antagonist, E21R, was earlier shown to inhibit JMML-cell growth in vitro and JMML-cell engraftment in immunodeficient mice.[17,18] Another feasible way to inhibit JMML-cell development is to block the GM-CSFR–RAS signaling intracellular pathway. The first obligatory step in the signal transduction, which is essential for RAS activity, is the membrane localization of RAS accomplished through a prenylation reaction mediated by farnesyltransferase (FTase), which involves the covalent linking of a 15-carbon isoprenyl (farnesyl) group to RAS.[19-21] FTase inhibitors (FTIs) have been evaluated in several in vitro and in vivo

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preclinical systems and demonstrated some antitumor effects.[22,23] However, even when FTase is inhibited, RAS can be transferred to the membrane by an alternative pathway using geranylgeranyl transferase-1.[24] Bisphosphonate (BP) has been used to treat bone diseases and acted as an anticancer drug by inhibiting the activation of RAS through the suppression of both farnesylation and geranylgeranylation.[25,26] More recently, zoledronic acid (ZOL), a third-generation BP, has been shown to be more effective in blocking RAS activity.[27] We then investigated the effect of ZOL on JMML-cell growth in vitro to clarify the function of continuously activated RAS in JMML cells and to evaluate the feasibility of using ZOL as an antileukemia drug for JMML.

Materials and methods

Zoledronic acid

Zoledronic acid (2-(imidazol-1-yl)-hydroxy-ethylidene-1, 1-bisphosphonic acid, disodium salt, 4.75 hydrate) was supplied by Novartis Pharma AG (Basel, Switzerland), dissolved in a stock solution of water at a concentration of 100 mM and stored at −20°C in plastic (not glass) containers.

Acquisition of donor samples

With personal and parental consent, bone marrow (BM) samples were obtained from children with JMML diagnosed based on the criteria of the International JMML Working Group.[28] Normal controls were healthy adult volunteers who donated BM samples after providing informed consent. The study protocol was approved by the MDS Committee of the Japanese Society of Pediatric Hematology. Plastic syringes (10 mL) coated with preservative-free heparin were used for the acquisition of BM samples. Nonphagocytic mononuclear cells (NPMNCs) were separated by Ficoll-Hypaque density centrifugation after the depletion of phagocytes with Silica (Immuno Biological Laboratories, Fujioka, Japan). The NPMNCs were washed twice and suspended in α-medium (Flow Laboratories, Rockville, MD). Nonerythroid cells were obtained by lysis with NH_4CL.

Clonal culture

The isolated BM NPMNCs were incubated in methylcellulose culture in triplicate using a technique described previously[29] with some modifications. Briefly, 1 mL culture mixture containing 2×10^4 cells, α-medium, 0.9% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% deionized fraction V bovine serum albumin (BSA; Sigma, Saint Louis, MO), 5×10^{-5} M mercaptoethanol, and various concentrations of ZOL was plated in each 35-mm standard nontissue culture dish (Nunc, Roskilde, Denmark) and incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. In some experiments, GM-CSF (Stem Cell Technologies, Vancouver, Canada) was added to the culture mixture at a concentration (10 ng/mL) that induced an optimal response in methylcellulose culture of human BM hematopoietic cells.[30] The size and type of colonies (> 40 cells) formed in the culture were assessed at day 14 of culture. The size of small colonies was determined by direct cell counting in situ under an inverted microscope. When colonies contained more than 200 cells, they were removed individually with an Eppendorf micropipette and prepared as single-cell suspensions. Colony size was estimated using a counting chamber with the cell suspension. Colony types were determined according to criteria reported previously[31]: granulocyte (G), monocyte/macrophage (M), and GM colonies that consist of granulocytes, including neutrophils, eosinophils, and basophils, monocytes/macrophages, and both granulocytes and monocytes/macrophages, respectively. To assess the accuracy of the in situ identification for the colony types, individual colonies were lifted, spread on glass slides using a cytocentrifuge (Cytospin 2; Shandon Southern Instruments, Sewickley, PA), and treated with May-Grünwald-Giemsa staining and cytochemical staining with α-naphthyl butyrate esterase (α-NBE) according to conventional methods. Differential counts of the cells were done with more than 100 cells on the cytospin smears in all experiments.

Suspension culture

Twenty thousand nonerythroid BM cells were incubated in a suspension culture using a modified version of a technique described previously.[32,33] Briefly, 1 mL culture mixture containing 5×10^4 cells, α-medium, 30% FBS, 1% deionized fraction V BSA, and various concentrations of ZOL was incubated in 12-well tissue plates (Nunc) at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. The number of cultured cells and the cellular composition were determined at days 0, 5, and 10. The cell number was assessed by direct cell counting of liquid culture aliquots in a hemocytometer. The cellular composition was determined on cytospin smears of the cultured cells stained and by flow cytometric analysis (see "Flow cytometry"). In the special experiment, colonies developed in clonal culture at day 5 were individually collected in a microtube containing 100 μL α-medium and then divided into 2 aliquots. Each half was incubated in a suspension culture with or without 10 μM ZOL for 10 days after the addition of 50 μL respective culture medium.

Flow cytometry

Flow cytometric analysis was carried out using myelomonocytic cell-differentiation–related antigens; fluorescein isothiocyanate (FITC)–conjugated CD14, CD16, and human leukocyte antigen (HLA)–DR; phycoerythrin (PE)–conjugated CD11b and CD13; and peridininchlorophyll (PerCP)–conjugated CD45. All monoclonal antibodies used were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). The staining procedure was described previously.[34] All incubations with antibodies were carried out for 30 minutes on ice. Flow cytometric data were acquired on a fluorescence-activated cell scan (Becton Dickinson) using the Lysis-II software program. In a standard 3-color immunofluorescence protocol, forward and side scatters (FSC and SSC, respectively) were collected along with 3-color antibody combinations. Electronic gating on the basis of FSC and SSC excluded cellular debris and nonviable cells. Cell population percentages of monocytes/macrophages, granulocytes, lymphocytes, and blastic cells in nonerythroid cells were assessed by SSC and CD45 staining as described.[35] The flow cytometric profile was obtained by either directly gating discrete populations identified by SSC and PerCP-conjugated CD45 staining, or by back-gating using FITC- and PE-conjugated antibody combinations to identify cells of interest.

Statistical analysis

Student t test was used to determine the significance of differences among groups of unpaired samples in all experiments. P values were derived from 2-sided tests, and P less than .05 was considered statistically significant.

Results

Effect of ZOL on colony growth from JMML and normal BM cells in clonal culture

Eight children with JMML were enrolled in this study (6 boys and 2 girls). Their median age was 3 years and 1 month (range, 1 month to 5 years and 9 months).

In clonal culture, BM cells of all 8 patients produced a large number of spontaneous colonies in the absence of hematopoietic factors, and the number of colonies was comparable to that formed in the presence of 10 ng/mL GM-CSF in 6 of 8 patients (Table 1). In cases 3 and 6, however, the number of colonies increased with the addition of 10 ng/mL GM-CSF to the culture. Most colonies formed in the absence or presence of 10 ng/mL GM-CSF were GM colonies that consisted of both granulocytes and macrophages

Table 1. Colony formation from BM cells of patients with JMML and healthy adults

Case no.	No. of colonies formed, 2 × 10^4 BM NPMNCs		GM colonies in total colonies, %	
	GM-CSF absent	GM-CSF present*	GM-CSF absent	GM-CSF present*
Patients with JMML				
1	135 ± 23	160 ± 14	97	99
2	153 ± 33	190 ± 22	100	100
3	44 ± 12	121 ± 13	98	100
4	193 ± 34	168 ± 33	97	99
5	131 ± 32	177 ± 32	95	99
6	46 ± 11	94 ± 21	100	100
7	152 ± 21	206 ± 16	96	99
8	217 ± 31	198 ± 22	99	98
Mean ± SD	137 ± 41	160 ± 36	98 ± 2	99 ± 0.2
Healthy adults				
1	0 ± 0	155 ± 22	NA	93
2	0 ± 0	28 ± 13	NA	65
3	0 ± 0	234 ± 35	NA	84
4	0 ± 0	104 ± 21	NA	91
5	0 ± 0	200 ± 22	NA	95
6	0 ± 0	203 ± 19	NA	97
Mean ± SD	0 ± 0	154 ± 23	NA	87 ± 11

NA indicates not applicable.
*GM-CSF was used at a concentration of 10 ng/mL, which induced an optimal response among normal BM cells.

(Table 1; Figure 1A). By contrast, BM cells of healthy adults produced no colonies in the absence of hematopoietic factors. On the addition of GM-CSF, normal BM cells produced colonies. Eighty-seven percent of the colonies were GM colonies whose appearance revealed a diminished number of macrophages (Figure 1B) as compared with spontaneous GM colonies generated from JMML BM cells, and the rest were G colonies (12% ± 11%, n = 6) with only a few M colonies (< 1%).

When added to the culture, ZOL suppressed the spontaneous colony formation from BM cells of all 8 patients with JMML in a dose-dependent manner (Figure 2A). The number of spontaneous colonies formed from JMML BM cells decreased to a 3rd and a 15th at concentrations of 10 and 100 μM ZOL, respectively. In particular, the formation of GM colonies was much decreased at 10 μM ZOL (3% ± 3%), and no GM colonies were produced at 100 μM ZOL. Furthermore, the size of spontaneous colonies was smaller at greater than 10 μM ZOL. As shown in Figure 2B, the numbers of constituent cells in individual spontaneous colonies (n = 15 at each concentration of ZOL) randomly chosen from the cultures of BM cells of 3 patients with JMML (cases 4, 6, and 7) with 10 and 100 μM ZOL were much smaller than those with no or 1 μM ZOL. Interestingly, the addition of ZOL enhanced the formation of small G colonies consisting of only granulocytes, with no macrophages (Figures 1C and 2A). The effect of ZOL on the formation of colonies from BM cells of patients with JMML (cases 4 and 7) in the clonal culture containing 10 ng/mL GM-CSF was also examined. We again observed that 10 μM ZOL inhibited GM colony formation and enhanced G colony formation in a manner similar to that observed for the spontaneous colony formation (data not shown).

However, when 10 and 100 μM ZOL were added to the culture of normal BM cells with 10 ng/mL GM-CSF, the number of colonies decreased to approximately 60% and 20%, respectively (Figure 2A), but at 10 μM ZOL, GM colonies still occupied the larger proportion of the total colonies retained (69% ± 14%, significantly different from the percentage [8% ± 7%] in spontaneous GM colony formation by JMML BM cells [$P < .01$]). ZOL did not have a significant effect on the formation of G colonies from normal BM cells in the presence of 10 ng/mL GM-CSF. The size of the GM colonies also decreased in a dose-dependent manner, but it was much larger than that of the spontaneous colonies from JMML samples at 10 μM ZOL ($P < .01$) (Figure 2B).

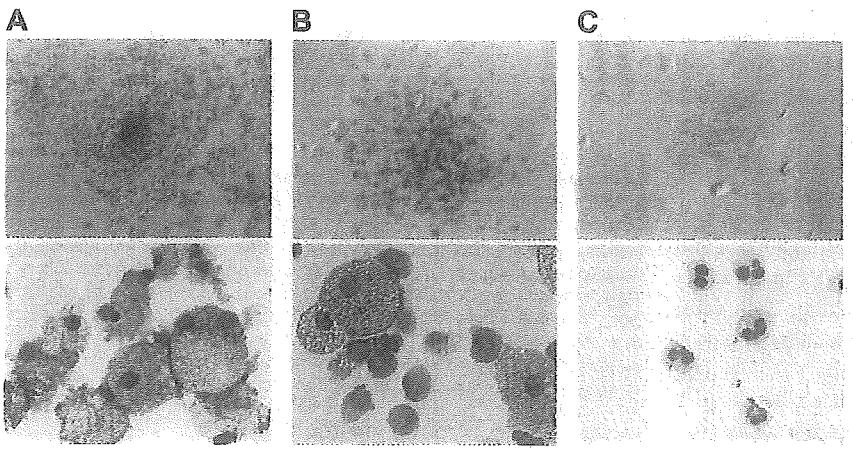

Figure 1. Colonies formed under various conditions. Top and bottom panels show the appearance of colonies under an inverted microscope (ITM; Olympus, Tokyo, Japan) equipped with a camera module (DP50; Olympus) (objective lens, SPlan 4PL; numerical aperture, 0.13; magnification, × 4) and their constituent cells stained with May-Grünwald-Giemsa solution (microscope, BX51, Olympus; camera module, XC-003, Sony, Tokyo, Japan; objective lens, UPlan F1, Olympus; numerical aperture, 1.3; magnification, × 100), respectively. (A) A spontaneous GM colony in the culture of JMML BM cells without hematopoietic factors. (B) A GM colony in the culture of normal BM cells with 10 ng/mL GM-CSF. (C) A spontaneous G colony in the culture of JMML BM cells with 10 μM ZOL.

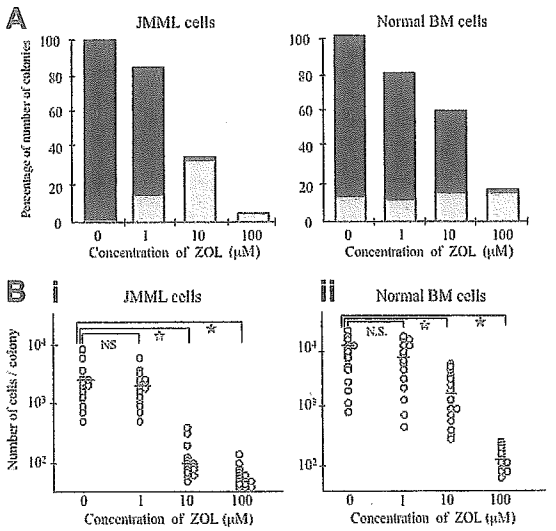

Figure 2. Effect of ZOL on colony formation from JMML and normal BM cells. (A) The percentages of the number of colonies in the culture of JMML and normal BM cells without and with 10 ng/mL GM-CSF, respectively, in the presence of various concentrations of ZOL to that in the absence of ZOL, and the proportion of G and GM colonies in total colonies under the respective conditions. The values indicate the means calculated from the data for 8 patients with JMML and 6 healthy donors in Table 1. ■ indicates GM colonies; ▨, G colonies. (B) The numbers of cells contained in individual colonies cultured from JMML and normal BM cells without and with 10 ng/mL GM-CSF, respectively. Fifteen colonies were randomly chosen in the culture of BM cells of 3 patients with JMML and 3 healthy donors under the respective conditions. Bars indicate the means of cell numbers of the 15 colonies. In i, NS indicates not significant; ☆, $P < .01$; ★, $P < .001$. In ii, ☆ indicates $P < .05$; ★, $P < .001$.

Effect of ZOL on development of JMML and normal BM cells in suspension culture

The result of the clonal culture suggested that ZOL inhibited the proliferation and differentiation of both JMML and normal BM cells, but the inhibitory effect was stronger in the former. We then carried out suspension culture to examine the effect of ZOL on the development of BM cells of patients with JMML and healthy donors in more detail.

In suspension culture without hematopoietic factors, nonerythroid BM cells of 3 patients with JMML (cases 4, 6, and 7) proliferated, whereas no increase of the cells cultured from BM cells of 3 healthy donors (cases 1, 3, and 5) was observed. With the addition of 10 ng/mL GM-CSF to the culture, normal BM cells proliferated. When incubated for 15 days, both JMML and normal BM cells showed the development of a number of adherent cells in the suspension culture without and with 10 ng/mL GM-CSF, respectively, as shown in Figure 3A. Therefore, we could not accurately calculate the number of cells contained in the cultures at day 15. When ZOL was added, no development of adherent cells was observed at greater than 10 μM in the culture of JMML BM cells at day 15. In the culture of normal BM cells, however, adherent cells developed at 10 μM ZOL in the presence of 10 ng/mL GM-CSF although they did not develop at 100 μM.

Figure 3B shows the effect of ZOL on the proliferation of BM cells of 3 patients with JMML (cases 4, 6, and 7) and 3 healthy donors (cases 1, 3, and 5) in the suspension culture. Each value represents the mean ± SD of the percentages of the cells cultured at the respective concentrations of ZOL to those without ZOL at days 5 and 10 of culture. At day 5, 100 μM, but neither 1 nor 10 μM, ZOL inhibited the proliferation of JMML BM cells, but at day 10, 1 to 100 μM ZOL inhibited it. However, 1 and 10 μM ZOL revealed no inhibitory effect on the proliferation of normal BM cells even at day 10 of culture, whereas 100 μM ZOL inhibited it at days 5 and 10.

Figure 4A shows the effect of ZOL on the proportion of α-NBE–positive macrophages and –negative cells in the suspension culture of JMML and normal BM cells. JMML BM cells contained 34% ± 6% of monocytes/macrophages whose α-NBE–positive granules were fine as compared with those in normal BM monocytes/macrophages, and α-NBE–negative cells included granulocytes, lymphocytes, immature blastic cells, and nucleated erythroid cells. In the culture of JMML BM cells without ZOL and with 1 μM ZOL, most of the cells generated were α-NBE–positive macrophages at day 10, and α-NBE–negative populations contained a few granulocytes and immature blastic cells (Figure 4A-B). At 10 and 100 μM ZOL, however, the proportion of α-NBE–positive macrophages significantly decreased and that of α-NBE–negative cells increased. The majority of the α-NBE–negative cells were granulocytes, but there was a substantial number of blastic cells.

However, a large proportion of normal BM cells were α-NBE–negative granulocytes. The α-NBE–negative cells also included nucleated erythroid cells, lymphocytes, and a few blastic cells. In the culture of normal BM cells with 10 ng/mL GM-CSF, α-NBE–negative granulocytes accounted for three quarters of the cells generated, and the other quarter were α-NBE–positive macrophages (Figure 4A-B). The addition of ZOL did not affect the

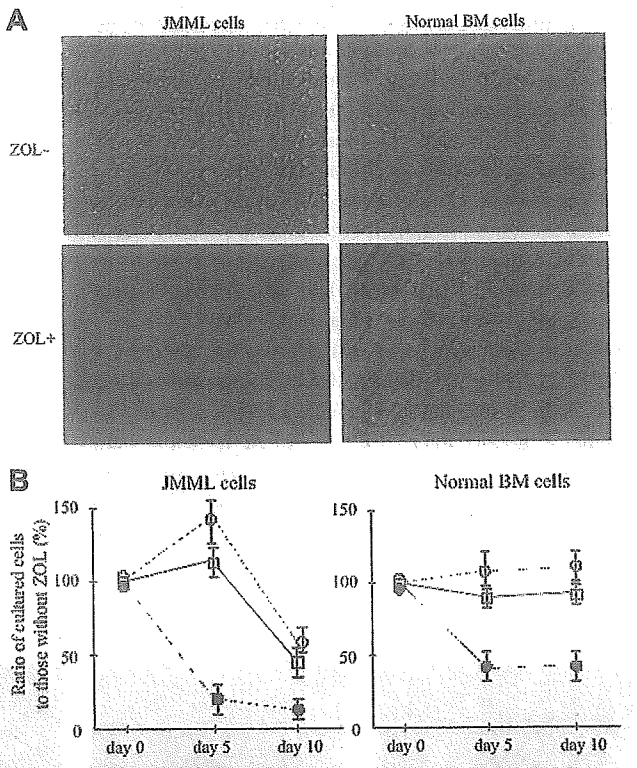

Figure 3. Effect of ZOL on the proliferation of JMML and normal BM cells in the suspension culture. (A) Phase microscopy of adherent cells developed from BM cells of a patient with JMML (case 4) and a healthy donor (case 3) at day 15 of suspension culture without and with 10 ng/mL ZOL (microscope, ITM2; camera module, DP50; objective lens, DPlan Ap10 UV, Olympus; numerical aperture, 0.4; magnification, × 40). A number of adherent cells were observed in the culture of JMML cells without ZOL, but their development was suppressed by the addition of ZOL. The growth of adherent cells in the culture of normal BM cells was not affected by the addition of ZOL. (B) The inhibition rate by 1, 10, and 100 μM ZOL in the proliferation of JMML and normal BM cells in the suspension culture without and with 10 ng/mL GM-CSF, respectively, at days 5 and 10. Each value indicates the mean ± SD calculated from the data in 3 patients with JMML and 3 healthy donors. □ indicates ZOL 1 μM; ○, ZOL 10 μM; ●, ZOL 100 μM.

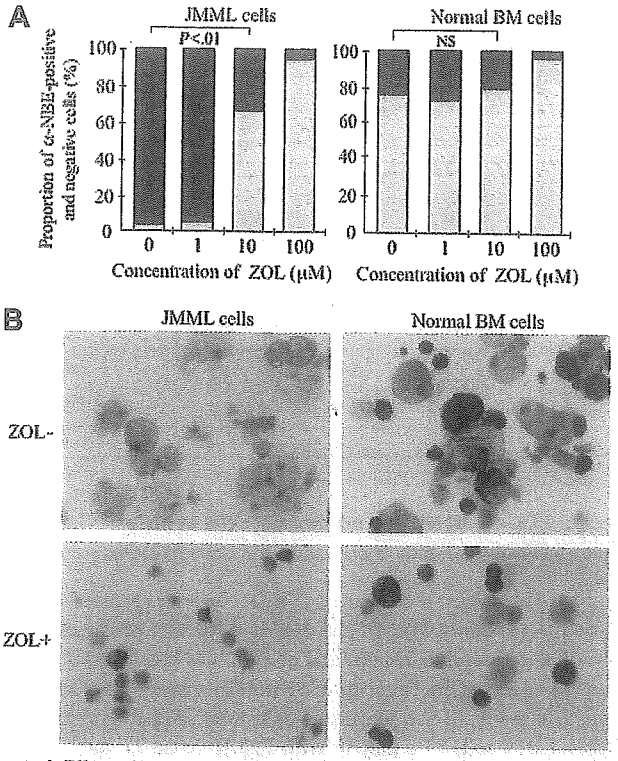

Figure 4. Effect of ZOL on the differentiation of JMML and normal BM cells in the suspension culture. (A) Effect of ZOL on the proportion of α-NBE–positive and –negative cells in the suspension culture of JMML and normal BM cells. Each value indicates the mean calculated from the data for 3 patients with JMML and 3 healthy donors. NS indicates not significant. ■ indicates α-NBE-positive cells; ▨, α-NBE-negative cells. (B) Appearance of α-NBE–positive and –negative cells in the suspension culture of BM cells of a patient with JMML (case 4) and a healthy donor (case 5) without and with 10 ng/mL GM-CSF (microscope, BH2, Olympus; camera module, DP70, Olympus; objective lens, Span, Olympus; numerical aperture, 0.7; magnification, × 40). α-NBE–positive macrophages possess brown granules in their cytoplasm.

proportion of α-NBE–positive macrophages and α-NBE–negative granulocytes at concentrations of 1 and 10 μM, but, in the culture with 100 μM ZOL, most of the cultured cells were α-NBE–negative granulocytes (Figure 4A-B).

The results in the suspension culture indicate that 100 μM ZOL inhibits the development of both JMML and normal BM cells, but 10 μM ZOL predominantly inhibits the spontaneous proliferation and differentiation along monocyte/macrophage lineage of JMML BM cells.

Flow cytometric analysis of the cells in the suspension culture of JMML and normal BM cells

The effect of ZOL on the differentiation of JMML and normal BM cells in the suspension culture was analyzed by flow cytometry. Figure 5 shows a representative flow cytometric profile of nonerythroid BM cells of a patient with JMML (case 4) before and after 10 days of suspension culture with and without 10 μM ZOL, and Table 2 summarizes the ratio of granulocytes, monocytes/macrophages, lymphocyte, and blastic cells in the population excluding CD45-negative nucleated erythroid cells before and after 10 days of the suspension culture of BM cells from 3 patients with JMML (cases 4, 6, and 7) and 3 healthy donors (cases 1, 3, and 5), which was determined by using SSC and CD45 staining.

Approximately one third of the nonerythroid cells obtained from BM cells of patients with JMML were monocytes/macrophages, characterized by high SSC and bright CD45 expression, which also expressed CD11b, CD13, CD14, CD16, and HLA-DR but no CD34, and half were high SSC and lower-density CD45-expressing granulocytes that were $CD13^+$, $CD16^+$, and $CD33^+$, but $CD14^-$ and $CD34^-$ (Figure 5; Table 2; data not shown). There was also a substantial number of lower SSC and bright CD45-expressing lymphocytes and lower SSC and lower CD45-expressing blastic cells that expressed HLA-DR and CD34 but neither CD11b, CD13, CD14, CD16, nor CD33. When cultured for 10 days, JMML BM cells generated a large number of mature macrophages revealing more bright CD45 expression. Granulocytes accounted for only 2% ± 1%, but 6% ± 2% of the population were blastic cells. On the addition of greater than 10 μM ZOL, however, the production of monocytes/macrophages was significantly suppressed, and the monocyte/macrophage population contained a proportion of lower CD11b-expressing immature monocytes/macrophages. A third of the cultured cells were granulocytes, and a substantial number of blastic cells were still present (8% ± 1%). Nonerythroid cells obtained from normal BM cells consisted of 77% ± 9% granulocytes, 6% ± 4% $CD11b^{bright}$/$HLA\text{-}DR^+$ mature monocytes/macrophages, 12% ± 4% lymphocytes, and a few blastic cells expressing CD34 (Table 2; data not shown). In the culture of normal BM cells with GM-CSF, macrophages also increased, but the percentage was 28% ± 3%. A large number of granulocytes existed, but no or few blastic cells did. The addition of

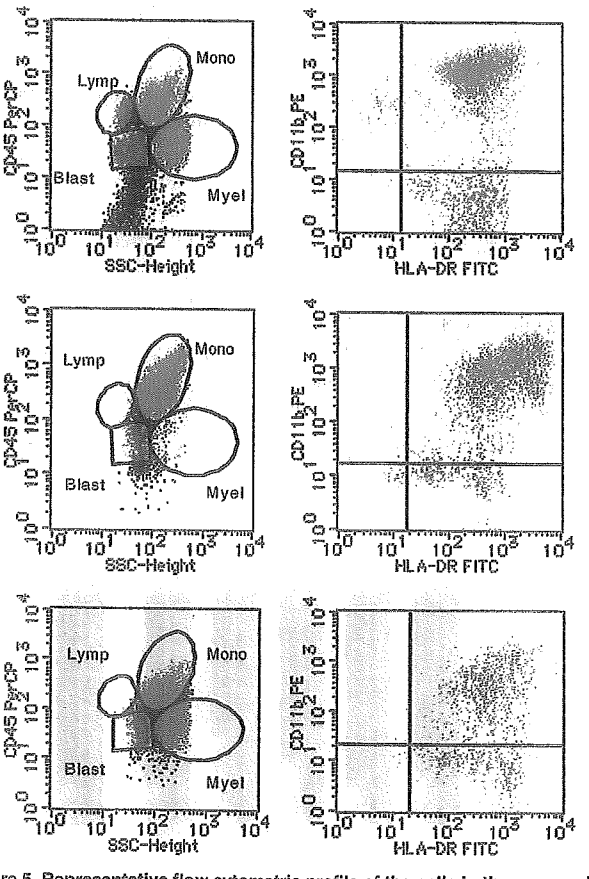

Figure 5. Representative flow cytometric profile of the cells in the suspension culture of JMML BM cells. The nonerythroid BM cells of a patient with JMML (case 4) before (top row) and after 10 days of culture without and with 10 μM ZOL (middle and bottom rows, respectively) were examined by flow cytometry. Populations of granulocytes (Myel, dark blue dots), monocytes/macrophages (Mono, green dots), lymphocytes (Lymp, bright blue dots), and blastic cells (Blast, red dots) were identified by a combination of SSC and CD45 staining (left). CD11b and HLA-DR expression in monocytes/macrophages and blastic cells was further analyzed (right).