initial rationale of allogeneic stem cell transplantation (allo-SCT) was based on a concept that SCT could provide the patients with HSCs to reconstitute their bone marrow hematopoiesis that was devastatingly damaged by such intensive treatments. Recently, allo-SCT is referred to as immunotherapy for leukemia rather than solely a vehicle to delivery intensive therapy because donor lymphocytes transplanted with HSCs function as cytotoxic T lymphocytes (CTLs) against the patient's leukemic cells (4-6). Although only a few successful cases have demonstrated the existence of CTLs against leukemic cells (7), a strong graft-versus-leukemia (GVL) effect demonstrated by indirect evidence that relapse rates increase if T cells are depleted from transplanted cells or in recipients of identical twin transplants have made infusions of donor lymphocytes (DLI) an standard treatment for patients with relapsed leukemia after allo-SCT, especially those with relapse of chronic myelogenous leukemia (CML) (8-12). Because CML is the most sensitive to DLI among relapsed leukemias, infusions of a large number of donor lymphocytes have proven to restore full donor chimerism and produce long-term complete molecular genetic remissions in relapsed CML cases. However, 50-60% of patients treated with DLI developed possible side effects such as graft-versus-host disease (GVHD) leading to a considerable amount of transplant-related mortality (TRM) (13). Although steroid (prednisolone) is used as first-line therapy against severe GVHD, the combination therapy with various immunosuppressive agents such as cyclosporine, ant-thymocyte globulin (ATG), and mycophenolate mefetil (MMF) is not frequently unsatisfactory in steroid-resistant GVHD (14). One of effective strategies to control severe GVHD while maintaining the GVL effect is the escalating dose regimen (EDR) in which the number of cells infused into the patients increases in a step-wise manner until the disease responds or GVHD occurs (15). Indeed, the EDR can significantly reduce the rate of severe GVHD with equal GVL effects for CML patients. Since the way to infuse donor lymphocytes in a step-wise manner takes much time to acquire GVL effects, however, the ERD is not suited for treatment of other acute leukemias with rapid progression. Another approach to decrease the risk of GVHD is to transfer specific effector cells that proliferate in response to leukemic cells into patients (16, 17). However, most of T cells exerting GVL effects recognize patient's allo-antigens and infusions of heterogenous lymphocytes show stronger GVL effects than do those of the specific T cells.

Control of severe GVHD using the suicide gene

To overcome the problems, a strategy of genetic manipulation of donor lymphocytes using retroviral vectors expressing the herpes simplex virus thymidine kinase (HSV-TK) gene has been devised to control severe GVHD (18-22). The HSV-TK protein converts the prodrug ganciclovir (GCV) to its monophosphate intermediate derivative that is further phosphorylated to di- and triphosphate (GCV-3P) compound by cellular kinases (23). The GCP-3P is incorporate into DNA, resulting in inhibition of DNA chain elongation. In the trial, donor lymphocytes are transduced with the HSV-TK gene using retroviral vectors and infused into the patients [Figure 1]. In a situation of no GCV, the transduced lymphocytes are expected to function as CTLs against the patient's leukemic cells. If severe GVHD occurs, GCV is administrated into the patients to eradicate donor lymphocytes. In 1997, the Italian group demonstrated successful cases of gene therapy clinical trials (TK-DLI) (18). They performed TK-DLI for 23 high-risk patients with hematological relapse after allo-

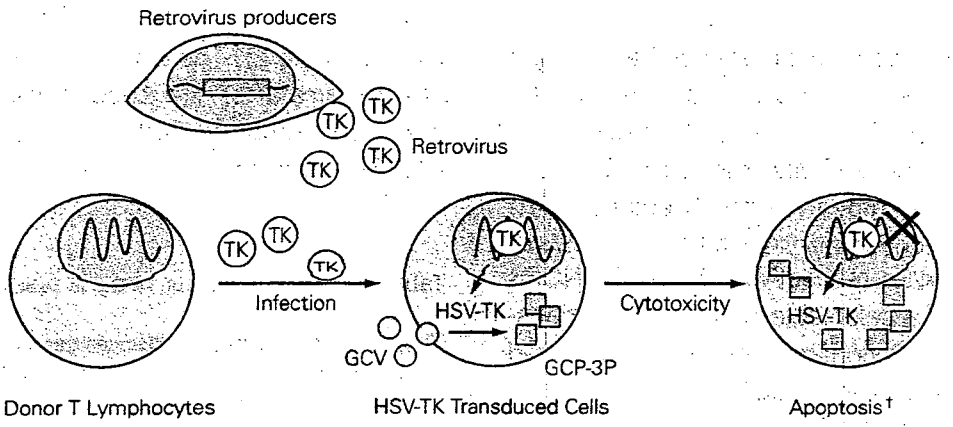

Figure 1 A strategy of TK-DLI using the HSV-TK / GCV suicide system. Peripheral lymphocytes collected from donors are transduced with the HSV-TK gene using retroviral vectors and infused into patients with relapsed leukemia. In a situation of no GCV, the cells are expected to function as CTLs against patients' leukemic cells. In case of occurrence of severe GVHD, GCV is administrated into the patients to eradicate the transduced cells. GCV is phosphorylated in the only transduced cells and incorporated into into genomic DNA as a GCV-3P compound, resulting in inhibition of DNA chain elongation and apoptosis of the transduced cells.

SCT and reported clinical results of 17 patients who were alive more than 30 days after receiving the therapy. The cell number infused, although it varied among patients, was approximately $4x10^7$ transduced cells per kilogram of body weight. Eleven patients (65%) experienced substantial clinical benefits, resulting in six complete remissions (35%, 2 CML, 1 AML, 2 NHL, and 1 multiple myeloma) and five partial responses (29%, 2 CML, 1 AML, 1 NHL, and 1 MM). Four patients developing GVHD (3 acute and 1 chronic) received GCV resulting in elimination of the transduced cells and control of severe GVHD.

Based on successful results of the clinical trial, they have extended the strategy to haplo-SCT for hematologic malignancies (24). Haplo-SCT is the last option for patients who lack an HLA-identical donor but it increases rates of morbidity and mortality due to severe GVHD. Infusions of donor lymphocytes transduced with the HSV-TK gene in an incremental manner after haplo-SCT (TK add-back) would help rapid immune recovery with protection from infection and prevent relapse of leukemia, and could control severe GVHD by administration of GCV if it occurs. Eight patients with high-risk hematologic malignancies who underwent haplo-SCT were enrolled; three patients received $1x10^6$ and five patients received $1x10^7$ transduced cells per kilogram of body weight. Although no immune reconstitution was observed in patients who received $1x10^6$ / kg, three out of five receiving $1x10^7$ transduced cells / kg recovered full immune reconstitution and showed significant reduction of incidence of viral infections. Especially, two out of these three patients have been free from leukemic relapse. Regarding GVHD, one patient out of the three developed a grade II acute GVHD that was quickly controlled by administration of GCV. A phase II multi-center trial (MM TK007) in which infusion of 1×10^7 transduced cells per kilogram of body weight is considered to be an effective dose to prevent disease relapse and promote immune reconstitution after haplo-SCT is ongoing.

TK-DLI in the Tsukuba Hospital

In collaboration with Dr. Bordignon at H. S. Raffaele, we started a phase I/II clinical trial of

TK-DLI for patients with relapsed hematologic malignancies after allo-SCT at the Tsukuba University in 2004 [Table 1]. A retroviral vector used is the SFCMM-3 in which the HSV-TK and nerve growth factor receptor (NGFR) genes are driven by the viral LTR and SV40 early promoter, respectively [Figure 2]. NGFR is used as a selectable marker of the transduced cells. Peripheral lymphocytes collected from donors using CS3000 are stimulated with a high dose of recombinant human interleukin-2 (rhIL-2, 600U/ml) and anti-CD3 antibody (OKT3, 30ng/ml) for 72 hours and transduced with the HSV-TK gene by exposure of the viral supernatant of SFCMM-3 twice, and then the transduced cells are isolated with the Isolex 50 using anti-NGFR antibody. The cells are cultured to expand for additional 3 to 5 days and stocked in −150°C until used. So far, nine transduction procedures have been done for eight patients enrolled in the trial and the transduced cells were prepared in a range from 8.8×10^7 to 3.1×10^8 per kilogram of body weight [Table 2]. The transduction efficiency at 48 hours after the second transduction was approximately 20% and the purity of NGFR expressing cells isolated using anti-

Table 1 Clinical protocol of TK-DLI in Tsukuba University Hospital

Title : Infusions of donor lymphocytes transduced with the herpes simplex virus thymidine kinase gene into patients with relapsed leukemia after allogeneic stem cell transplantation

Population : Patients > 2years of age with relapsed hematologic malignancies after allo-SCT

Sample size : Five patients for 2 years

Treatment : Donor peripheral lymphocytes transduced with the HSV-TK gene are administrated into patients with relapsed hematologic malignancies at 5 to 10×10^7 transduced cells per kilogram of body weight. GVHD, if it occurs over grade III, will be treated with ganciclovir (GCV) given by intravenous infusion at 5mg/kg x2 / day for 7 days, during which time no other immunosuppressive regents such as steroid or cyclosporine are used. Conventional treatments for GVHD are considered if the 7 days GCV treatment does not control GVHD completely.

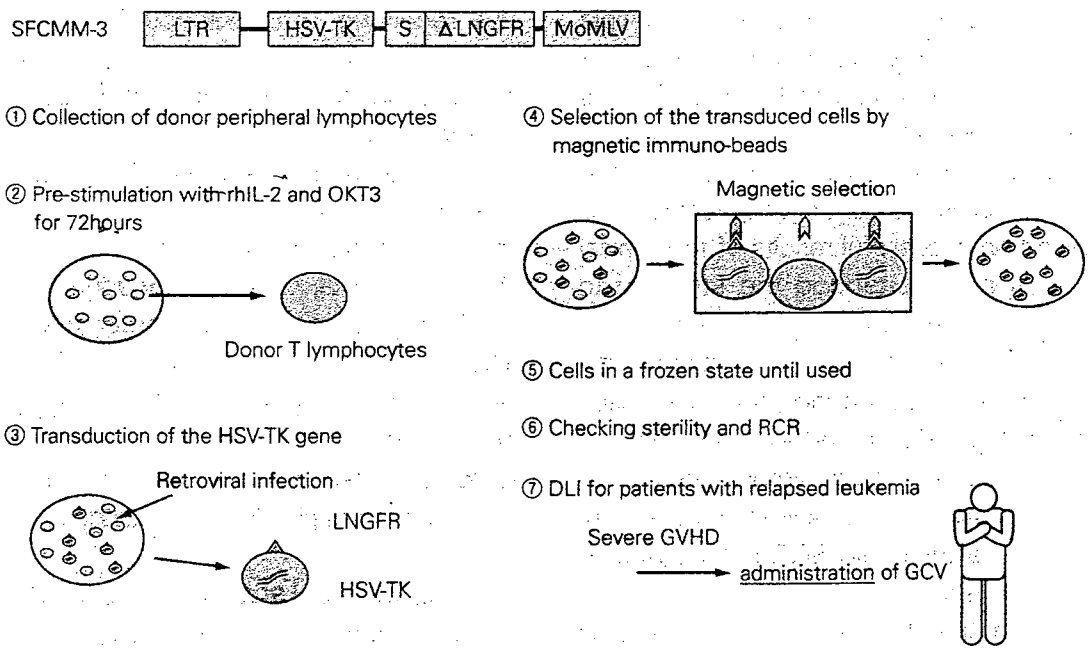

Figure 2 A structure of the retroviral vector SFCMM-3 and a Tsukuba TK-DLI protocol

Table 2 Clinical results of Tsukuba TK-DLI Trial

UPN	Diag	Age, Sex	# of prep (/kg)	NGFR+	# of infused (/kg)	GVL	GVHD	GCV
1	MDS (RAEB)	42, M	1.0×10^{10} (1.8×10^{8})	93.5%				
2	ALL (Ph1+)	15, F	4.6×10^{9} (1.2×10^{8})	97.8%				
3	AML	60, M	1.0×10^{10} (2.3×10^{8})	97.2%	3.8×10^{9} (7.7×10^{7})	+ Disease control	acute	+ (controlled)
4*	ALL	20, M	0.1×10^{9} (0.2×10^{7})	37.7%				
5	ALL		4.4×10^{9} (8.8×10^{7})	93.1%				
6	MDS (RAEB)	58, M	2.4×10^{10} (3.1×10^{8})	95.1%	9.7×10^{8} (9.5×10^{7})	+ Remission	chronic	–
7-1	ALL	14, M	7.9×10^{9} (2.6×10^{8})	94.9%	2.0×10^{9} (6.7×10^{7})	–		
-2					5.0×10^{9} (8.5×10^{7})	–		
8-1	AML	46, M	1.8×10^{10} (2.3×10^{8})	90.7%	9.0×10^{9} (8.5×10^{7})	+ WT1↓		
-2					9.0×10^{9} (8.5×10^{7})	–		
9	ALL	50, M	7.2×10^{9} (1.8×10^{8})	90.7%	4.4×10^{9} (8.6×10^{7})	+ L/N swelling↓		

(* an identical patient)

NGFR antibody exceeded 90% in all the cases except for UPN 4. All samples cleared safety tests including cell viability, sterility, existence of replication competent retrovirus (RCR), and sensitivity to GCV.

Five (2 AML, 2 ALL, and 1 MDS) out of 8 patients were treated with TK-DLI and two patients among them (UPN 7 and 8) received infusions of the transduced cells twice [Table 2]. The cell count infused, although it varied among patients, was approximately 8×10^7 cells per kilogram of body weight. Four patients showed some clinical responses; inhibition of leukemic cell growth in UPN 3 and UPN 6, decrease of WT1 values in UPN 8, and mitigation of lymph node swelling in UPN 9. In particular, UPN 6 that had needed frequent blood transfusions due to severe anemia retrieved bone marrow hematopoiesis and has been in complete remission over 1 year after the treatment. However, the second infusions of UPN 8 as well as both infusions of UPN 7 did not show any GVL effects (discussed later). In terms of GVHD, UPN 3 developed the grade III acute GVHD (the total bilirubin levels exceeded 3 mg/dl) that was successfully controlled by administration of GCV in the absence of immunosuppressive drugs, and UPN 6 showed chronic GVHD that has been observed without any treatments.

Problems and safety issues in TK-DLI

To determine the reason why some cases did not show any GVL effects, we analyzed kinetics of in vivo elimination of HSV-TK expressing lymphocytes using the real-time PCR (qPCR) [Figure 3]. In cases with clinical benefits (UPN 6 and 9), the transduced cells survived relatively longer in the patients' peripheral blood. On the other hand, the cells rapidly disappeared from the peripheral blood of patients without GVL effects and the ten-

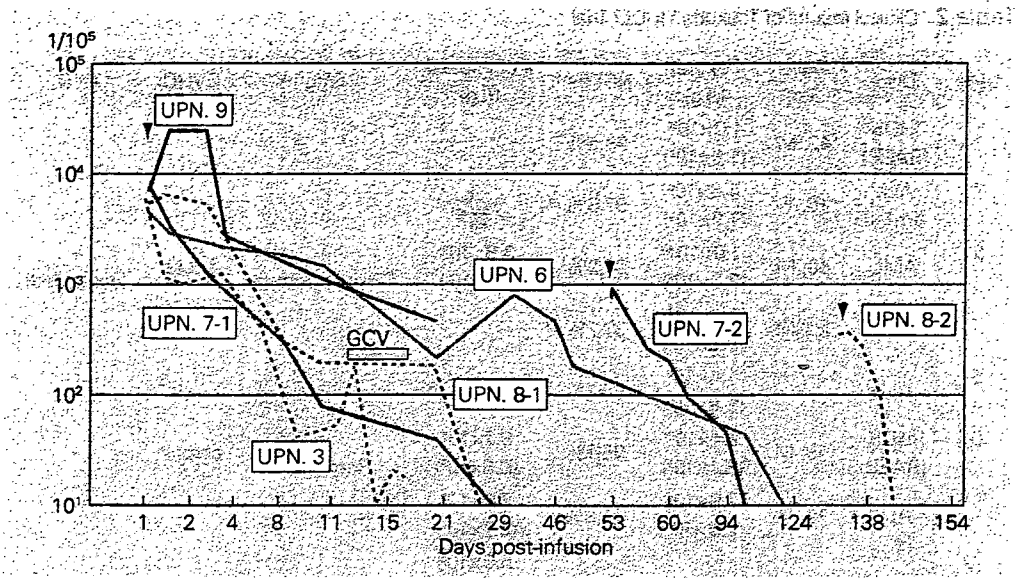

Figure 3 In vivo elimination kinetics of the transduced cells. Genomic DNA obtained from the patients (UPN 3, 6, 7, 8, and 9) was tested with the real-time PCR (qPCR) to determine DNA values of transduced cells in the patients. The values in the Y-axis represent the number of transduced cells / 10^5 cells of peripheral blood cells. UPN 7 and 8 received the transduced cells twice (7-1, 7-2, 8-1, and 8-2) and GCV was administrated for 7 days in UPN 3.

dency was especially more remarkable in second infusions, suggesting development of CTLs against HSV-TK expressing cells (25, 26). With regard to clonality of transduced cells (27-29), we determined the provirus copy number and integration sites by LAM-PCR using genomic DNA extracted from the patients' peripheral lymphocytes (30, 31). Samples before infusions showed DNA smear patterns suggesting that multiple provirus copies were integrated into host genomes. Consistent with results of real-time PCR (qPCR), a few bands that were observed until 2 to 3 weeks post-infusions were not detected by LAM-PCR over 30days. Consequently, a trial to determine the integration sites was unsuccessful.

In our trial, two cases developed GVHD (acute and chronic) suggesting that donor lymphocytes infused maintained their ability to respond to allogeneic antigens. However, the frequency of severe GVHD occurrence seemed to be lower than that observed in DLI using unmanipulated donor lymphocytes despite of infusions of the large number of donor lymphocytes. In addition to development of CTLs against HSV-TK expressing cells in the patients, it was likely that in vitro culture to genetically manipulate donor lymphocytes impaired their immune functions (32, 33). Improved culture conditions could preserve the T-cell repertoire and improve their viability (34).

Out of five patients receiving the TK-DLI, four are alive and one patient (UPN 3) was dead of multiple organ failure due to infiltration of leukemic cells. No adverse effects related to gene therapy have been observed.

Conclusions

The clinical trial confirmed the safety and therapeutic effects of the suicide-gene transduced lymphocytes for relapsed leukemia after allo-SCT. Furthermore, acute GVHD developed by infusions of the donor lymphocytes was controlled by administration of

GCV in the absence of immunosuppressive drugs. At the same time, rapid disappearance of the donor lymphocytes was observed in non-negligible numbers of infusions. The potential problem that the suicide gene derived from viruses could elicit strong immune responses against the transduced cells may be overcome by using genes derived from human (34, 35). As an alternative, the TK-DLI will be combined with allo-SCT in which the patient's immune functions are severely damaged to impair T cell priming against foreign antigens (24).

With further modifications including vector constructs (36, 37), culture conditions (38), and the timing of infusions, the suicide-gene strategy would offer the safe and effective immune cell therapy for patients with hematologic malignancies.

Acknowledgment

On behalf of the Gene and Cell Therapy Group in the Tsukuba University, the author appreciates Drs. C. Bonini, S. Toma and C. Bordgnon for providing us with the SFCMM-3 viral supernatants and important information about their clinical trials, and all medical staffs for caring for patients enrolled in the trial. The gene therapy clinical trial is ongoing in the Tsukuba University Hospital.

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Granulocyte Colony-Stimulating Factor Prevents Progression of Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats

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Background Regeneration of the lung microvasculature and replacing pulmonary artery lesions with functional endothelial cells could be a novel and effective therapeutic strategy for treating advanced pulmonary arterial hypertension (PAH). In the present study it was postulated that granulocyte colony-stimulating factor (G-CFS), which induces the proliferation of endothelial cells, would stimulate endothelial regeneration in situ at sites of impaired lung vasculature and prevent the development of PAH.

Methods and Results Daily administration of G-CSF for 48 days did not affect the hemodynamism of normal Fischer 344 rats. PAH was induced with monocrotaline (60 mg/kg) and G-CSF was administered daily (100 μg/kg per day). Echocardiographic findings and an invasive catheter study indicated a significant decrease in the progression of PAH in rats given G-CSF. Furthermore, G-CSF increased Ki-67 positivity in the pulmonary arteries of PAH rats but did not accelerate c-kit positive cell recruitment into peripheral blood. Daily doses of G-CSF at both 2 and 100 μg/kg improved the survival and body weight gain of PAH rats.

Conclusions G-CSF improved the progression of PAH in a rat model, possibly by stimulating pulmonary endothelial cells to proliferate at sites of impaired lung vasculature. These findings show that cytokine therapy for PAH is valid based on the concept of vascular regeneration. (*Circ J* 2007; **71:** 138–143)

Key Words: Cytokines; Endothelial cells; Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is characterized by increased thickening of the walls of the pulmonary arteries, narrowing of the pulmonary artery (PA) lumen, increased pulmonary vascular resistance, and right-sided heart failure[1] Environmental stress in a genetically predisposed host might trigger endothelial cell apoptosis, which could lead to arteriolar occlusion either directly, perhaps by initiating microvascular degeneration, or indirectly by promoting the emergence of hyperproliferative, apoptosis-resistant vascular cells[2] Because transplantation with exogenous endothelial progenitor cells (EPCs) rescues the survival of rats with monocrotaline (MCT)-induced PAH[2,3] the regeneration of lung vasculature or the replacement of PA lesions by functional endothelial cells might be effective therapeutic strategies to improve pulmonary hemodynamics in patients with advanced PAH. These findings suggest that disordered antiproliferative and vasoregulatory functions of endothelial cells play an important role in PAH progression. However, a clinically useful number of autologous EPCs is difficult to obtain from donors or from patients with progressive PAH. Therefore, we postulated that granulocyte colony-stimulating factor (G-CSF), which induces endothelial cells to migrate and proliferate[4,5] could stimulate regeneration of impaired lung microvasculature and improve the survival of PAH rats.

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Methods

Animals were purchased from CLEA Japan (Tokyo). The Institutional Animal Care and Use Committee of the University of Tsukuba approved the animal protocols and the experiments proceeded according to institutional guidelines.

To estimate the basal effect of G-CSF in intact rats (without MCT injection), G-CSF (100 μg/kg per day) or saline was subcutaneously injected into 9-week-old male Fischer 344 rats. On day 40 of injection, peripheral blood cells sampled via the tail vein were counted and on day 48 we performed an invasive catheter study. After euthanasia, the right ventricle (RV) was excised and weighed.

One subcutaneous injection of MCT (Sigma, St Louis, MO, USA; 60 mg/kg) was sufficient to induce PAH in the 4- to 6-week-old male Fischer 344 rats[3] and then recombinant human G-CSF (100 or 2 μg/kg per day; Kirin, Tokyo, Japan) or saline was subcutaneously injected from day 14 (for echocardiographic study, catheter study, histopathologic study) or day 21 (for survival analysis) thereafter. Intact adult male Fischer 344 rats served as normal controls.

Echocardiography and the catheter study proceeded after 1 week of G-CSF administration. Echocardiographic studies were performed using a 12 MHz transducer (Sonos4500, Philips). At the aortic valve (AV) level in the short-axis

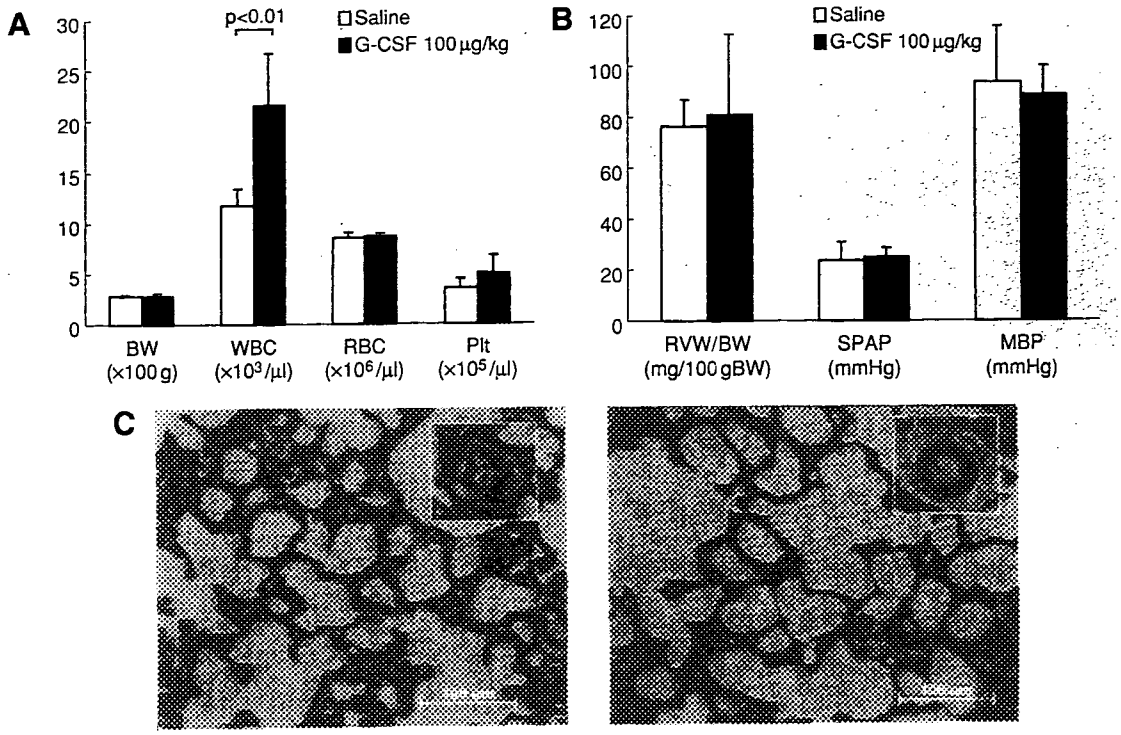

Fig 1. Hemodynamic and hematological effects of G-CSF in normal rats. (A) BW and peripheral blood cell count after 40 days of daily G-CSF injections (n=6 per group). (B). Hemodynamic assessment after 48 days of daily G-CSF injections shows no significant differences (n=4 per group). Error bars in (A) and (B) indicate SD. (C) Histological examination of normal rat lung stained with hematoxylin–eosin. Pulmonary arterioles of the same scale are inserted upper right. Left, normal rat given G-CSF for 48 days; Right, control. Original magnification×20. BW, body weight; G-CFS, granulocyte colony-stimulating factor; WBC, white blood cells; RBC, red blood cells; Plt, platelets; RVW/BW, right ventricular weight adjusted to body weight; SPAP, systolic pulmonary arterial pressure; MBP, mean systemic blood pressure.

view, the RV outflow tract dimension (RVOTD) and aortic dimension (AoD) were recorded[6] The eccentricity index was measured at end-diastole from the parasternal short-axis views of the left ventricle at the level of the chordae tendinae. The diastolic eccentricity index was defined as the D2/D1 ratio at end-diastole, where D2 is the minor-axis dimension of the left ventricle parallel to the septum and D1 is the minor-axis diameter perpendicular to and bisecting the septum[7] Pulsed Doppler PA flow velocities were recorded at the AV level in the short-axis view. Acceleration time (AT: time from onset of pulmonary flow to peak pulmonary outflow velocity) and ejection time (ET: time from onset to completion of systolic pulmonary flow) of PA flow velocity tracings were measured, and the ratio of AT to ET (AT/ET) was calculated[6]

After anesthesia was induced with pentobarbital IP (50 mg/kg), RV systolic pressure (RVSP) and mean aortic pressure (MAoP) were recorded using a polyethylene catheter inserted into the RV and ascending aorta via the right jugular vein and right carotid artery, respectively.

Formalin-fixed rat lungs were paraffin-embedded, sectioned and stained with hematoxylin–eosin, as well as with the elastic van Gieson technique. A blinded observer measured all vessels with perceptible media (>35 muscular arteries/rat), under ×20 magnification, and the average was obtained for vessels of 25–50 and 50–100 μm in external diameter for each rat. The medial wall thickness is expressed as follows: % wall thickness = [(medial thickness × 2)/external diameter] ×100[3]

For immunohistochemical localization of Ki-67[8] endogenous peroxidase in lung tissue sections was quenched and the sections were heated in antigen retrieval solution (0.01 mol/L citrate buffer, pH 6.0) for 20 min in a microwave oven. After incubation in a casein block, mouse MAb anti-rat Ki-67 (clone MIB-5; Dako) was added to the sections at a dilution of 1:50 and incubated overnight at 4°C. We used a secondary detection kit (Histofine Simplestain Rat Max-Po Multi; Nichirei, Japan) to visualize antibody binding. Staining was developed using 3-amino-9-ethylcarbazole, and then sections were counterstained with hematoxylin. Ki-67 positivity was expressed as %Ki-67 labeling by calculating the average ratio (%) of Ki-67 positive endothelial cells within 20 pulmonary arteries per rat. Histopathological photomicrographs were captured using Axioplan 2/AxioVision 3.1 (Carl Zeiss).

Three weeks after MCT injection, peripheral blood was sampled via the tail vein. Red blood cells were lysed using buffered ammonium chloride. The samples were incubated with rabbit anti-c-kit antibodies[9] (H-300, Santa Cruz Biotechm Santa Cruz, CA, USA) for 30 min at 4°C, washed with phosphate-buffered saline, and incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG goat antibodies as secondary antibodies for 30 min at 4°C. The samples were washed, propidium iodide was added and cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA).

Survival was estimated from the date of MCT injection until death or 6 weeks after MCT injection.

Data are presented as means ± SD. Data were statistically analyzed using Student's t-test. The significance of differ-

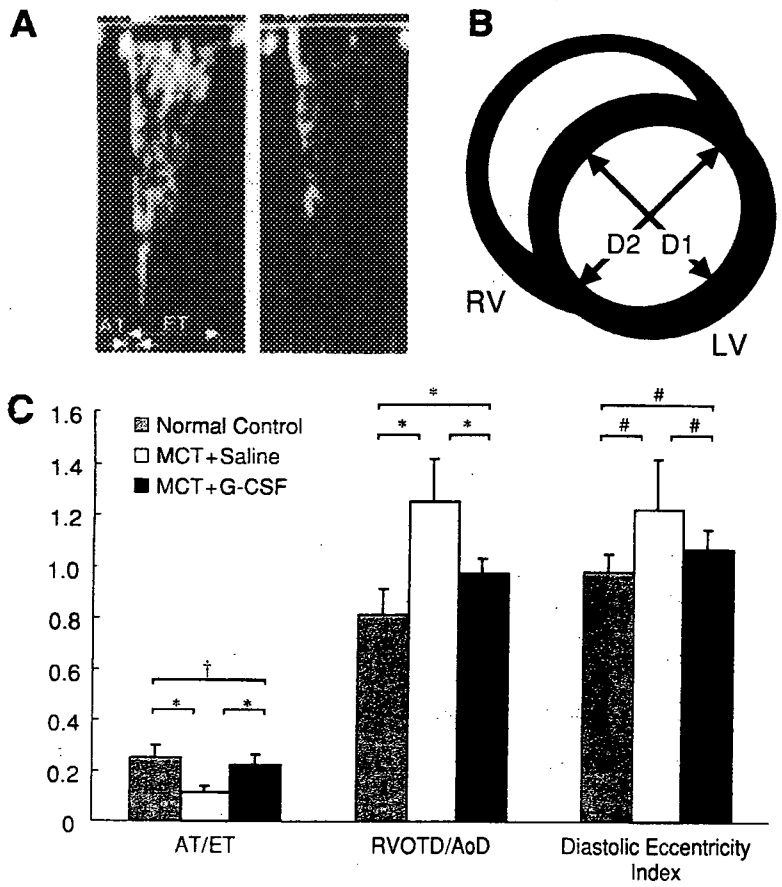

Fig 2. Echocardiographic findings. (A) Representative pulsed Doppler echocardiographic recordings of pulmonary artery flow 3 weeks after monocrotaline (MCT) injection. Left: MCT+Saline group, Right: MCT+G-CSF group. (B) Schematic representation of parasternal short-axis views at chordae tendinae level. Eccentricity index=D2/D1. (C) Quantitative echocardiographic evaluations. Normal control. n=5; MCT+Saline, n=12; MCT+G-CSF, n=13. *p<0.01; #p<0.05; †p=NS. AT, acceleration time; ET, ejection time; RV, right ventricle; LV, left ventricle; G-CFS, granulocyte colony-stimulating factor; RVOTD, RV outflow tract dimension; AoD, aortic dimension.

ences in survival data was determined using the Kaplan-Meier analysis. A value of $p<0.05$ was considered statistically significant.

Results and Discussion

After 40 daily G-CSF injections, the number of leukocytes (WBC) in the peripheral blood approximately doubled in intact rats (without MCT injection) compared with control rats. However, body weight (BW) and peripheral blood cell count of erythrocytes and platelets did not significantly differ (Fig 1A). On day 48, significant differences between the 2 groups in mean systemic pressure, systolic pulmonary arterial pressure and right ventricular weight adjusted to BW (RVW/BW),[10] which reflects chronic overload of pulmonary arterial pressure, were undetectable (Fig 1B). The alveolar walls in most lung tissue samples contained slightly more mature neutrophils without vascular and peribronchial infiltrates and no emphysema or pleural thickening as described by Chang et al[11] (Fig 1C). These findings indicated that G-CSF administered over the semi-long term does not affect the hemodynamism of normal intact rats.

Echocardiography, invasive catheter and histopathologic studies proceeded 3 weeks after MCT injection, with daily injections of G-CSF (100μg/kg; MCT+G-CSF group) or saline (MCT+Saline group) during the last week. Fig 2A shows that flow velocity accelerated rapidly to a peak in early systole, followed by rapid deceleration to the midsystolic notch in the MCT+Saline group, but not particularly in the MCT+G-CSF group. The diastolic eccentricity index was defined as the D2/D1 ratio at end-diastole, where D2 is the minor-axis dimension of the left ventricle parallel to the septum and D1 is the minor-axis diameter perpendicular to and bisecting the septum (Fig 2B).[7] Fig 2C shows quantitative echocardiographic evaluations. The AT/ET values significantly decreased in the MCT+Saline group compared with the normal control group (0.11±0.02 vs 0.25±0.05, $p<0.01$). In MCT+G-CSF group, AT/ET returned to the level of normal control group (0.22±0.04 vs 0.25±0.05, NS). The ratio of RVOTD to AoD (RVOTD/AoD) and diastolic eccentricity index were significantly greater in the MCT+Saline group than in the normal control group (1.26±0.14 vs 0.81±0.10, $p<0.01$ and 1.22±0.20 vs 0.98±0.07, $p<0.05$, respectively). These increases were suppressed in the MCT+G-CSF group (0.97±0.06, $p<0.01$ and 1.07±0.08, $p<0.05$ vs MCT+Saline group, respectively). The AT/ET value correlated inversively to mean PA pressure and resistance.[12] The ratio of RVOTD/AoD is thought to be a parameter of RV dilatation[6] and the diastolic eccentricity index reportedly increases in patients with both right ventricular volume and pressure overload.[7] These findings suggest that MCT injection promotes, whereas G-CSF improves pulmonary hypertension under noninvasive conditions.

Three weeks after MCT injection, RVSP was increased from 20.7±6.3 mmHg in the normal control group to 43.6±9.5 mmHg in the MCT+Saline group (<0.01, Fig 3A), which was consistent with the development of PAH. In rats treated with G-CSF, RVSP was significantly reduced to 30.0±5.3 mmHg ($p<0.01$ vs MCT+Saline group). However, this strategy did not completely restore the RVSP to normal values ($p<0.01$ vs normal control group). The MAoP was slightly but significantly decreased from 116.0±15.7 mmHg in the normal control group to 93.8±25.3 mmHg in the

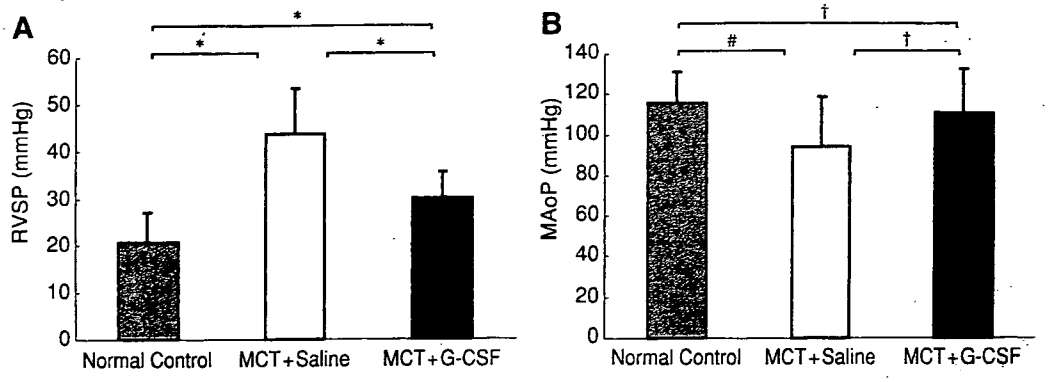

Fig 3. Hemodynamic evaluations. Data of invasive hemodynamic study 3 weeks after monocrotaline (MCT) injection. (A) Right ventricular systolic pressure (RVSP). normal control, n=9; MCT+Saline, n=8; MCT+G-CSF, n=12. (B) mean aortic pressure (MAoP). Normal control, n=9; MCT+Saline, n=10; MCT+G-CSF, n=9. *p<0.01; #p<0.05; †p=NS. G-CFS, granulocyte colony-stimulating factor.

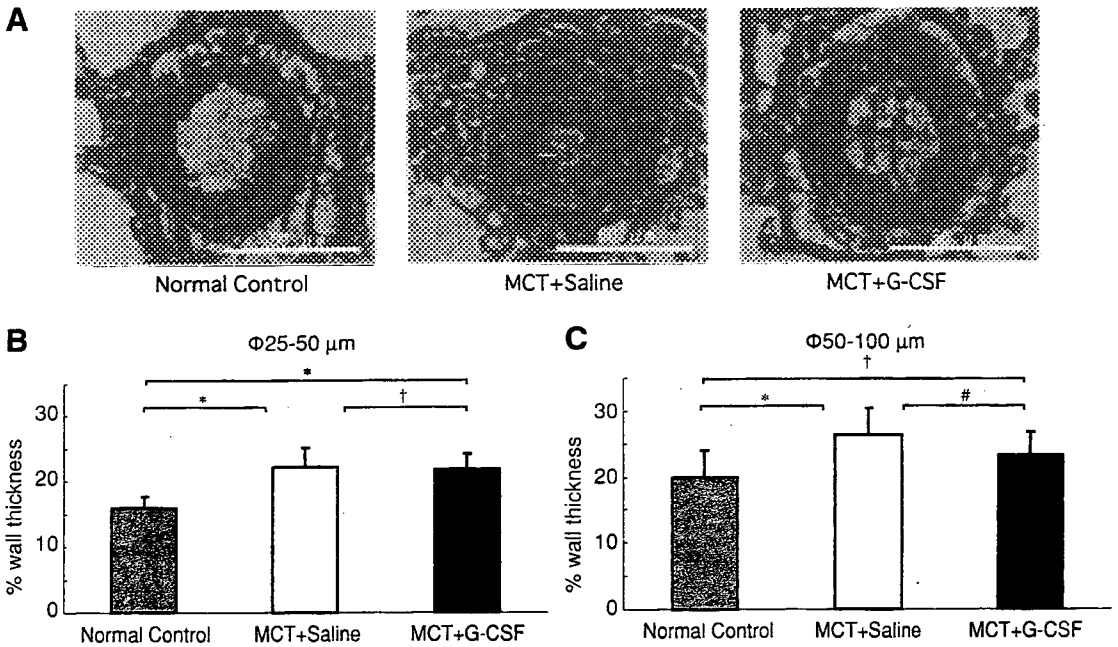

Fig 4. Histopathological study of pulmonary arteries. (A) Representative photomicrographs of peripheral pulmonary arteries of rats 3 weeks after monocrotaline (MCT) injection (elastica van Gieson, ×20). (B). Quantitative analysis of %wall thickness of peripheral pulmonary arteries. Normal control, n=10; MCT+Saline, n=12; MCT+G-CSF, n=14. *p<0.01; #p<0.05; †p=NS. G-CFS, granulocyte colony-stimulating factor.

MCT+Saline group (p<0.05, Fig 3B), and G-CSF restored the MAoP to normal values (110.9±22.2 mmHg, NS vs normal control group). During this examination, heart rates did not differ statistically among the groups (Normal control, 462±45 beats/min; MCT+Saline, 457±54 beats/min, MCT+G-CSF, 447±45 beats/min; NS).

Fig 4A shows representative sections of lung tissue from normal controls (Left) and from rats in the MCT+Saline (Center) and MCT+G-CSF (Right) groups. Hypertrophy of the media of peripheral muscular arteries was apparent in the MCT+Saline group. Quantitative analysis also demonstrated a significant increase in % wall thickness after MCT injection, but G-CSF obviously attenuated this change in pulmonary vessels of 50–100 μm but not in those of 25–50 μm in diameter (Figs 4B,C).

We immunohistochemically examined Ki-67 in paraffin-embedded rat lung tissue sections (Fig 5). Nuclear immunoreactivity, as revealed by Ki-67 staining, usually has a reddish violet granular appearance. Few endothelial cells were positive for Ki-67 in normal control rats, whereas positivity was increased in groups injected with MCT (MCT+Saline; MCT+G-CSF). Fig 5B shows the %Ki-67 labeling within the endothelial cells of pulmonary arteries (PAECs). Injected MCT increased Ki-67 positivity approximately 4-fold compared with normal rats (8.3±1.1 vs 2.2±0.6, p<0.01). Furthermore, G-CSF slightly but significantly enhanced the increase (11.1±1.4, p<0.01 vs MCT+Saline). Ki-67 detection is an established immunohistochemical technique used to assess cell proliferation[8] Our findings suggest that MCT injection induces apoptosis within PAECs,[13] which involves PAH progression and consequently induces replenishment, but insufficient proliferation, of PAECs and that G-CSF accelerated proliferation to a physiologically effective level.

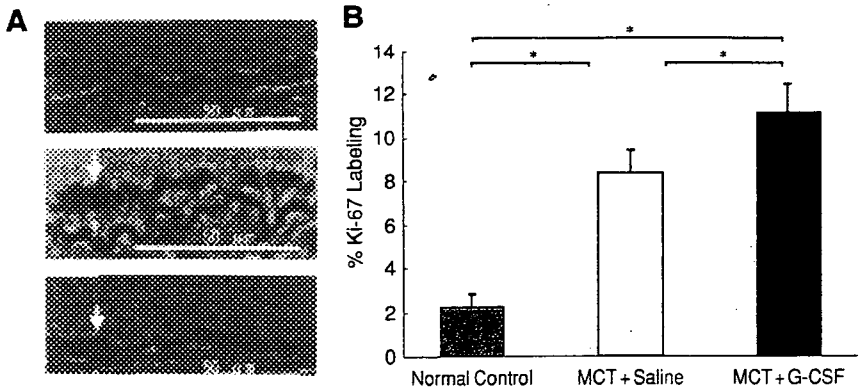

Fig 5. (A) representative photomicrographs of immunohistochemistry of Ki-67. Nuclear immunoreactivity has a reddish violet granular appearance (arrows). Control rats (Upper), MCT+Saline (Middle), MCT+G-CSF (Lower). Original magnification ×40. (B) %Ki-67 labeling within endothelial cells of pulmonary arteries. *p<0.01. G-CFS, granulocyte colony-stimulating factor; MCT, monocrotaline.

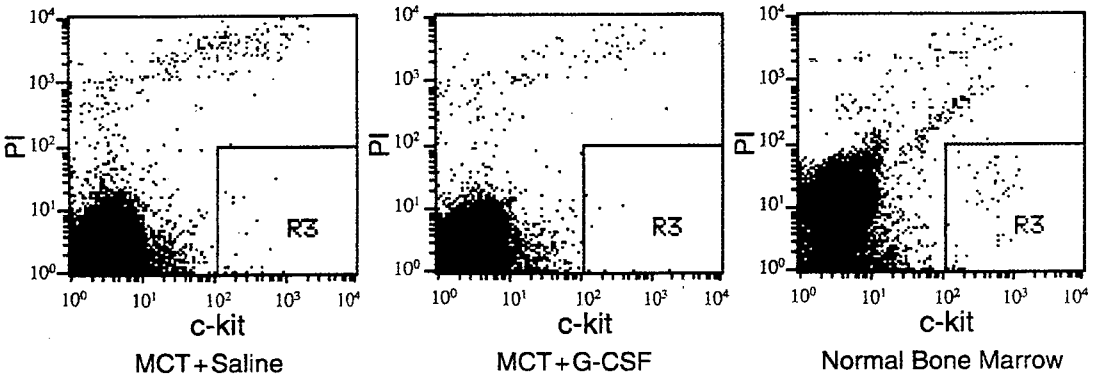

Fig 6. Flow cytometric analysis of peripheral blood. Peripheral blood samples after 10 days of daily G-CSF injections to PAH rats were analyzed by expression of c-kit and propidium iodide (PI) incorporation after gating for WBC fraction. Dot-plots show representative data of peripheral blood from MCT+Saline and MCT+G-CSF groups and bone marrow of normal rats (positive control). Viable c-kit positive cells are fractionated in R3 region. G-CFS, granulocyte colony-stimulating factor; MCT, monocrotaline; PAH, pulmonary arterial hypertension; WBC, white blood cells.

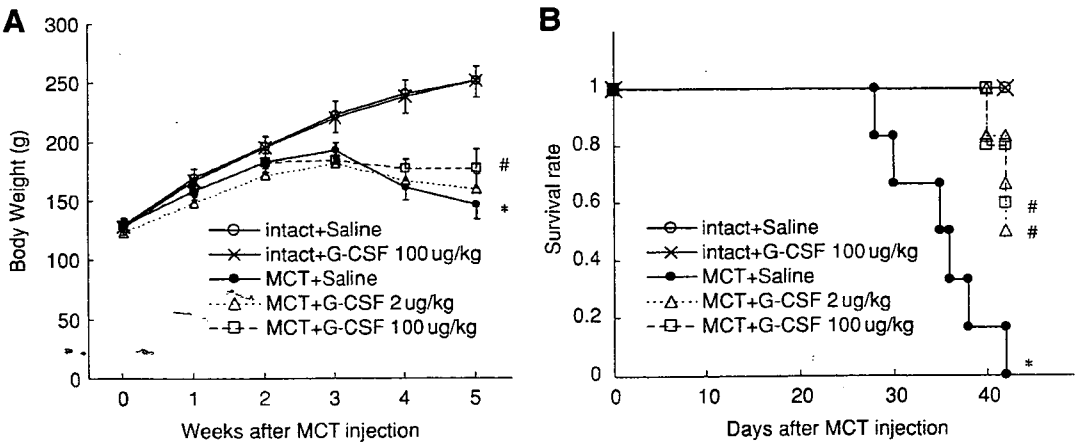

Fig 7. Hemodynamic assessment, body weight change and survival curve of PAH rats given G-CSF from day 21 of MCT injection. (A) Body weight change of surviving PAH rats. Number of rats per group on day 0 is shown. Error bars indicate SD. *p<0.05 compared with intact rats; #p<0.05 compared with PAH control rat (MCT+Saline). (B) Survival curve of PAH rats. *p<0.05 compared with intact rats; #p<0.05 compared with PAH control rats (MCT+Saline). G-CFS, granulocyte colony-stimulating factor; MCT, monocrotaline; PAH, pulmonary arterial hypertension.

Markers for rat EPCs have not been established. We therefore evaluated circulating angiogenic stem/progenitor cells presumably recruited from the bone marrow (BM) using flow cytometry to detect c-kit, which is a marker of hemangioblasts and hematogenic angioblasts (Fig 6)[14] The findings indicate that the numbers of c-kit positive cells in the WBC fraction of peripheral blood did not statistically differ between the MCT+Saline and MCT+G-CSF groups (0.12±0.01% and 0.07±0.04%, respectively).

The gain in body weight becomes impaired as PAH progresses[6,15] and surviving PAH rats weighed significantly less than intact rats (Fig 7A). Treatment with G-CSF at 100 μg/kg per day from day 21 significantly inhibited the reduction in body weight gain. Treatment with G-CSF at

2μg/kg per day elicited a similar trend, but the difference was not statistically significant. The administration of G-CSF did not affect the body weight gain of intact rats. All intact rats regardless of G-CSF administration survived the observation period. Saline-injected control PAH rats began to die from day 28 and none survived beyond day 42 (Fig 7B). Treatment with both doses of G-CSF from day 21 significantly improved the survival of PAH rats. Interestingly, the survival of rats started on G-CSF (100μg/kg per day) from day 0 of the MCT injection did not improve (unpubl. data), suggesting that G-CSF enhances the acute inflammatory response to develop pulmonary arterial muscularization[16] after MCT-induced injury of the pulmonary vessels. Because 1 subcutaneous injection of MCT could produce alterations in the pulmonary vessels and right ventricular hypertrophy after 3 weeks;[10] the present results showed that G-CSF administration starting from day 21 improves the prognosis of progressive PAH but does not inhibit MCT toxicity.

Lee et al recently reported that a low dose of G-CSF (2–20μg/kg) in rats directly stimulates mature endothelial cells to migrate without mobilizing endothelial stem/progenitor cells.[17] The present study found that a low dose of G-CSF (2μg/kg) improved the survival of PAH rats, and that daily G-CSF administration did not increase circulating c-kit positive stem cells, which comprise hemangioblasts and hematogenic angioblasts. These findings suggest that in our model, G-CSF did not mobilize angiogenic stem/progenitor cells, which would engraft to injured pulmonary arteries from the BM into the circulation, but directly stimulated endothelial cells to proliferate and migrate in PA lesions in situ.

This is the first report to show that G-CSF improves the progression of MCT-induced PAH, and that direct administration (without gene transfer) of a cytokine to PAH, based on the concept of vascular regeneration, is valid. Between 200 and 300μg/kg per day of recombinant human G-CSF injected for 5 days into rats induced hematopoietic effects that are equipotent to those observed in humans given 10–20μg/kg per day for a similar period.[17] We consider that 100μg/kg per day of rhG-CSF corresponded to 5μg/kg per day in humans, which is used as a clinical dose.[8–21] Further investigation of other tissue-protective properties of G-CSF in this model, such as anti-apoptotic effects[22] of right ventricular cardiomyocytes that undergo apoptosis because of pressure overload[23] and of renal protective effects[24] against MCT toxicity, should be informative.

Acknowledgments

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Original Article

Stem cell transplantation in primary immunodeficiency disease patients

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Abstract

Background: Primary immunodeficiency diseases (PID) are rare but have a high associated risk of death from overwhelming infection in early childhood. Stem cell transplantation (SCT) can be curative for PID, but standardized protocols for each disease have not yet been established.

Methods: Between May 1995 and May 2005, nine patients diagnosed with a PID received SCT at the Department of Pediatrics, Hokkaido University Hospital. The median age of the patients (eight boys and one girl) was 1.0 year (range: 6 months–4 years). Five patients had Wiskott–Aldrich syndrome (WAS), three had severe combined immunodeficiency (SCID), and one had X-linked hyper-IgM syndrome (X-HIGM). Four patients received bone marrow transplantation (BMT), and five received cord blood stem cell transplantation (CBSCT). All patients, including those with SCID, received a conditioning regimen: six (WAS and X-HIGM) received a myeloablative conditioning regimen, and three (SCID) received a reduced-intensity conditioning regimen.

Results: All the patients are alive and have stable, complete chimerism, based on a median follow-up period of 4 years. Moreover, all patients have good immune reconstitution, and none required immunoglobulin replacement therapy. Two patients had significant acute graft-versus-host disease (GVHD), and three patients had chronic GVHD. Four of the nine patients developed cytomegalovirus (CMV) infection after SCT.

Conclusion: The transplantation procedures appear to have provided a permanent cure in nine PID patients. Early diagnosis and prompt performance of SCT with an optimal donor and conditioning regimen contributed to the favorable outcomes.

Key words complication, outcome, preparative conditioning, primary immunodeficiency disease, stem cell transplantation.

Enormous progress has been made in the field of primary immunodeficiency disease (PID) using natural mutant models for study of the immune system. PID are rare, but some diseases in this class are fatal unless early optimal treatment is performed. Stem cell transplantation (SCT) and gene therapy can be curative for PID; indeed, SCT has improved the survival rate of PID patients, as well as for those with malignant diseases.[1] In some recent PID cases, favorable outcomes of SCT using reduced-intensity conditioning regimens have been reported.[2] However, because PID comprises various types of disease, the optimal SCT conditions for each disease should be considered separately. Furthermore, the protocol may need to be individualized depending on the patient's condition, phenotype/genotype or donor source, even for patients with the same PID. In the present study we analyzed the outcomes of SCT performed in nine consecutive PID patients over 10 years, as a step towards establishment of a standardized SCT protocol for treatment of PID.

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Methods

Patient characteristics

Between May 1995 and May 2005, a total of 133 patients received SCT at the Department of Pediatrics, Hokkaido University Hospital, with a follow-up period after SCT of between 0.4 and 10 years (median: 4.0 years). Of these patients, nine diagnosed with PID were included in the present study (Table 1). The median age of the nine patients was 1.0 year (range: 6 months–4 years), and eight were boys and one was a girl. Five patients had Wiskott–Aldrich syndrome (WAS), two

Table 1 Stem cell transplantation profiles of the nine patients

Case no.	Age at diagnosis /sex (months)	Age at SCT (years)	Diagnosis	Source	Donor	HLA matching	Preparative regimen	GVHD prophylaxis	Donor NCC ($\times 10^8$/kg)	Engraftment	Chimerism/Status
1	12/M	4	WAS	BM	Mother (carrier)	6/6	$BU^2 + CY^1 + ALG$	CsA + short MTX	6.2	Yes	Complete/alive
2	10/M	1	WAS	BM	Sibling (non-carrier)	6/6	$BU^2 + CY^2$	CsA + short MTX	10.7	Yes	Complete/alive
3	2/M	1	WAS	BM	Unrelated	6/6	$BU^2 + CY^2 + ALG$	CsA + short MTX	3.8	Yes	Complete/alive
4	1/M	1	WAS	CB	Unrelated	6/6	$BU^2 + CY^2$	CsA + short MTX	0.64	Yes	Complete/alive
5	3/M	0.5	WAS	CB	Unrelated	6/6	$BU^2 + CY^2 + ALG$	CsA + mPSL	0.31	Yes	Complete/alive
6	36/M	4	X-HIGM	BM	Unrelated	6/6	$BU^2 + CY^2 + ALG$	FK506 + short MTX	5.3	Yes	Complete/alive
7	8/M	0.8	X-SCID	CB	Unrelated	6/6	BU^1 + Flu	CsA + mPSL	1.12	Yes	Complete/alive
8	4/M	0.5	X-SCID	CB	Unrelated	6/6	BU^1 + Flu	CsA + mPSL	2.04	Yes	Complete/alive
9	11/F	1	SCID (unknown)	CB	Unrelated	5/6	BU^1 + Flu + ALG	CsA + mPSL	0.96	Yes	Complete/alive

ALG, anti-lymphocyte globulin; B, B cell; BM, bone marrow; BU, busulfan; BU^1, busulfan 8 mg/kg; BU^2, busulfan 16 mg/kg; CB, cord blood; CsA, cyclosporin A; CY, cyclophosphamide; CY^1, cyclophosphamide 120 mg/kg; CY^2, cyclophosphamide 200 mg/kg; F, female; FK506, tacrolimus; Flu, fludarabine; HIGM, hyper IgM syndrome; M, male; mPSL, methylprednisolone; MTX, methotrexate; NCC, number of nucleated cells transplanted; NK, natural killer cell; SCID, severe combined immunodeficiency; SCT, stem cell transplantation; T, T cell; WAS, Wiskott–Aldrich syndrome.

patients had X-linked severe combined immunodeficiency (X-SCID, $T^-B^+NK^-$), one patient had unknown SCID ($T^-B^+NK^-$), and one patient had X-linked hyper-IgM syndrome (X-HIGM). Diagnosis was confirmed at the molecular level for all except the SCID patient. In particular, the WAS patients were diagnosed rapidly on flow cytometric analysis of WAS protein expression (FCM-WASP) in peripheral blood mononuclear cells, as previously reported.[3]

Except for the patient with X-HIGM, all the patients were diagnosed within 1 year after birth and underwent SCT before 4 years of age. The median period from diagnosis of PID to SCT was 8 months (range: 1–39 months). Lower respiratory infections and otitis media due to bacteria were frequently observed as pre-transplant infections. In addition, one patient with SCID had pulmonary aspergillosis, two patients with WAS had cytomegalovirus (CMV) hepatitis, and one patient with SCID had CMV pneumonitis.

Transplant procedure

Four patients received bone marrow transplantation (BMT), and five patients received cord blood stem cell transplantation (CBSCT). The transplantation donors were a human leukocyte antigen (HLA)-matched mother (carrier) for one WAS patient, a matched sibling (non-carrier) for one WAS patient, matched unrelated donors for six patients, and a single locus-mismatched unrelated donor for one patient with SCID. For conditioning, busulfan (BU)-containing regimens were used in all patients, as follows. Patient 1, BU 16 mg/kg + cyclophosphamide (CY) 120 mg/kg; patients 2–6, BU 16 mg/kg + CY 200 mg/kg; patients 7,8, fludarabine (Flu) 180 mg/m^2 + BU 8 mg/kg; and patient 9, Flu 180 mg/m^2 + BU 8 mg/kg + anti-lymphocyte globulin (ALG) 60 mg/kg. Six patients were given BU orally at a daily dose of 4 mg/kg for 4 days, and three patients were given a daily dose of 4 mg/kg for 2 days (Table 1). That is, a myeloablative conditioning regimen was used for five WAS patients and one X-HIGM patient, and a non-myeloablative conditioning regimen was used for the three SCID patients. As prophylaxis for graft-versus-host disease (GVHD), four patients received cyclosporin A (CsA) and short-term methotrexate (MTX), four patients received CsA and methyl prednisolone, and one patient received tacrolimus (FK506) and short-term MTX. The median number of transfused cells from BMT donors was 5.75×10^8/kg of the recipient's bodyweight (range: $3.75–10.70 \times 10^7$/kg), and the median number of transfused cells from CBSCT donors was 9.60×10^7/kg of the recipient's bodyweight (range: $3.10–20.40 \times 10^7$/kg).

Engraftment and chimerism

Neutrophil engraftment was defined as an absolute neutrophil count of 500/μL for 3 consecutive days, platelet engraftment

as a platelet count >5.0 × $10^4/\mu L$ without transfusion, and red cell engraftment as a reticulocyte level >1%. Chimerism was analyzed in the peripheral blood using XY fluorescence *in situ* hybridization (FISH; sex-mismatched patient–donor), transition of donor blood type (sex-matched and blood type-mismatched patient–donor), or a short tandem repeat polymerase chain reaction (STR-PCR; sex-matched and blood type-matched patient–donor). FCM-WASP was used for WAS patients.[4]

Supportive care and acute/chronic GVHD

Oral polymyxin B and amphotericin B or i.v. micafungin were administered for antibacterial and antifungal prophylaxis, and oral acyclovir and i.v. γ-globulin were given for antiviral prophylaxis. Granulocyte colony-stimulating factor (G-CSF) was given i.v. at 5 μg/kg from day +5 until engraftment. Acute GVHD was diagnosed and graded according to the published criteria, and was treated with prednisolone (2 mg/kg per day), CsA or FK506.[5] Chronic GVHD was assessed using established criteria for patients who survive until at least day +100 after SCT.[6]

Statistical analysis

All statistical tests were carried out using two-tailed, 5% levels of significance. The Mann–Whitney *U*-test was used for comparison of continuous variables. The association between categorical variables was investigated using χ^2-test or Fisher exact probability test.

Results

Engraftment and survival

Engraftment with a complete chimera occurred in all nine patients, and all were alive and well at the time of writing. The median times for neutrophil engraftment, platelet engraftment, and reticulocyte engraftment were 17 days (range: 11–38 days), 42 days (range: 11–126 days), and 21 days (range: 13–63 days), respectively. The median time to neutrophil engraftment was significantly shorter in patients who underwent BMT, compared to those who received CBSCT ($P = 0.016$). The median time to neutrophil engraftment was significantly longer for the three patients with SCID who underwent a non-myeloablative conditioning regimen, compared to patients who underwent myeloablative regimen ($P = 0.024$). There were no significant correlations between engraftment times for platelets or reticulocytes and the source of stem cells or the nature of the conditioning regimen.

GVHD and post-transplant complications

Two patients developed acute skin and gut GVHD of grade 3 (Table 2): one patient with WAS who underwent a myeloablative conditioning regimen, and a patient with SCID who underwent a non-myeloablative conditioning regimen. Both the patients remitted with first-line steroid therapy. Additionally, three patients developed chronic GVHD: one patient had oral mucosa and liver GVHD, one had liver GVHD, and one patient with SCID who underwent a non-myeloablative regimen developed immune thrombocytopenic purpura and hemolytic anemia. No significant differences were observed in the severity of GVHD for the myeloablative or non-myeloablative conditioning regimens. Five of the nine patients suffered from post-transplant infections: two patients had sepsis including central venous catheter infection, and four patients developed CMV infection. Interestingly, all three patients who had suffered from CMV infection before SCT (patients 3, 4 and 9) developed CMV infection after SCT. The patient with X-HIGM was suffering from viral encephalitis of unknown cause on day +100 after SCT, presenting with dysarthria, lethargy, and labile gait. However, he recovered spontaneously without sequelae. One patient with WAS developed hyponatremia due to syndrome of inappropriate secretion of anti-diuretic hormone (SIADH) and pancreatitis after SCT.

Immune reconstitution after transplantation

The recovery of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells in peripheral blood after SCT is shown in Figure 1. During a 12 month follow-up period, all the patients who did not receive steroid therapy showed good recovery of T-cell and B-cell counts. For patients 1, 4, 7 and 9, who were treated with steroids for GVHD, the post-transplantation T-cell count only increased slowly. However, these patients all showed prompt recovery of T-cell and B-cell counts after discontinuation of steroid therapy. Furthermore, all the patients had normal serum immunoglobulin G, A and M levels at the time of writing (data not shown), and none of the patients have required i.v. immunoglobulin replacement therapy. No significant differences in immune reconstitution were seen between patients who underwent bone marrow transplantation and those who received cord blood transplantation.

Discussion

Our data show that SCT was performed successfully in nine PID patients in a single Japanese center over a period of 10 years. In each case, a preparative conditioning regimen corresponding to the type of PID was used: myeloablative conditioning for patients with WAS and X-HIGM, and

Table 2 GVHD and transplant complications

Case no.	Pre-transplant complication	Post-transplant complication	aGVHD (grade)	cGVHD
1	Zoster, HSV infection, Otitis media, Thrush	Catheter infection, Sepsis	(-)	Oral mucosa, liver
2	Gonarthritis, Catheter infection	None	(-)	(-)
3	CMV hepatitis, Enteropathy	CMV infection	(-)	(-)
4	CMV hepatitis	Sepsis, CMV infection, SIADH, Pancreatitis	3	Liver
5	None	None	(-)	(-)
6	Bacterial pneumonia, Otitis media	Encephalitis, CMV infection	(-)	(-)
7	Aspergillosis (lung), Thrush, Bacterial bronchitis	None	(-)	(-)
8	Bronchiolitis, Bronchitis, Thrush, Otitis media, Enteropathy	None	3	(-)
9	Bronchitis, CMV pneumonitis, Otitis media, Bacterial pneumonia	CMV infection	(-)	Immune thrombocytopenic purpura, Hemolytic anemia

aGVHD, acute graft-versus-host disease; CMV, cytomegalovirus; GVHD, graft-versus-host disease; cGVHD, chronic graft-versus-host disease; HSV, herpes simplex virus; SIADH, syndrome of inappropriate secretion of antidiuretic hormone.

non-myeloablative conditioning for SCID patients. In cases of non-malignant diseases, the main purpose of preparative conditioning is to immunosuppress the host to allow engraftment of donor cells; there is no advantage regarding avoidance of clinical GVHD in such patients with respect to the graft-versus-leukemia effect. Acute GVHD of more than grade 2 has been noted as a poor prognostic factor for survival following transplantation in PID patients.[7] In the present series no significant difference in the severity of GVHD was identified between the myeloablative and non-myeloablative conditioning regimens. More patients suffered from GVHD following CBSCT than BMT, but previous reports suggest that reduced-intensity SCT is appropriate for patients with non-malignant diseases,[8] and it has also been suggested that CBSCT is useful because of a reduced risk for GVHD and a reduced severity of GVHD that does occur.[9,10] Collectively, the available data suggest that determination of optimal conditioning regimens is of importance, especially in CBSCT, to reduce the occurrence of GVHD in PID patients, and establishment of these conditions will require further study.

Patients with X-HIGM or WAS are likely to reject donor cells, and therefore the conditioning regimen for these patients should be designed to avoid graft rejection after SCT. In the present study we demonstrated that all patients who received a conditioning regimen of BU + CY with or without ALG survived and did not require immunoglobulin replacement therapy. Several reports have demonstrated that a conditioning regimen consisting of BU + CY with or without ALG is well tolerated, and that full engraftment and normal expression levels of CD40 ligand (CD40L) and WASP can be established with such a regimen.[4,11–13] Thus, using BU+CY with or without ALG for preparative conditioning in patients with WAS or X-HIGM appears to be the current choice to achieve a favorable outcome and full immune reconstitution. Moreover, Ziegner *et al.* reported successful CBSCT in a patient with X-HIGM.[14] However, the longer-term prognosis in these patients needs to be evaluated before confirmation of these conditioning regimens as appropriate for SCT in WAS and X-HIGM patients.

There are several different scenarios for SCT in patients with SCID. Because SCID patients do not reject donor cells, SCT in most SCID patients is successful without preparative conditioning; indeed, Buckley *et al.* reported successful SCT in 89 patients with SCID, and none of the recipients received preparative conditioning before transplantation (except for two patients who received cord blood).[15] However, it remains controversial whether patients with SCID should undergo a conditioning regimen before SCT. All the SCID patients in the present series underwent SCT with preparative conditioning, and subsequently they did not require immunoglobulin replacement therapy and are alive and in good condition. Insufficient recovery of B-cell function has been reported after SCT in SCID patients, and especially following most HLA-mismatched transplantations; therefore, some SCID patients

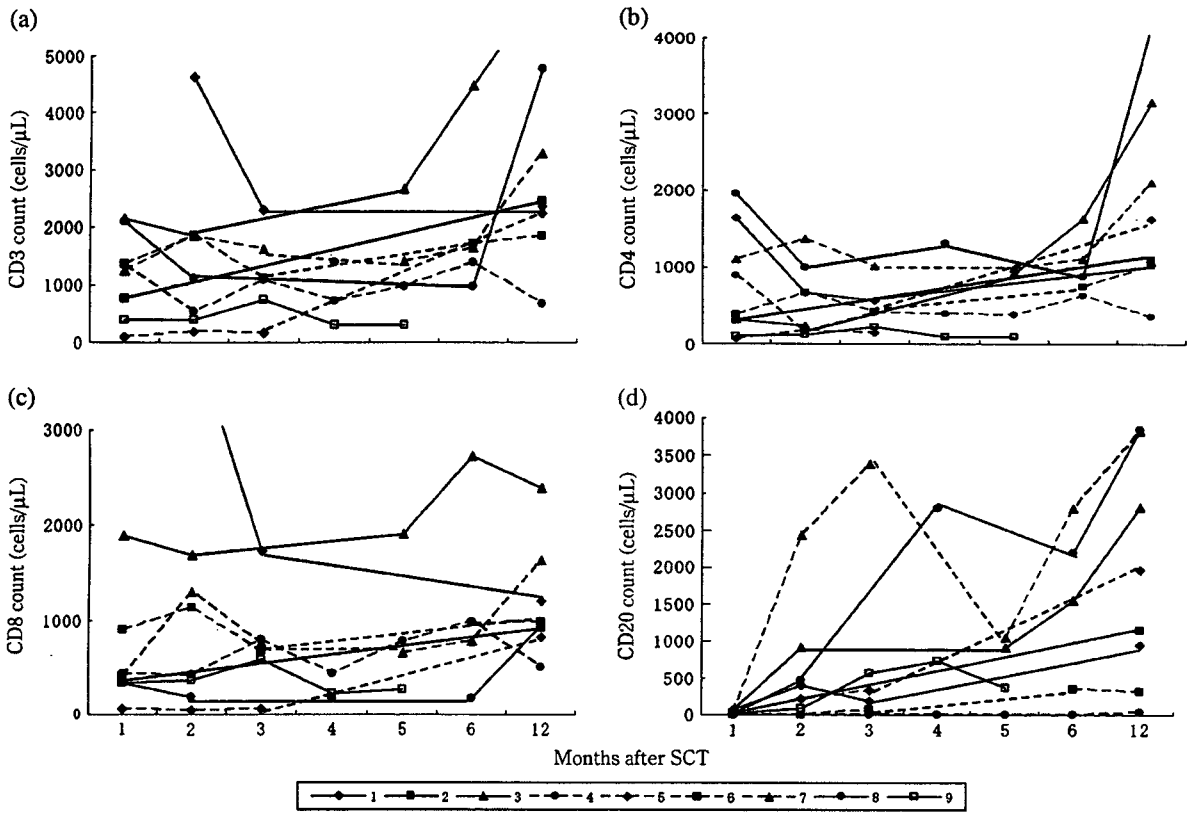

Fig. 1 Immune reconstitution after stem cell transplantation. Recovery of (a) $CD3^+$, (b) $CD4^+$ and (c) $CD8^+$ T cells, and (d) $CD20^+$ B cells in peripheral blood. 1–9, patient number.

require post-transplantation immunoglobulin replacement therapy to enhance recovery of B-cell function. In fact, Buckley *et al.* reported that in 45 of 72 surviving SCID children after SCT, the majority of whom received T-cell-depleted haploidentical bone marrow SCT without preparative conditioning, were receiving immunoglobulin replacement therapy.[15] However, preparative conditioning before transplantation does not always guarantee full development of donor B cells, and therefore the real reason for the favorable outcome of SCT in the present SCID patients remains unclear.[16] However, reduced-intensity conditioning including Flu, which has strong immunosuppressive properties, as reported by Classen *et al.*, and performance of SCT at an early age may be of importance.[17] Thus, the optimal preparative conditioning for patients with SCID remains to be determined.

In the present study four of nine patients (44.4%) developed CMV infection. In the analysis of 170 patients who underwent SCT at Hokkaido University Hospital, no significant differences were identified in the prevalence of CMV infection following SCT for PID patients and in patients with other malignant/non-malignant diseases such as aplastic anemia and congenital metabolic disorder (Kobayashi R, unpubl. data, 2006: $P = 0.099$ and $P = 0.165$, respectively). Interestingly, however, all patients who had suffered from CMV infection before SCT also developed CMV infection after SCT. Haastrup *et al.* reported that in 110 consecutive pediatric and adolescent transplant patients, CMV reactivation occurred in 29% of the CMV-seropositive patients, and found that a positive CMV serology was a risk factor for post-transplant CMV infection.[18] Furthermore, it was suggested that CMV infection induces immunosuppression after SCT and increases the risk of other bacterial and fungal infection.[18] Therefore, in SCT for CMV-seropositive PID patients, regular monitoring for CMV after SCT should be performed using antigenemia, and CMV infection should be treated promptly using ganciclovir.

In summary, it is important to perform SCT for patients with PID before the patient develops complications such as CMV. For this reason, early diagnosis and shortening of the interval between diagnosis and transplantation are very important for successfully helping patients with life-threatening diseases. SCT has become an established treatment procedure for patients with PID, but more progressive and large-scale studies are needed to determine the optimal preconditioning regimen that allows stable engraftment and full immune reconstitution