(MNCs) from CB samples were isolated using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) density gradient centrifugation and washed three times in phosphate-buffered saline (PBS). MNCs were then enriched for $CD34^+$ cells using the CD34 Progenitor Cell Selection System (Dynal, Oslo, Norway) according to the manufacturer's instructions. The purity of $CD34^+$ cells (>90%) was determined by flow cytometry.

Cell cultures

Enriched $CD34^+$ cell populations were divided into three aliquots. They were cultured with or without AMD3100 (10 mM; Sigma Chemicals) for 2 h in RPMI-1640 (Sigma Chemicals, St. Louis, MO) without serum at 1×10^6 cells/ml (10^5 to 2×10^5 cells per well) in 96-well round bottom microtiter plates (Costar; Corning, Inc., Corning, NY). All cultures were performed at 37°C under a humidified atmosphere of 5% CO_2. A third aliquot was neither treated nor cultured and used immediately for all experiments without preservation.

Flow cytometric analysis

The presence of cell surface antigens was determined with a FACSCalibur (BD Biosciences, San Jose, CA) using the following fluorescein-conjugated monoclonal antibodies (mAbs): FITC-labeled anti-CD45 (2D1; BD Biosciences), PE-labeled anti-CD34 (AC136; Miltenyi Biotec, Bergisch-Gladbach, Germany), APC-labeled anti-CXCR4 (12G5; BD Biosciences), PE-labeled anti-CD26 (M-A261; BD Biosciences), APC-labeled anti-CD38 (HIT2; BD Biosciences), PE-labeled anti-CD33 (WM53; BD Biosciences), PE-labeled anti-CD19 (SJ25C1; BD Biosciences), and PE-labeled anti-CD3 (UCHT1; BD Biosciences). To identify and disregard dead cells from our analyses, cell cultures were stained with propidium iodide (PI; Sigma Chemicals, St. Louis, MO) at a concentration of 1 μg/mL.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to determine *CXCR4* mRNA expression in cultured versus noncultured cells. Total RNA from cultured $CD34^+$ cells was extracted using the acid–phenol technique. RT-PCR was performed according to the manufacturer's protocol (Takara Bio, Inc., Shiga, Japan). The following specific primers were used to amplify *CXCR4*: sense, 5′-ggc cct caa gac cac agt ca-3′; antisense, 5′-tta gct gga gtg aaa act tga ag-3′ [12]. As an internal control, we also examined the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in all samples. Amplified DNA fragments were then electrophoresed on an agarose gel and visualized by ethidium bromide staining.

Migration assay

Cells were incubated for 30 min at 37°C in 5% CO_2 in the upper chambers of a 96-well transwell apparatus (1×10^5 cells per well; QCM™ Chemotaxis 5 μm 96-Well Cell Migration Assay; Chemicon, Temecula, CA) containing RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; StemCell Technologies, Vancouver, BC, Canada) and then allowed to migrate to the lower chamber containing 125 ng/ml SDF-1α (Sigma Chemicals). The number of cells that migrated to the lower chamber was scored visually with a light microscope.

Transplantation into mice

NOD/Shi-scid jic mice obtained from CLEA (Kawasaki, Japan) were bred and maintained under controlled conditions in individually ventilated (high-efficiency particle-arresting filtered air), sterile microisolator cages at the Hiroshima University Animal Institute. Before transplantation, 6- to 8-week-old mice were subjected to a sublethal dose of 300 cGy total-body irradiation. Then, 1×10^5 cultured or noncultured $CD34^+$ cells were injected into the tail veins of irradiated mice.

Homing assay

Sixteen hours after tail vein injection, the mice were killed and PBS was used to flush bone marrow cells from femurs and tibias. The cells obtained in this manner were analyzed by flow cytometry for the presence of human cells using human-specific anti-CD45-FITC and anti-CD34-PE. At least 1×10^6 cells were analyzed.

Engraftment analysis

To assess the engraftment of human cells into murine bone marrow, cell populations obtained from the murine marrows were incubated with antihuman, fluorescein-conjugated mAbs against CD45, CD34, CD33, CD19, and CD3 and then analyzed via flow cytometry. Engraftment analysis was performed 8 weeks after transplantation.

Secondary transplantation

Bone marrow cells were harvested from NOD/SCID mice in which CB $CD34^+$ cells had previously engrafted. Three mice were injected with 1×10^7 MNCs after sublethal irradiation at 300 cGy. Eight weeks after secondary transplantation, human cells in murine bone marrow were labeled with the same antihuman mAbs used in the primary engraftment analysis and subjected to flow cytometry.

Statistics

All data are expressed as the mean ±standard deviation (SD). Statistically significant differences within the data set were detected using the Student's *t*-test and Wilcoxon's signed-ranks test. All analyses were performed using the StatView software (version 5.0; SAS Institute, Cary, NC).

Results

Flow cytometric analysis of CXCR4 expression on $CD34^+$ cells from umbilical cord blood

Consistent with previous studies, a subpopulation of $CD34^+$ cells from umbilical CB constantly expressed CXCR4

TABLE 1. PERCENTAGE OF CB CD34$^+$ CELLS THAT ARE CXCR4$^+$, CD26$^+$, AND CD38$^-$

	Incubation		
	(−)	(+)	
		AMD3100	
Surface markers		(−)	(+)
CXCR4$^+$	8.0 ± 5.0	19.8 ± 8.7*	5.2 ± 3.4
CD26$^+$	7.7 ± 3.5	7.7 ± 4.7	7.6 ± 3.6
CD38$^+$	4.3 ± 2.0	5.5 ± 3.2	5.3 ± 2.8

Data represent the mean ±SD of six independent experiments. CD34$^+$ cells from CB were incubated with or without 10 mM AMD3100 for 2 h. *$p < 0.05$, compared to noncultured and AMD3100-treated cells.

on the cell surface (7.7 ± 3.6%). The cell surface expression of CXCR4 increased after culturing CD34$^+$ cells for 2 h (19.8 – 8.6%, Fig. 1, $p < 0.05$). It increased to >30% after 6 h in culture, but PI staining showed that the nonviable cells at 6 h were >30% relative to <10% at 2 h. Viability of the cultured cells at for 2 h was not different from that of the noncultured cells. Culturing for 6 h, therefore, was not done after this first experiment. The increase in the CXCR4 level was not exhibited in the presence of AMD3100, a CXCR4 antagonist. Next, we cultured CD34+ cells in the presence or absence of AMD3100 and examined the expression of CD26, CD38, and CXCR4. As shown in Table 1, a 2-h incubation with AMD3100 did not affect the expression of CD26 or CD38.

RT-PCR analysis of CXCR4 expression

The expression of *CXCR4* mRNA during short-term incubation was examined via RT-PCR. As shown in Figure 2, the addition of AMD3100 had no affect on *CXCR4* mRNA expression after a 2-h incubation. A quantitative real-time RT-PCR analysis using the same samples showed no difference in *CXCR4* expression after a 2-h incubation (data not shown). Thus, the observed increase in cell surface expression was not the result of increased mRNA expression.

Migration and homing activity of cultured cells

Because 2-h incubation increased CXCR4 expression on the surface of CD34$^+$ cells, we examined the in vitro migration of these cells toward the CXCR4 ligand, SDF-1. As shown in Figure 3, over 25% of CD34$^+$ cells cultured for 2 h showed transmigration in response to SDF-1. However, transmigrational activity was significantly lower in noncultured cells and in cells which had been cultured in the presence of AMD3100 ($p < 0.05$). Furthermore, the effect of increased CXCR4 expression on homing activity was determined by evaluating the presence of human CD45+/CD34+ cells in mouse bone marrow 16 h after tail vein injection. As shown in Figure 4A, a cluster of CD45+/CD34+ cells (R1) was detected in the bone marrow of mice injected with cultured CD34+ cells. Homing activity was compared among cells cultured in the presence and absence of AMD3100 and

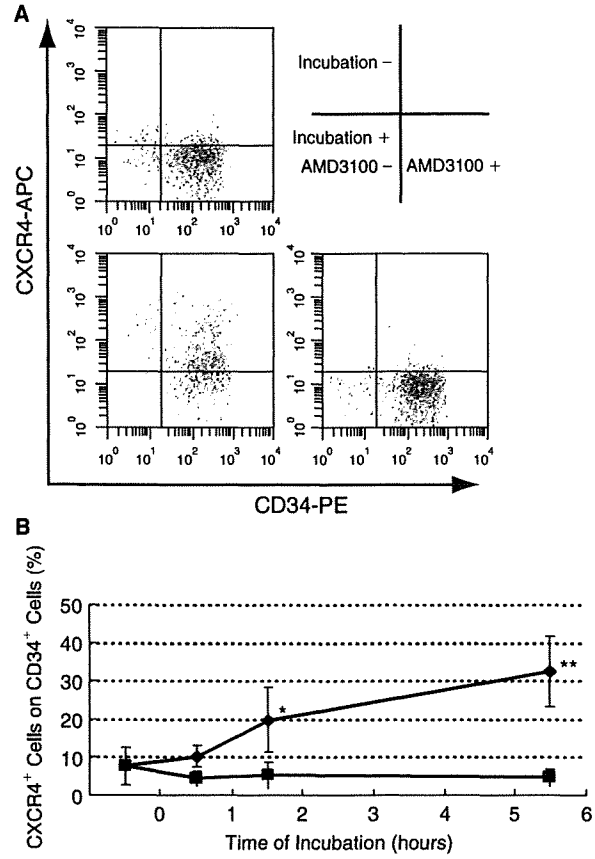

FIG. 1. Effect of short-term culture with or without AMD3100 treatment on CXCR4 expression on CD34$^+$ cells. Representative flow cytometry profiles for CXCR4 expression in cultured cells with or without AMD3100 treatment (**A**). CXCR4 expression on CD34$^+$ cells during short-term incubation with AMD3100 (◆) or without AMD3100 (◆) (**B**). Data represent the mean ±SD of six independent experiments. And, CXCR4 expression increased significantly on the surface of CD34$^+$ cells (*$p < 0.05$, **$p < 0.01$).

noncultured cells. Cultured CD34+ cells demonstrated significantly greater homing activity compared to noncultured cells and to cells cultured in the presence of AMD3100 ($p < 0.05$ in each case).

Engraftment of cultured CD34$^+$ cells into NOD/SCID mice

Figure 5 shows a representative FACS analysis of human marrow cells in bone marrow from identical NOD/SCID mice after CD34$^+$ cell transplantation. The presence of human cells was detected by the cell surface expression of CD45, CD34, CD33, CD19, and CD3. The recipients exhibited CD45$^+$/CD19$^+$ B-lymphoid cells, CD45$^+$/CD33$^+$ granulomonopoietic cells, and CD45$^+$/CD34$^+$ immature cells, but not CD3$^+$ T cells. The percentage of CD34$^+$/CD19$^+$ positive cells

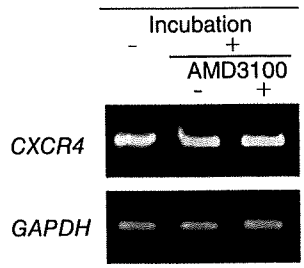

FIG. 2. Effect of short-term incubation on *CXCR4* mRNA expression in CD34⁺ cells. Total RNA was extracted from cells after the cells were incubated under the indicated culture conditions, RT-PCR was performed. *GAPDH* was used as an internal control. Short-term culture had no effect on *CXCR4* mRNA expression in CD34⁺ cells.

in the CD45⁺ cells was 6–12%, consistent with the report of Kobari et al. [13]. Next, at 8 weeks, we performed a secondary transplant of engrafted cells from previously transplanted mice to determine if long-term engraftment of self-renewing cultured CB cells had occurred. Successful secondary engraftment occurred as shown by the presence of CD45⁺/CD19⁺ cells and possibly CD45⁺/CD34⁺ cells (Fig. 5B).

To assess the effect of short-term culture and AMD3100 interference on engraftment, CD34⁺ cells were cultured with or without AMD3100 as indicated and injected into NOD/SCID mice via the tail vein. Eight weeks after transplantation, engraftment was assessed based on the expression of the human CD45 antigen. As shown in Figure 6, cultured CD34⁺ cells showed increased engraftment into murine bone marrow compared to noncultured CD34⁺ cells (32.0 ± 21.4% vs. 17.1 ± 15.7%), $p < 0.01$, or to CD34⁺ cells from AMD3100-treated cultures (12.9 ± 11.1%), $p < 0.01$, as determined by Wilcoxon's signed-ranks test.

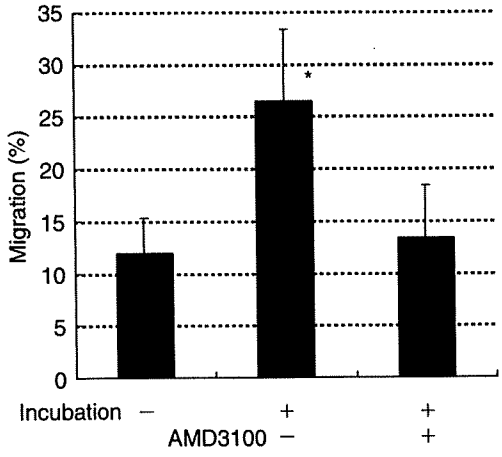

FIG. 3. Effect of short-term incubation and AMD3100 treatment on transmigrational activity in CD34⁺ cells. CD34⁺ cells were loaded into the upper wells of a transwell apparatus and incubated for 2 h. Migration was assessed according to the percentage of cells migrated to lower wells containing SDF-1. The data represent the mean ± SD of six independent experiments. * $p < 0.05$, significant increase compared to noncultured and AMD3100-treated cells.

Discussion

Umbilical CB contains hematopoietic cells capable of engrafting into NOD/SCID mice in vivo. Our results demonstrate that short-term culture in the absence of serum and/or cytokines induced the expression of CXCR4 on umbilical CB-derived CD34⁺ cells, resulting in enhanced homing activity and subsequently improved engraftment into NOD/SCID mice. The enhanced homing and engraftment of short-term cultured CD34+ cells was abolished by the addition of AMD3100 to the cultures, suggesting that improved engraftment was dependent upon increased CXCR4 expression.

Lataillade et al. [14] reported that cell surface expression of CXCR4 increased significantly when purified progenitor cells were incubated overnight in serum. Peled et al. [9] demonstrated that human CD34⁺ cells incubated with stem cell factor and IL-6 for 48 h showed increased CXCR4 expression, resulting in enhanced in vitro migration toward an

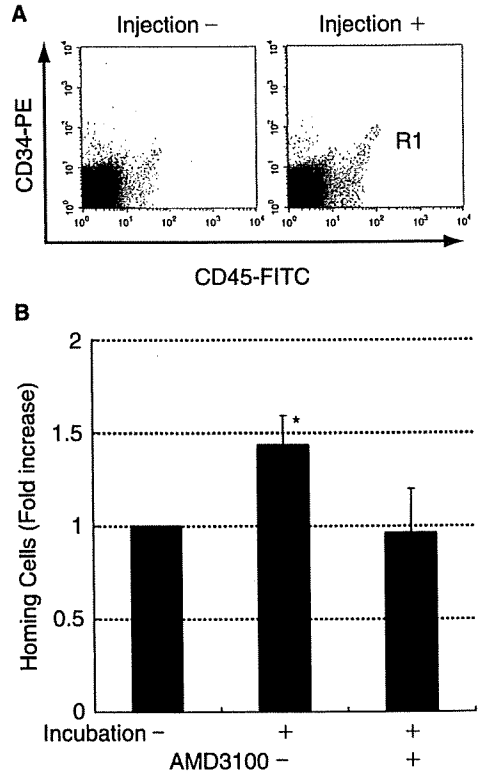

FIG. 4. Homing activity of CD34⁺ cells in NOD/SCID mice. CD34⁺ cells were cultured as indicated and then injected into the tail veins of NOD/SCID mice. After 16 h, the mice were killed and human-derived cells in the bone marrow were quantified based on the presence of a CD45⁺/CD34⁺ cluster (R1) in flow cytometry (**A**). The fold-increase compared to noninjected mice was calculated (**B**). The data represent the mean ±SD of six independent experiments. *$p < 0.05$, significant increase compared to noncultured and AMD3100-treated cells.

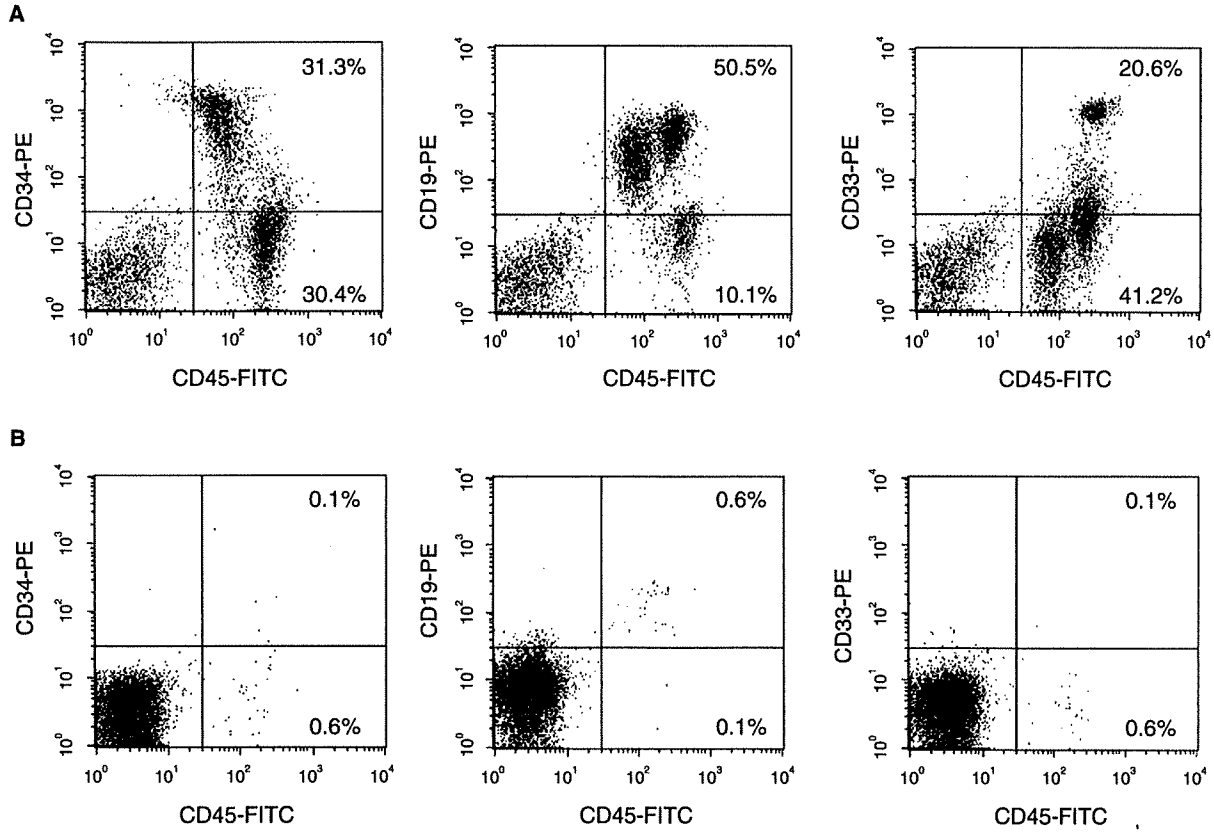

FIG. 5. Representative flow cytometry profiles of human CD45⁺, CD34⁺, CD33⁺, and CD19⁺ cells obtained at 8 weeks after transplantation. The following fluors were coupled to each antibody: CD45-FITC, CD34-PE, CD33-PE, and CD19-PE. Similar results were obtained in 12 additional experiments (**A**). Secondary transplantation of bone marrow cells obtained from previously transplanted mice (**B**). Marrow cells obtained at 8 weeks.

SDF-1 gradient and increased homing and repopulating potential in NOD/SCID and NOD/SCID/beta2-microglobulin null mice. In contrast, the long-term culture of CD34⁺ cells in a cytokine cocktail decreased CXCR4 expression and led to reduced repopulating potential in immunodeficient mice [15]. The mechanism underlying CXCR4 up-regulation during incubation remains unclear, although various factors, including cell surface adhesion molecules, cell–cell contact between CD34⁺ and low-density MNCs, growth factor production, and adhesion molecule interactions, may be involved [4,5,16]. Our RT-PCR results showed that cell surface CXCR4 expression increased in cultured CD34⁺ cells without concomitant increases in mRNA expression.

CXCR4 is thought to play a crucial role in the homing and retention of HSCs in murine bone marrow [9] and is required for the retention of B-lineage and myeloid precursors in the bone marrow [16]. Recently, Kahn et al. [11] reported that the overexpression of CXCR4 in human CD34⁺ cells via gene transfer increased proliferation, migration, and NOD/SCID repopulation. Similarly, we confirmed the importance of CXCR4 expression in CD34⁺ cell in NOD/SCID marrow homing through the inhibitory effect of AMD3100. AMD3100, a small bicyclam molecule, reversibly inhibits SDF-1–CXCR4 binding [17].

In conclusion, we demonstrated that short-term culture of CB-derived CD34⁺ cells increased CXCR4 expression on these cells, resulting in increases in in vivo homing and engraftment into NOD/SCID mice. Thus, short-term culture of hematopoietic stem/progenitor cells in the absence of sera and cytokines may serve as a simple but effective way to enhance their repopulating activity.

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There is no possible conflict of interest (including financial and other relationship) for each author.

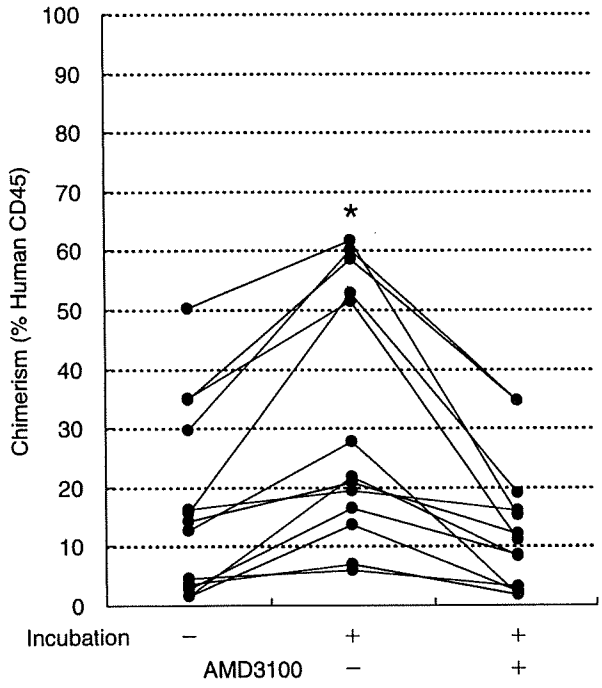

FIG. 6. Engraftment of CD34⁺ cells with or without the presence of AMD3100 into NOD/SCID mice. CD34⁺ cells were cultured as indicated and then injected into the tail veins of NOD/SCID mice. Eight weeks after transplantation, the mice were killed and percentage of human CD45⁺ cells was determined. The data represent the percentage of human CD45⁺ cells in 13 independent experiments. Statistically significant differences among cultured, noncultured, and AMD3100-treated cells were analyzed using Wilcoxon's signed-ranks test. $^*p < 0.01$, significant increase compared to noncultured and AMD3100-treated cells.

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Successful bone marrow transplantation in chronic granulomatous disease

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Key words bone marrow transplantation, life-threatening infections, mixed chimerism, neutrophil function, non-myeloablative conditioning.

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Chronic granulomatous disease (CGD) is an inherited disorder of phagocyte function, characterized by recurrent, often life-threatening, bacterial and fungal infections and granulomas in several organs.[1] In CGD, phagocytes cannot generate microbicidal oxygen metabolites due to a defect in one of the four genes encoding the four subunits of nicotinamide adenine dinucleotide

phosphate oxidase (*gp91phox*, *p47phox*, *p67phox*, and *p22phox*).[2] The standard prophylaxis against infections in patients with CGD consists of antibiotics, antimycotics, and interferon (IFN)-γ.[3,4] Despite prophylaxis, patients remain susceptible to life-threatening infections, and the annual mortality is still between 2% and 5%.[5] Recently, allogeneic stem cell transplantation with myeloablative or non-myeloablative conditioning was shown to be a feasible treatment for patients with CGD.[5–8] Herein we present a patient with CGD who had critical infections refractory to conventional treatment, including multiple abscesses in the brain and multiple osteomyelitis. The patient underwent allogeneic bone marrow transplantation (BMT) from a human leukocyte antigen (HLA)-identical sibling using a non-myeloablative conditioning protocol. The appearance of functional neutrophils ameliorated the life-threatening infections.

Case report

A 20-year-old man with X-linked CGD (Leu342Gln in exon 9, Gly472Ser in exon 11, and complete gp91-phox deficiency) was hospitalized due to intractable infection. He had been diagnosed with CGD at the age of 5 years because of recurrent bacterial infections and had been on trimethoprim–sulfamethoxazole prophylaxis. He had suffered from a perianal abscess with a fistula to the rectum since the age of 15 years, and osteomyelitis of the right tibia was discovered 3 months before admission to hospital. He underwent curettage of the lesion followed by the administration of antibiotics, antifungal agents, and IFN-γ for 1 month. No pathogens were isolated from the bone lesion. Despite these treatments, he suffered from recurrent headache and fever. Multiple intracranial abscesses and multiple osteomyelitis were found on computed tomography (CT) of the head and ^{99m}Tc scintigraphy (Fig. 1).

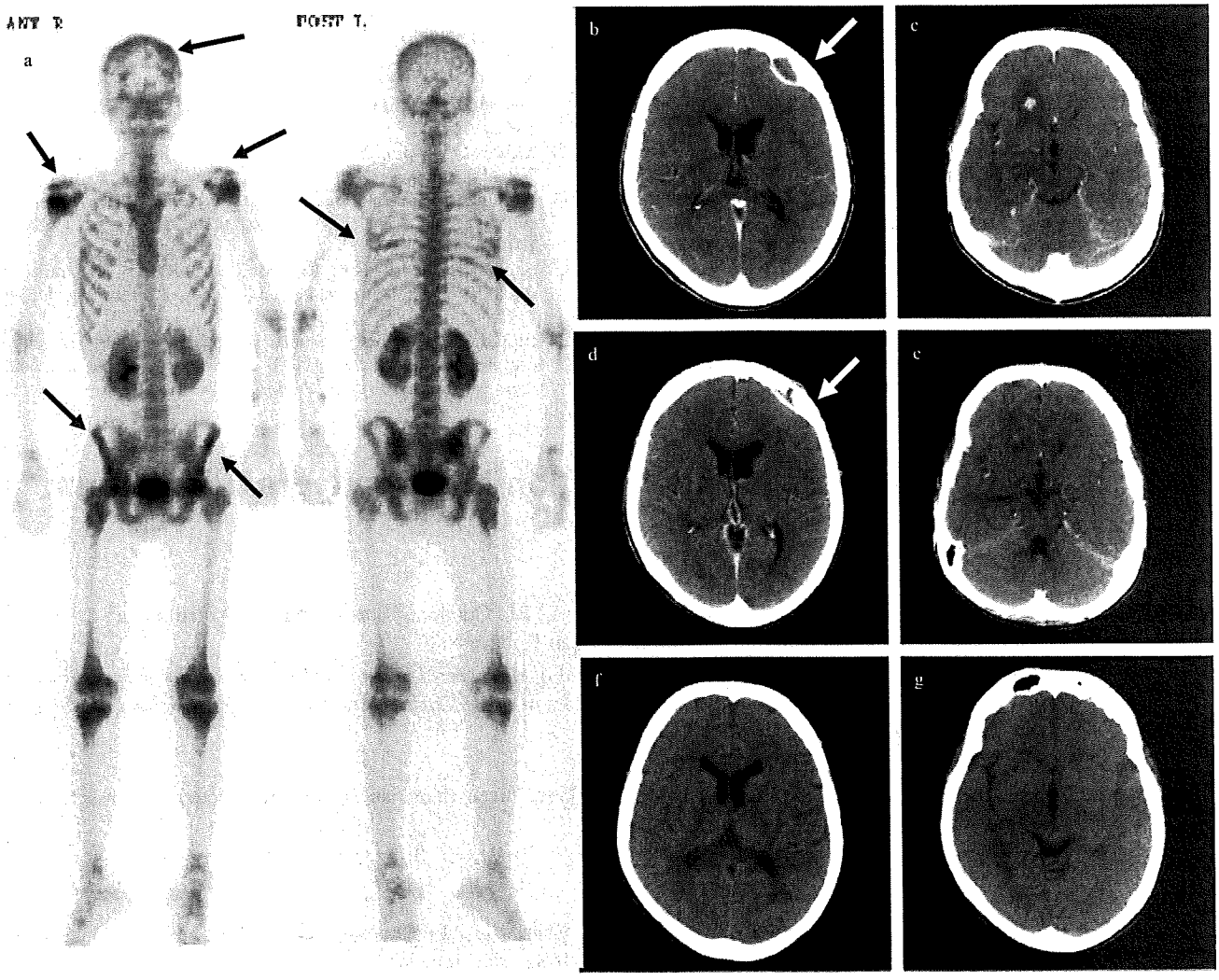

Fig. 1 (a) ^{99m}Tc scintigraphy showing multiple osteomyelitis. (b,c) Computed tomography of the head before transplantation showed a subdural abscess with an osteolytic lesion, an abscess beside the right anterior horn of the lateral ventricle (arrows), and multiple brain abscesses. (d,e) Twenty-three days after transplantation, a subdural abscess (arrows) with an osteolytic lesion ruptured. (f,g) Forty-five days after transplantation, all the brain abscesses had disappeared.

Table 1 Laboratory data on admission

Peripheral blood		Biochemistry	
White blood cell count	14 400/μL	Total bilirubin	0.5 mg/dL
Neutrophilic granulocyte	74.9%	Aspartate aminotransferase	27 IU/L
Lymphocyte	9.0%	Alanine aminotransferase	31 IU/L
Monocyte	12.8%	Lactic dehydrogenase	148 IU/L
Red blood cell count	386/μL	Alkaline phosphatase	2742 IU/L
Hemoglobin	9.3 g/dL	Urea nitrogen	10 mg/dL
Hematocrit	27.5%	Creatinine	0.37 mg/dL
Platelet count	394 × 10^3/μL	Total protein	7.5 g/dL
Reticulocyte	0.93%	Albumin	3.1 g/dL
		Sodium	131 mEq/L
Serological test		Potassium	3.5 mEq/L
C-reactive protein	11.4 mg/dL	Chloride	94 mEq/L
IgG	2030 mg/dL	Calcium	4.4 mEq/L
IgA	374 mg/dL	Phosphate	4.7 mEq/L
IgM	89 mg/dL	Iron	19 μg/dL
Ferritin	261 ng/mL		
β-D-glucan	Negative		

On admission to hospital the patient complained of mild dysphagia, muscle weakness in his right arm, neck pain and stiffness, headaches, and persistent fever. Laboratory data indicated leukocytosis with an increased proportion of neutrophils and persistent high levels of C-reactive protein (>10 mg/dL; Table 1). Given his uncontrolled infections and the absence of indications for surgical treatment, we decided on hematopoietic stem cell transplantation using a reduced-intensity conditioning protocol from an HLA-identical brother. Informed consent for BMT and donor lymphocyte infusion (DLI) were obtained from the patient and his brother prior to transplantation.

Due to his serious infections, non-myeloablative conditioning was applied, according to the method of Horwitz *et al.* with some modifications.[7] The conditioning regimen consisted of cyclophosphamide 25 mg/kg (days −7 to −5), fludarabine phosphate 25 mg/m^2 (days −7 to −3), antilymphoglobulin 15 mg/kg (days −7 to −5), and total body irradiation (3 Gy, day −2). The prophylaxis for graft-versus-host disease (GVHD) consisted of cyclosporine A and short-term methotrexate. Bone marrow was transfused with a total of 4 × 10^8/kg nucleated cells/kg (4.5 × 10^6/kg CD34-positive cells were observed). The white blood cell count reached 1000/μL with >500/μL neutrophils on day 19, and the platelet count increased to 20 000/μL on day 24. Reticulocytes appeared on day 28, and C-reactive protein decreased to <0.3 mg/dL on day 26. Prior to the increase in neutrophil count, CT showed a decrease in the size of the brain abscesses, which disappeared on day 40 after BMT.

We examined donor–recipient chimerism serially using a variable number of tandem repeats of lymphocytes and *gp91phox* expression on neutrophils.[9] H_2O_2 production from neutrophils was also examined using flow cytometry, based on the reduction of dihydrorhodamine 123.[10] Figure 2 shows the percentages of donor-derived lymphocytes and neutrophils, and neutrophils producing H_2O_2 before and after transplantation. The percentage of donor lymphocytes was >60%, whereas that of donor neutrophils was <40% on days 30 and 90 after transplantation. Based on the report of Horwitz *et al.* we infused donor peripheral lymphocytes containing 5 × 10^6 and 10 × 10^6 CD3-positive cells /kg on days 38 and 105 after transplantation, respectively, which resulted in the presence of a high proportion of donor cells.[7] Figure 1 shows the improvement in multiple intracranial abscesses in the present patient after transplantation.

After the first DLI the patient had mild acute GVHD of the skin (grade I) on day 42. The skin lesion immediately disappeared following short-term administration of corticosteroid hormone. The patient experienced no acute and/or chronic GVHD after the second DLI on day 105. Six months after transplantation, complete replacement of the donor-derived lymphocytes and neutrophils was observed. At 30 months after BMT, he had no transplantation-related or intractable infection-related complications, with a 100% Karnofsky index.

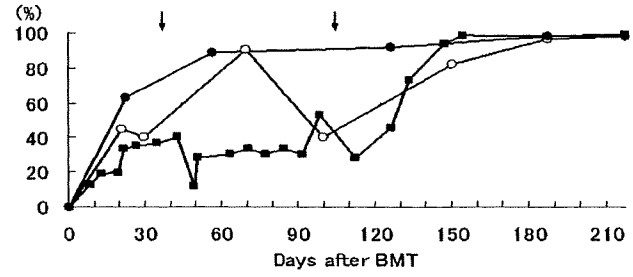

Fig. 2 Engraftment of donor cells and the frequency of functional neutrophils. The percentages of (●) donor lymphocytes and (○) neutrophils were determined based on the variable number of tandem repeats and the expression of *gp91phox* using the monoclonal antibody 7D5. The frequency of (■) H_2O_2-producing neutrophils was determined using the dihydrorhodamine oxidation assay with flow cytometry. Arrows indicate donor lymphocyte infusion.

Discussion

We report successful BMT in a patient with CGD who had life-threatening, refractory infections. Recent studies have reported that allogeneic hematopoietic stem cell transplantation is effective not only as curative therapy, but also as treatment for active infections refractory to conventional therapy in patients with CGD.[5–8] After the development of a non-myeloablative conditioning regimen, allogeneic stem cell transplantation was demonstrated to be safe from toxicity. Horwitz *et al.* reported the efficacy of hematopoietic stem cell transplantation with non-myeloablative conditioning in patients having CGD without conditioning-related morbidity and mortality.[7] The present patient tolerated non-myeloablative conditioning, which led to the induction of a mixed chimerism of neutrophils in the peripheral blood. This resulted in relief from intractable infection, without major transplantation-related complications.

We serially monitored the frequency of H_2O_2-producing neutrophils using flow cytometry before and after transplantation. Neutrophils producing H_2O_2 first appeared on day 14 after transplantation. As shown in Figure 1, the frequency of neutrophils producing H_2O_2 was maintained at 30–40% for the first 3 months, and then gradually increased to normal levels. In accordance with the presence of 30–40% functional neutrophils, the patient's infections improved gradually. In particular, the size of the brain abscesses decreased immediately, leading to amelioration of his neurological symptoms. Female carriers of the X-linked form of CGD do not develop recurrent, refractory infections, despite the presence of approximately 50% of neutrophils producing active oxygen species. This implies that even partial engraftment of donor stem cells should restore normal phagocyte function in the recipient. The clinical course in the present case strongly implies that a small percentage of functional neutrophils are sufficient to improve infections that are refractory to conventional treatment.

We were able to ascertain the donor–recipient chimerism in detail due to the prevention of graft rejection. As reported previously, donor lymphocytes can facilitate the engraftment of donor stem cells.[7] Donor lymphocytes were infused in the present patient 1 month after transplantation, because <60% of the donor lymphocytes and neutrophils expressed gp91. Consequently, complete chimerism was achieved on day 190 (6 months). Thus, it is important to observe donor–recipient chimerism until complete chimerism is achieved following stem cell transplantation using non-myeloablative conditioning.

Collectively, the present findings suggest that the emergency transplantation of hematopoietic stem cells from an HLA-identical sibling with non-myeloablative conditioning is effective for improving intractable infections without severe conditioning-related complications. Therefore, hematopoietic stem cell transplantation might serve as an alternative therapeutic approach in patients with CGD who have organ dysfunction, overt infections, and inflammatory or post-inflammatory complications.

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SHORT COMMUNICATION

Respiratory complications after haematopoietic stem cell transplantation in a patient with chronic granulomatous disease

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SUMMARY. Chronic granulomatous disease (CGD) is an inherited immunodeficiency disorder caused by defects in NADPH oxidase and characterized by recurrent life-threatening bacterial and fungal infections. Although CGD has been considered to be a target for gene therapy, bone marrow transplantation (BMT) is now selected as the radical treatment in most cases. We performed BMT in a patient with CGD with severe infections and experienced respiratory complications of diffuse alveolar haemorrhage and/or infection-associated alveolar haemorrhage. We suggest that attention be paid to signs of onset of alveolar haemorrhage during BMT in CGD patients.

Key words: chronic granulomatous disease, diffuse alveolar haemorrhage, haematopoietic stem cell transplantation.

Most of the chronic granulomatous disease (CGD) patients experience recurrent infections since infancy. The most frequent infections for CGD cases include pneumonia (*Aspergillus* sp.), purulent lymphadenitis (*Staphylococcus* sp.), subcutaneous abscess (*Staphylococcus* sp.), liver abscess (*Staphylococcus* sp.), osteomyelitis (*Serratia* sp.) and sepsis (*Salmonella* sp.); these infections often prove fatal to the patients (Winkelstein *et al.*, 2000). It is reported that in one third of the CGD patients who were administered interferon-γ as a treatment, the effectiveness of prevention against severe infection was demonstrated (International Chronic Granulomatous Disease Cooperative Study Group, 1991). Following the recent advances in post-transplantation management and the prevalent use of transplantation with a non-myeloablative conditioning regimen, haematopoietic stem cell transplantation (HSCT) is becoming the standard radical treatment for CGD (Horwitz *et al.*, 2001). In this study, we present a case of a 17-year-old male CGD patient whom we treated. As he was suffering from a severe infection, the only treatment option for him was HSCT. Generally, most CGD patients are still infectious when they undergo HSCT. In our case too, HSCT was performed in the patient while he was still infectious. Our patient died of an associated complication such as diffuse alveolar haemorrhage (DAH) and/or infection-associated alveolar haemorrhage (IAH). Therefore, we suggest that it is necessary to consider the complication of alveolar haemorrhage for CGD patients undergoing bone marrow transplantation (BMT).

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CASE REPORT

Our patient was a 17-year-old male with a positive family history; his older brother had been diagnosed with CGD and had died of severe pneumonia 2 years before the patient was referred to our hospital. Therefore, our patient was promptly diagnosed with CGD (deficiency of gp91; a subunit of NADPH oxidase) at birth itself. He has been repeatedly hospitalized for a skin abscess, severe pneumonia and aspergillosis since infancy. We selected HSCT as the radical treatment for CGD. He demonstrated certain infectious symptoms when he was admitted to our hospital. In particular, severe damage to his right lung because of aspergillosis was identified (Fig. 1a). Therefore, a variety of antibiotics along with amphotericin B were used for treating the patient before the transplantation to reduce the number and intensity of infectious foci.

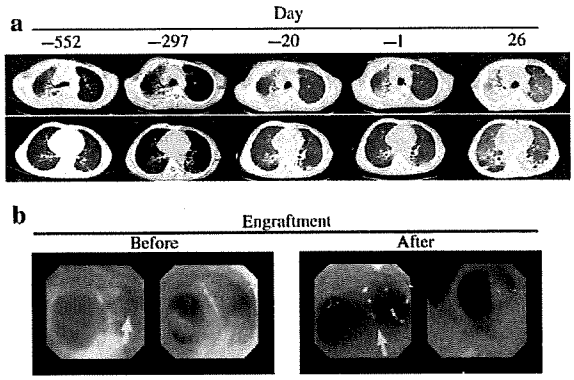

Fig. 1. Chest computed tomography (CT) changes over time and bronchoscopy findings. (a) High-density areas were found all over the lungs more than a year before BMT. A cavity was also identified in the right upper lobe. The lungs appeared to worsen with time; however, a day before the transplantation, a temporary improvement was observed. On day 26, ground-glass opacity was observed in both lungs covering each lung. (b) A small amount of bronchial haemorrhage was observed before engraftment. Although bleeding from the right bronchial tube was also identified, this haemorrhage was arrested within a few days (left panel). Bronchial haemorrhage started again after engraftment. At this time, a blood clot (indicated by arrow) was deposited in the bifurcation of the trachea, and active bleeding was identified in the right S6 area (right panel).

As he did not have an human leukocyto antigen (HLA) full-match sibling donor, he received reduced-intensity stem cell transplantation from an unrelated one-locus HLA-mismatched donor. Briefly, the patient received conditioning therapy (3 Gy of total body irradiation, 750 mg m^{-2} day^{-1} cyclophosphamide for 4 days, 25 mg m^{-2} day^{-1} fludarabine for 5 days, 70 mg m^{-2} melphalan for 1 day and 15 mg kg^{-1} day^{-1} antithymocyte globulin for 4 days) before transplantation.

Although the patient did not show any symptoms for the first few days after HSCT, his haematopoietic recovery was gradually delayed and the signs of infection became worse, particularly his respiratory status progressively deteriorated. Blood gas analysis showed that the CO_2 levels were extremely high on day 26. When the respiratory status became worse, 3–8 L min^{-1} of oxygen was given to patient with oxygen mask. Therefore, mechanical ventilation was used to alleviate the respiratory failure in the intensive care unit. As shown in Fig. 1a, worsened pneumonia was identified on day 26. On day 33, the white blood cell count from peripheral blood was 70 μL^{-1}, and his haematopoietic recovery was considered to have begun. Around the same time, bloody secretions were suctioned through the intubation tube, and we identified the cause as a small tracheal haemorrhage. Meanwhile, the FiO_2 was observed to be 0·4, and the SpO_2 was 100%. On day 44, although the white blood cell count from peripheral blood was above 500 μL^{-1} and engraftment was identified, he had extensive tracheal haemorrhage with progressive worsening of his respiratory status. Bronchoscopy demonstrated haemorrhage from the right upper and lower lobes (Fig. 1b). His respiratory status further worsened; it was not possible to maintain 90% of SpO_2 with 1·0 of FiO_2. Furthermore, CO_2 accumulation occurred, and acidosis was observed. On day 54, his blood pressure decreased and ventricular tachycardia appeared. His heart rate rapidly slowed down, and death occurred soon after. Figure 2 shows the summary of the process. Any bacteria and fungi had been not detected by blood cultures or through bronchoscope.

The autopsy showed traces of haemorrhage all over the lungs; there was no air infiltration area (Fig. 3a–c). Based on these findings, we diagnosed it as a case of DAH and/or IAH. Figure 3d shows the cavity in the right upper lobe from which *Aspergillus* was isolated (Fig. 3e–g).

DISCUSSION

During the 1980s and 1990s, the incidence of pulmonary complications after BMT was 30–60% (Cordonnier *et al.*, 1986; Jules-Elysee *et al.*, 1992). Although most of the complications involved infectious diseases such as pneumonia, this incidence decreased considerably

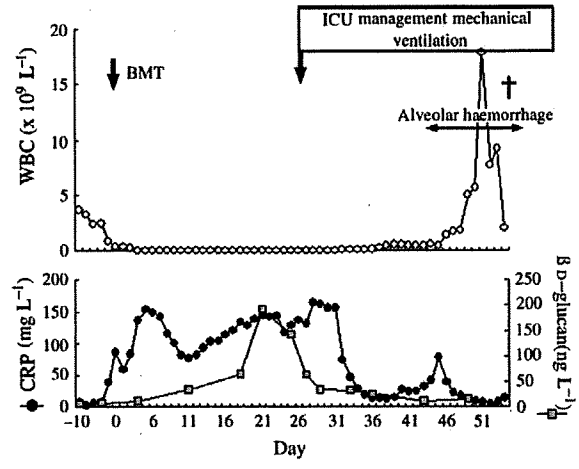

Fig. 2. Significant changes in laboratory results following HSCT. The delay in the haematopoietic recovery is shown. Because of the delay, the mycotic infection appears to have worsened. Alveolar hemorrhage appeared following the increase in the white blood cell (WBC) count. CRP, C-reactive-protein; ICU, intensive care unit. †, death of patient.

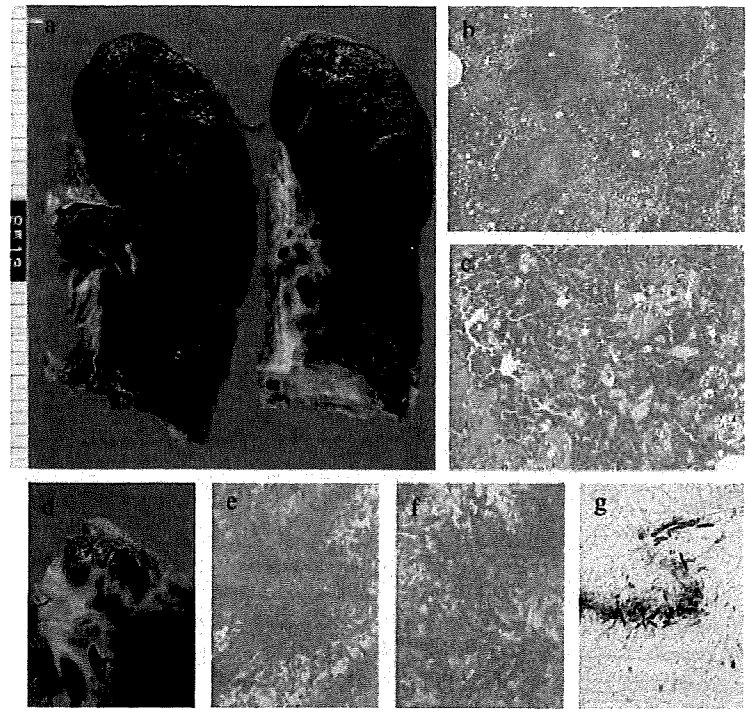

Fig. 3. Autopsy findings of the lungs. (a) The entire lungs are observed to be filled with blood with a complete absence of air-infiltrated areas. (b) All alveoli are also filled with blood. (c) A number of red blood cells are identified in the alveoli under high magnification. (d) There is a cavity in the right upper lobe. (e) Under high magnification, stick-like *Aspergillus* can be observed with haematoxylin and eosin staining. (f) *Aspergillus* observed with periodic acid-Schiff (PAS) staining. (g) *Aspergillus* observed with Grocott staining.

because of the development of new medicines and facilities. DAH is an associated complication in 5% of BMT patients (Afessa *et al.*, 2002b), and there is no significant difference in incidence between adults and children (Heggen *et al.*, 2002). The frequency of alveolar haemorrhage in our facility is 2·78%.

Non-infectious inflammation at the time of engraftment following HSCT is known as engraftment syndrome (ES) (Cahill *et al.*, 1996; Moreb *et al.*, 1997; Marin *et al.*, 1998; Ravenel *et al.*, 2002), and DAH is one of the typical findings of ES. Although the causes of DAH are not completely known, angiopathy because of conditioning therapy or radiation and immunogenicity caused by graft-vs.-host disease have been considered as causes. However, when symptoms caused by infectious diseases are observed, the condition is called IAH. Although it is difficult to differentiate between DAH and IAH, a case in which no infection is identified within 7 days of the occurrence of alveolar haemorrhage is diagnosed as DAH, and a case in which a pathogen is identified by either a blood test or a bronchoalveolar lavage (BAL) is diagnosed as IAH (Majhail *et al.*, 2006). A previous report has stated that high levels of certain types of cytokines – such as IL-1β, IL-6, IL-8, TNFα, MIP1α and G-CSF – identified by BAL after BMT are characteristic of DAH (Kharbanda *et al.*, 2006). Our patient already had a respiratory tract infection by aspergillosis when he underwent BMT. However, the mycotic infection had improved a week before the bronchial bleeding began (day 33), and when bronchial haemorrhage was observed (day 44), all other infections also showed improvement. For these reasons, although we diagnosed the patient as a case of DAH, it may be described as IAH because *Aspergilli* were observed by the autopsy.

It has been reported that certain underlying diseases are strongly associated with DAH; e.g. breast cancer patients are categorized as a high-risk group for DAH (Robbins *et al.*, 1989), and myeloma patients are in a higher risk group for DAH than are patients of malignant lymphoma (Capizzi *et al.*, 2001). HSCT has not been reported for many immunodeficient patients such as CGD patients, and few reports discuss this treatment. It has been reported that a delay in haematopoietic recovery and the administration of amphotericin B are considered as risk factors for ES, manifesting as DAH (Afessa *et al.*, 2002b; Gorak *et al.*, 2005). In this case, our patient had several risk factors. Most CGD patients are expected to undergo HSCT while already having certain risk factors. Therefore, caution should be exercised in patients who are considered to be at risk for ES. Although the administration of steroid hormones, particularly high doses of methylprednisolone, is effective for treating DAH, the patient outcome is not promising; the mortality rate is approximately 80% (Afessa *et al.*, 2002a,b; Robbins *et al.*, 1989; Lewis *et al.*, 2000; Huaringa *et al.*, 2000). In

particular, 61% of the patients who died had an onset of DAH 30 days or more after BMT (Afessa *et al.*, 2002a); the onset appears to be related to the delay in haematopoietic recovery. Other than steroid hormones, it has been reported that recombinant factor VIIa (Pastores *et al.*, 2003) and aminocaproic acid (Wanko *et al.*, 2006) are also effective as treatments for DAH. There are bound to be some cases where HSCT is needed as an emergency procedure such as in immunocompromised patients with CGD. However, in the case of non-emergency patients, we strongly suggest that the infectious disease that could delay the haematopoietic recovery be cured or adequately treated before HSCT.

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Cardiac infiltration in early-onset sarcoidosis associated with a novel heterozygous mutation, G481D, in *CARD15*

Sir, Early-onset sarcoidosis (EOS) and Blau syndrome (BS) are rare multi-organ granulomatous inflammatory disorders clinically characterized by the distinct triad of skin, joint and eye lesions without any apparent cardio-pulmonary involvement [1]. Gain-of-function mutations in *CARD15* (NM_022162) cause EOS and/or BS (EOS/BS) [2–4], but not the development of adult-type sarcoidosis [5, 6]. We identified a novel heterozygous gain-of-function mutation, G481D, in *CARD15* from a patient with EOS, who was suffering from recurrent episodes of congestive heart failure. Cardiac infiltration is a common clinical manifestation in adult-type sarcoidosis, but is rare and atypical in EOS/BS. Notably, the cardiac manifestations of this patient are quite similar to those in adult sarcoidosis. This is the first report demonstrating the precise manifestations of cardiac infiltration of sarcoidosis in a patient with a *CARD15* mutation.

The patient was an 18-year-old female. At 3 months of age she developed a miliaria-like skin rash. Thereafter, she presented various manifestations, such as uveitis, joint involvement, hepatosplenomegaly, arterial hypertension and congestive heart failure. A lymph node biopsy showed fresh-looking multiple granulomas, indicating a diagnosis of EOS. The administration of glucocorticoids improved her symptoms. However, extended treatments were required because of recurrent episodes of congestive heart failure accompanied with the activation of an autoinflammatory reaction. Echocardiography showed intraventricular septum thickness [Fig. 1A (a, b)]. A histopathological examination of the right ventricle endocardium revealed inflammatory cell infiltration, ballooning of myocardium and mild fibrosis (Fig. 1B). The histopathological findings were similar to those of cardiac sarcoidosis in adults. Other immunosuppressive treatments, e.g. NSAIDs, MTX, AZA and/or CSA, did not sufficiently improve her symptoms. The administration of TNF-α inhibitor, infliximab, in combination with MTX effectively inhibited the autoinflammation, thus resulting in an improvement of the intraventricular septum thickness [Fig. 1A (c, d)].

Under approval by the Ethics Committee/Internal Review Board of Hiroshima University and informed patient consent, we analysed the nucleotide sequence of *CARD15*, and found a novel heterozygous single base-pair substitution, 1442G>A (G481D), in exon 4. This mutation was located within the nucleotide-binding domain. NF-κB reporter assay revealed that MDP-independent NF-κB transactivation in the G481D, C495Y and H496L mutants in *CARD15* showed significantly higher levels than that in wild-type (WT) (Fig. 1C), thus suggesting the G481D mutation to be a gain-of-function mutation [3].

Cardiac infiltration is a rare and atypical manifestation among the patients with EOS/BS. Only one report has shown cardiac infiltration among the patients with *CARD15* mutations [4]. The patient suffered from severe multi-organ involvement, arterial hypertension and myocardial hypertrophy, and was identified as being a heterozygous C495Y mutation. The C495Y mutation displayed the highest level of MDP-independent NF-κB activation (Fig. 1C), and notably the G481D mutation also demonstrated a relatively higher level of activity. Although no genotype–phenotype correlation in EOS/BS could be proven in the previous study, cardiac infiltration may therefore be associated with higher levels of MDP-independent NF-κB activation [3].

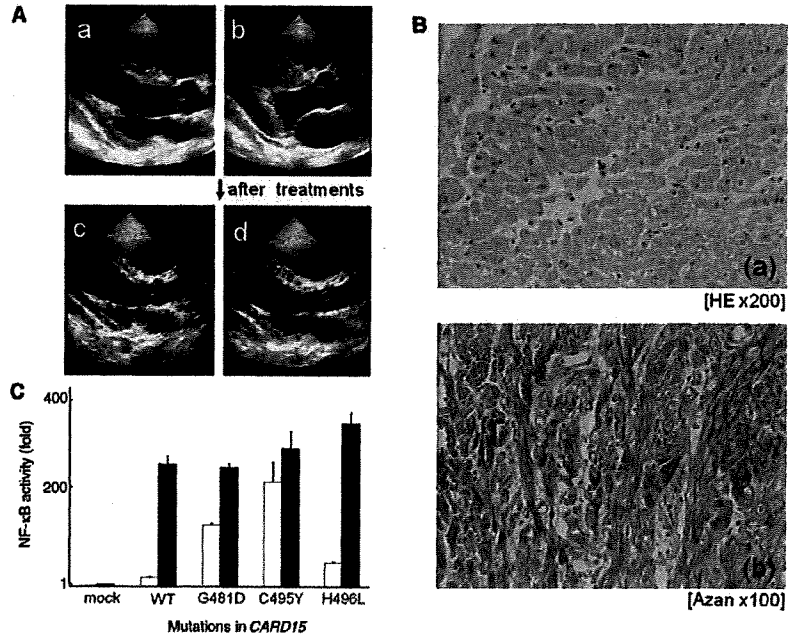

Fig. 1. (A) Echocardiography findings (parasternal long-axis view) before (a, b) and after (c, d) sufficient anti-inflammatory treatments. The systolic (a, c) and diastolic (b, d) image. (B) Histopathological findings of endocardium [(a) HE ×200, (b) Azan ×100]. (C) The MDP-independent (open columns) and MDP-dependent (filled columns) NF-κB transactivation. Values represent the mean of the normalized data (mock without MDP = 1) of triplicate cultures, and error bars indicate the S.D. Both C495Y and H496L are previously reported mutations in patients with EOS [3, 4].

Cardiac infiltration in adult-type sarcoidosis has shown a variety of manifestations, such as a conduction disorder, ventricular arrhythmias, congestive heart failure and sudden cardiac death. Matsumori *et al.* [7] reported that echocardiography revealed cardiac abnormalities in 6 of 82 patients with adult-type sarcoidosis. Four of six patients presented intraventricular septum thickness with mild to moderate myocardial cellular infiltration and fibrosis. The clinico-pathological findings in the current case resembled cardiac sarcoidosis in adults.

Recently, TNF-α inhibitors or IL-1 inhibitors have been tried on refractory cases, and were shown to improve the clinical manifestations [4, 8]. The efficacy of TNF-α inhibitors against cardiac sarcoidosis in adults has been also reported [9]. The administration of infliximab resulted in the reduction of the daily dose of prednisolone without disease progression. Furthermore, her cardiac manifestation was also improved clinically and morphologically in response to sufficient anti-inflammatory treatment. The intraventricular septum thickness was a reversible change, similar to a previous report in cardiac sarcoidosis in adults [10].

In this report, we identified a novel heterozygous gain-of-function mutation, G481D, in *CARD15* in a patient with EOS, suffering from cardiac infiltration of sarcoidosis. The clinical manifestation of cardiac sarcoidosis accompanied with EOS/BS is similar to that in observed in adults. Cardiac infiltration is an important manifestation in not only adult-type sarcoidosis, but also in patients with EOS/BS.

Rheumatology key message

- Cardiac infiltrations in an EOS patient associated with a novel gain-of-function *CARD15* mutation.

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Case report

Different neuroradiological findings during two stroke-like episodes in a patient with a congenital disorder of glycosylation type Ia

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Abstract

Congenital disorders of glycosylation type Ia (CDG-Ia) are the most common type of CDG and are characterized by liver dysfunction, coagulation disorders, mental retardation, hypotonia, cerebellar dysfunction, polyneuropathy, seizures, and stroke-like episodes. Stroke-like episodes occur in 40–55% of cases, but their etiology is not fully understood. Although it has been stated that an epileptic process may cause the stroke-like episodes, there is no clear evidence of ischemic stroke. Here, we describe two stroke-like episodes in a patient with CDG. We performed radiological studies during each episode and obtained two distinct magnetic resonance imaging (MRI) findings: one revealed an ischemic stroke, and the other demonstrated marked edema followed by focal necrosis. This is the first direct evidence of ischemic stroke, and we report that another process may affect the etiology in the same patient.

Keywords: Congenital disorder of glycosylation; Stroke-like episode; MRI; Ischemic stroke; Epilepsy

1. Introduction

Congenital disorders of glycosylation (CDG) type Ia is characterized by decreased activity of the enzyme phosphomutase owing to mutations in the *PMM2* gene [1,2]. The clinical features include liver dysfunction, coagulation disorders, mental retardation, hypotonia, cerebellar dysfunction, polyneuropathy, seizures, and stroke-like episodes. The stroke-like episodes occur in 40–55% of cases [3,4]. Despite much speculation [3,5–7], the mechanism of these stroke-like episodes is not fully understood.

We evaluated a boy with genetically confirmed CDG-Ia, who experienced repeated stroke-like episodes. The magnetic resonance imaging (MRI) findings during these two stroke-like episodes suggest that different etiologies caused these stroke-like episodes.

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2. Case report

An 11-month-old boy was referred to our hospital due to abnormal liver function and coagulation activities. At that time, he already showed developmental delay with hypotonia and ataxia, which gradually became more severe. MRI performed at 11 months of age demonstrated atrophy of the cerebellar hemispheres and vermis. We made a diagnosis of CDG-Ia based on the isoelectric focusing of serum transferrin and *PMM2* gene analysis (P113L/R194X) [8].

He experienced hemiplegia of the right extremities with vomiting and conjugate eye deviation to the left, with a high fever at the age of 5.2 years. Subsequently, intermittent clonic-convulsions of the right extremities occurred, which were stopped with a continuous midazolam infusion. He was not fully responsive at the first occurrence, which lasted for 1 h. An electroencephalogram (EEG) during the episode of hemiplegia showed a right hemispheric high-voltage slow wave superimposed on an inter-

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SUCCESSFUL TREATMENT OF KASABACH-MERRITT SYNDROME WITH VINCRISTINE AND DIAGNOSIS OF THE HEMANGIOMA USING THREE-DIMENSIONAL IMAGING

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□ *Kasabach-Merritt syndrome is a life-threatening congenital disorder characterized by an enlarging hemangioma, thrombocytopenia, and consumption coagulopathy. We report the case of a one-month male infant who presented with a large cutaneous tumor in his right axilla with ecchymosis, thrombocytopenia, and chronic consumption coagulopathy. Three-dimensional computed tomography was useful for accurate diagnosis of the cutaneous tumor and for determining the precise vascular constitution of the hemangioma, suggesting the efficacy of this method for diagnosing Kasabach-Merritt syndrome. Although administration of a corticosteroid was not effective, additional administration of vincristine resulted in the reversal of thrombocytopenia and coagulopathy with reduction of the hemangioma.*

Keywords Kasabach-Merritt syndrome, three-dimensional computed tomography, vincristine

Kasabach-Merritt syndrome is a congenital hemangioma that enlarges rapidly and is usually associated with complications such as thrombocytopenia and consumption coagulopathy. Kasabach and Merritt reported the first case in 1940 [1]. In the diagnosis of Kasabach-Merritt syndrome, it is necessary to find clinical evidence of hemangioma and thrombopenia. Here, we report a case of a pediatric patient with Kasabach-Merritt syndrome, for which three-dimensional computed tomography (CT) was useful for diagnosis. Although prednisolone and interferon-α therapy is the standard treatment for Kasabach-Merritt syndrome, we selected an anti-cancer drug, vincristine. Because vincristine was effective and had few side effects, we conclude that vincristine administration may be an effective treatment for steroid-resistant cases of Kasabach-Merritt syndrome.

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Experimental Hematology

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Reciprocal expression of Bmi1 and Mel-18 is associated with functioning of primitive hematopoietic cells

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Objective. **The *Polycomb*-group (*PcG*) genes regulate global gene expression in many biological processes, including hematopoiesis, by manipulating specific target genes. It is known that various *PcG* genes regulate self-renewal of hematopoietic stem cells (HSCs). Here we have shown that the reciprocal expression of PcG proteins regulates self-renewal and differentiation of HSCs.**

Methods. **We used murine and human bone marrow cells and evaluated the reciprocal expression of PcG proteins on the basis of their respective intranuclear distributions. *PcG*-gene expression in HSCs was knocked down by small interfering RNAs. The function of each gene in HSCs was analyzed in vitro and in vivo.**

Results. **Cells were either Bmi1-positive or Mel-18−positive. The Bmi1-positive cells contained very little amounts of Mel-18 and vice versa. The *bmi1*-knockdown marrow cells did not show HSC function, while the *mel-18*−knockdown marrow cells showed increased stem cell function. Results of the analysis on human cells were similar to those observed in case of murine cells. In a clinical investigation, transplantation using sources with a low Bmi1 to Mel-18 ratio was associated with early hematopoietic recovery.**

Conclusion. **Reciprocal expression of Bmi1 and Mel-18 regulated HSC function. Here, we observed that expression of the PcG genes—*bmi1* and *mel-18*—is correlated with self-renewal and differentiation of HSCs. Thus, it was suggested that the balance between Bmi1 and Mel-18 regulates self-renewal of HSCs.** © **2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Recently, a number of genes have been reported to be associated with the self-renewal of hematopoietic stem cells (HSCs). Path-breaking research studies have established *Hoxb4* as one such gene. *Hoxb4*, a homeotic gene, exerts a significant influence on HSC self-renewal [1,2]. A study showed that induction of the *Hoxb4* gene or the TAT-Hoxb4 protein in blood cells could lead to HSC proliferation [3]. The *Polycomb*-group (*PcG*) genes (*bmi1*, *rae28*, and *mel-18*), which are known as negative control factors of the *Hox* gene, also regulate differentiation and self-renewal of HSCs [4–8]. Mammalian PcG protein complexes can be classified into two distinct types—Polycomb repressive complex 1 (PRC1) and PRC2. The Mel-18 protein, together with M33, Bmi1, or Rae28/Mph1, and Scmh1, is a constituent of mammalian PRC1 [9–13]. The Mel-18 protein is composed of 342 amino acids, and the N-terminal region of the 102nd amino acid, which includes a particularly interesting new gene (RING) finger motif, shares 93% homology with the corresponding region of the Bmi1 protein [14]. In addition, the secondary structures of this region in the Mel-18 and Bmi1 proteins show a high homology. We previously reported the relationship between self-renewal of HSCs and functions of *mel-18* in these cells. In brief, the loss of *mel-18* results in promotion of HSC self-renewal and, inversely, increase in *mel-18* expression leads to HSC differentiation [8]. Other researchers have reported that the loss of *bmi1* and *rae28* does not lead to HSC self-renewal [4,5]. These findings suggest that *mel-18* and *bmi1* have reciprocal functions in HSCs. We hypothesized

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that Mel-18 and Bmi1 perform reciprocal functions because they share the same structure and compete when they are present in the complex form. Here, we observed that the reciprocal expression of Bmi1 and Mel-18 regulates self-renewal and differentiation of HSCs. Interestingly, this phenomenon was confirmed using human cells, rather than only mouse cells. Further, these findings may form the basis for assessments of hematopoietic recovery in clinical bone marrow transplantations.

Materials and methods

Mice

In this study, we used 5- to 8-week-old C57BL/6 mice (Ly5.1 and Ly5.2). Ly5.1 mice were obtained from the Sankyo Labo Service Corporation (Tokyo, Japan). All mice were bred and maintained in the animal facility at Hiroshima University.

Immunostaining of murine bone marrow cells

Bone marrow was flushed from the medullary cavities of murine bones by using Ca^{++}- or Mg^{++}-free phosphate-buffered saline. To purify the hematopoietic progenitor/stem cells, collected cells were labeled with an antibody cocktail consisting of biotinylated anti-Gr1, anti-Mac1, anti-B220, anti-CD4, anti-CD8, and anti-Ter119 mouse antibodies. The lineage-negative cells were purified with streptavidin microbeads by using a magnetically activated cell sorter (MACS) system (Miltenyi Biotech, Auburn, CA, USA). After the first MACS separation, cells were stained with anti-Sca1 microbeads and repurified. All antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). To stain the nucleus of the marrow cells, collected cells were fixed using an IntraPrep permeabilization kit (Beckman Coulter Inc., Fullerton, CA, USA), and fixed cells were stained with phycoerythrin (PE)- or Alexa Fluor 488-labeled anti-Bmi1 (Millipore Co., Billerica, MA, USA) and Alexa Fluor 488-labeled goat polyclonal Mel-18 antibodies (Abcam plc, Cambridge, UK). The Zenon labeling kit (Molecular Probes Inc., Eugene, OR, USA) was used for labeling. Flow-cytometric analysis was performed using a FACSCalibur system (Becton Dickinson, Bedford, MA, USA). 4′-6-Diamidino-2-phenylindole staining was used to clearly visualize the nucleus. A fluorescence microscope (BZ-8000; KEYENCE, Osaka, Japan) was used for fluorescence observation.

Knockdown of PcG gene expression by small interfering RNA

For the in vitro knockdown assays, we used small interfering RNA (siRNA) that binds to the targeted messenger RNA and DNA sequences (Dharmacon Inc., Lafayette, CO, USA). Predesigned siRNA reagents (*bmi1*, catalog: 065526; *rnf110*, catalog: 051261) were used. The final siRNA concentration was 100 nM. The efficiency of siRNA introduction into the cytosol and nucleus was increased by using each siRNA in combination with a delivery reagent (DharmaFECT; Dharmacon Inc.). For the colony-forming cell (CFC) assay and the in vivo transplantation of the HSCs, the siRNA solution was added to a Dulbecco's minimum essential medium–based culture medium and incubated for 48 hours. We added 20 ng/mL solutions of human Flt3 ligand (PeproTech, London, UK) and human thrombopoietin (Kirin Brewery Co., Tokyo, Japan) to Dulbecco's minimum essential medium containing a supplement, StemPro 34 (Invitrogen Inc., San Diego, CA, USA). The culture plates were incubated at 37 °C for 48 hours in a humidified atmosphere with 5% CO_2. The siRNA procedure was performed according to manufacturer's instructions.

Quantitative real-time reverse transcriptase polymerase chain reaction

To analyze expression of *bmi1* and *mel-18* in each cell fraction, reverse transcription was performed according to manufacturer's protocol by using ExScript reverse transcriptase (RT) and SYBR Premix Ex Taq (TAKARA BIO Inc., Shiga, Japan). Real-time polymerase chain reaction (PCR) was used for quantitative analysis of gene expression (Opticon; Bio-Rad Laboratories, Hercules, CA, USA). The following specific primers were used: mouse *bmi1*, sense primer 5′-GGA GAC CAG CAA GTA TTG TCC-3′ and antisense primer 5′-TTC TCC TCG GTC TTC ATT GG-3′; mouse *mel-18*, sense primer 5′-ACC ACC ATT GTG GAA TGC TT-3′ and antisense primer 5′-GTC GCC GTT TCA TTT CAT CT-3′; mouse glyceraldehyde-3-phosphate dehydrogenase, sense primer 5′-CCT GGA GAA ACC TGC CAA GTA TG-3′ and antisense primer 5′-AGA GTG GGA GTT GCT GTT GAA GTC-3′; mouse *ink4a*, sense primer 5′-TCA ACT ACG GTG CAG ATT CG-3′ and antisense primer 5′-ATT GGC CGC GAA GTT CC-3′; human *BMI1*, sense primer 5′-GAG GGT ACT TCA TTG ATG CCA CAA C-3′ and antisense primer 5′-GCT GGT CTC CAG GTA ACG AAC AAT A-3′; human *MEL-18*, sense primer 5′-CAT CGT GCG CTA CCT GGA GA-3′ and antisense primer 5′-AGC CCA GGG ACC AAT TTG TAG AC-3′; human β-actin, sense primer 5′-ATT GCC GAC AGG ATG CAG A-3′, and antisense primer 5′-GAG TAC TTG CGC TCA GGA GGA-3′.

Methylcellulose colony assay

In vitro CFC activity was assessed by performing a methylcellulose colony assay. Bone marrow cells (1000 cells/well), in which the expression of each gene was knocked down by siRNA, were cultured in a methylcellulose medium containing various cytokines (Methocult GF M3434; StemCell Technologies, Vancouver, BC, Canada).

Culture plates were incubated at 37 °C for 7 days in a humidified atmosphere with 5% CO_2. A colony was defined as a group of >50 cells. The erythroid, myeloid, and mixed erythroid-myeloid colonies were counted using an inverted microscope. A secondary CFC assay was performed by replating aliquots of the cells obtained by harvesting complete primary colonies. The secondary colonies were counted after an additional week of incubation.

In vivo bone marrow transplantation assay

We performed a transplantation experiment in which recipient mice were F1 hybrids of Ly5.1 and Ly5.2 mice. The donor marrow cells (2×10^5 cells) were intravenously transplanted into F1 recipients that were lethally irradiated with a dose of 9 Gy (rate of 1 Gy/min). In the donor Ly5.1 and Ly5.2 cells, each gene was knocked down by siRNA before being analyzed. After transplantation, peripheral blood samples were collected from the recipient mice every 4 weeks. The two competing transplant cell populations were distinguished by staining the cells with PE-conjugated anti-Ly5.1 and allophycocyanin-conjugated anti-Ly5.2. All the antibodies were purchased from Abcam plc.

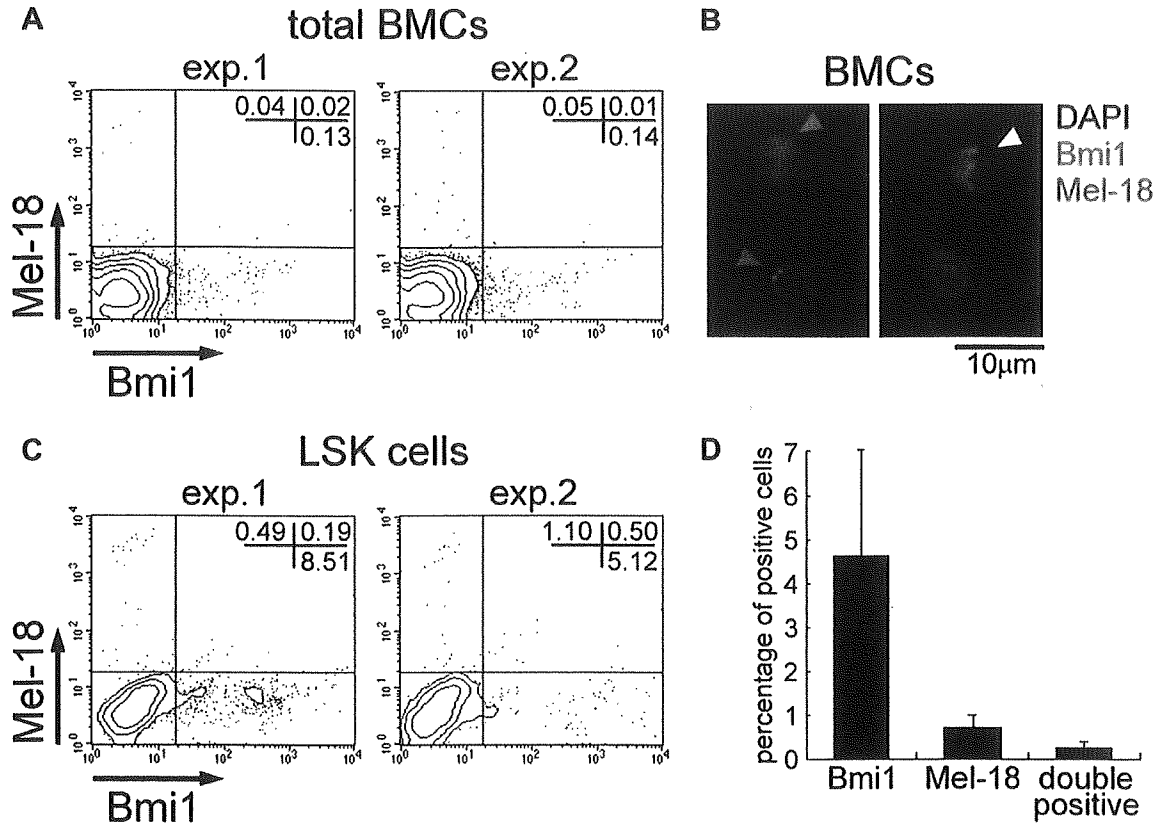

Figure 1. Reciprocal expression of Bmi1 and Mel-18 in murine bone marrow cells (BMCs). (**A**) Unpurified murine BMCs were fixed and stained with anti-Bmi1 and anti – Mel-18 antibodies. Approximately 0.1% marrow cells were Bmi1-positive, and approximately 0.05% cells were Mel-18–positive. A few cells expressed both Bmi1 and Mel-18. (**B**) Fluorescence microscopy confirmed the presence of Bmi1 and Mel-18 proteins in the nucleus. The Bmi1-positive cells are indicated with a red arrow. The green arrow shows Mel-18–positive cells. A very small fraction of Bmi1– and Mel-18–double-positive cells was detected, and this fraction is indicated with a white arrow. Bmi1 and Mel-18 seemed to exist distinctly in the cells. DAPI = 4′-6-diamidino-2-phenylindole. (**C**) Approximately 5% lineage-marker–negative, Sca1-positive, and cKit-positive cells (LSK) were Bmi1-positive, and approximately 1% LSK cells were Mel-18 positive. (**D**) The percentage of each type of positive cells is summarized. The cell population positive for both Bmi1 and Mel-18 was quite small (<0.5%).

Results

Expression of Bmi1 and/or Mel-18 in murine bone marrow cells

We examined expression of Bmi1 and Mel-18 in murine bone marrow cells by flow cytometry. Figure 1A shows the results of a representative flow-cytometric analysis of the bone marrow cells stained with Bmi1-PE and Mel-18-Alexa Fluor 488. The Bmi1- and Mel-18–positive bone marrow cells were clearly distinguished from each other. Few cells were positive for both Bmi1 and Mel-18. Immunostaining analysis confirmed the presence of polycomb-positive areas in the nuclei of the cells (Fig. 1B). We observed very few Bmi1– and Mel-18–double-positive cells in the immunostaining analysis, similar to the observations in the flow-cytometric analysis.

To study the expression patterns of Bmi1 and Mel-18 in primitive (early) hematopoietic cells, bone marrow cells were enriched for lineage-negative and Sca1-positive cells. Cells were then gated by cKit-allophycocyanin staining (Suppl. Fig. S1). Because the lineage-marker–negative, Sca1-positive, and cKit-positive (LSK) cells exhibit long-term multilineage reconstitution activity [15–17], we used this population for the experiments shown in Figure 1C and D.

To confirm the results of the flow-cytometric analysis, both Bmi1-positive and Bmi1-negative cells were purified, and expression of *bmi1* and *mel-18* was examined by RT-PCR. The Bmi1-positive and Bmi1-negative cells were purified from lineage-negative cells by using magnetic beads. As shown in Figure 2A, the Bmi1-positive cells showed *bmi1* expression, but they did not show *mel-18* expression. In contrast, the Bmi1-negative cells showed *mel-18* expression but did not show *bmi1* expression. Furthermore, we analyzed the cell cycles of Bmi1-positive and Bmi1-negative cells by using mouse embryonic fibroblasts (Fig. 2B). Because the sizes and characteristics of hematopoietic cells vary, we analyzed the cell cycles in mouse embryonic fibroblasts. The percentage of Bmi1-