6. You may puncture transplacentally when the placenta is located anteriorly. Bleeding from the placenta usually stops spontaneously. However, we recommend every effort to avoid this approach by manipulation.
7. The survival rate with this *in utero* transplantation technique is currently 100%, excluding those fetuses that died from massive teratoma formation.
8. Uterine atony requiring oxytocin administration is quite rare in primates.
9. The treatment with proteinase K may need longer time depending on samples.
10. The amount of master mix per slide is 25 μL for Takara cat. no. 9066 and 65 μL for cat. no. 9067.
11. Slides are attached to the Takara slide seal kit. Be careful not to trap air under films.
12. The PCR conditions should be optimized for each *in situ* PCR.
13. The results should be observed within the same day. On the following day, the tissue would peel off, making examination difficult.

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VII

Animal Models and Therapy

NOTE *Laboratory Animal Science*

Prevention of Immune Responses to Human Erythropoietin in Cynomolgus Monkeys (*Macaca fascicularis*)

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ABSTRACT. Genes and proteins of human origin are often administered to monkeys for research purposes, however, it can be difficult to obtain sufficient levels of the products *in vivo* due to immunological clearance. In this study, we showed that human erythropoietin (hEPO) induces generation of anti-hEPO antibody in cynomolgus macaques (n=2), although 92% of amino acid residues are common between the human and macaque EPO. The administered hEPO was thus eliminated from the animals. On the other hand, when an immunosuppressant, cyclosporin A (CyA), was administered (6 mg/kg) intramuscularly every other day in combination with hEPO (n=2), no anti-hEPO antibody was generated and high serum levels of hEPO were obtained during administration of hEPO, resulting in an increase in serum hemoglobin levels. No adverse effects associated with CyA were observed. Thus, CyA treatment is useful for prevention of immune responses associated with the administration of human proteins in monkeys.

KEY WORDS: cyclosporin A, cynomolgus monkey, erythropoietin.

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Macaque monkeys are widely used for preclinical testing of genes and proteins of human origin, taking advantage of their close phylogenetic relationship to humans [5, 11, 21]. Despite the genetic similarity between the two species, human gene products or proteins are often immunogenic to monkeys. An example is erythropoietin (EPO). EPO is a hematopoietic growth factor that stimulates the proliferation and differentiation of erythroid progenitor cells [10]. Recombinant human EPO (hEPO) has a variety of clinical uses [4, 6, 17, 22]. Although 92% of amino acid residues (142/166) are common between human and macaque EPO [12, 20], we showed here that hEPO induces potent immune responses in macaque monkeys, precluding its administration to monkeys.

Therefore, it is necessary to develop a method to prevent such immune responses following administration of hEPO. Among many immunosupressants available, cyclosporin A (CyA) is widely used to suppress detrimental immune reactions associated with allogenic bone marrow and organ transplantation [1–3, 19]. CyA is a calcineurin inhibitor that inhibits nuclear factor of activated T cells (NFAT) activity and induces immunosuppression [9, 13]. In this study, we showed that hEPO can be successfully administered to cynomolgus monkeys (*Macaca fascicularis*) without immunological clearance by using CyA.

Four cynomolgus monkeys (4–6 years old, 2.5–5.5 kg) bred in the Tsukuba Primate Research Center (Ibaraki, Japan) were used in this study (Table 1). The animals were free of intestinal parasites, herpes-B, simian type-D retrovirus, and simian varicella virus. This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [8] and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [14]. The protocols of the experimental procedures were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

First, we administered hEPO (Chugai, Tokyo, Japan) subcutaneously to a cynomolgus monkey (099054) at a dose of 3,000 IU/kg three times a week and assessed the hEPO concentrations in the serum by enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany). Low levels (< 1.0 *ng/ml*) of hEPO were detected for the first 3 weeks, but thereafter the levels decreased to the lowest limit of detection (0.01 *ng/ml*) despite continued administration of hEPO (Fig. 1A). Assessment by ELIZA revealed that anti-hEPO antibody was being generated [7] (Fig. 1A), and the hEPO was cleared from the serum. A second cynomolgus monkey (001051) was intravenously (instead of subcutaneously) given a much lower dose of hEPO (200 IU/kg, three times a week). During administration, very low levels (< 0.1 *ng/ml*) of hEPO were detected with the exception of one time point (1.0 *ng/ml* at day 28), and the levels eventually decreased to zero (Fig. 1B). Despite the lower dose, anti-hEPO antibody was generated again (Fig. 1B), leading to clearance of hEPO from the serum. Although we did not try subcutaneous administration of 200 IU/kg hEPO in the present study (Table 1), we assumed that subcutaneous administration of 200 IU/kg hEPO would also result in anti-hEPO antibody generation

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Table 1. Characteristics of cynomolgus monkeys subjected to hEPO administration

Group	Animal (Sex)	Age (years)	Body Weight (kg)	hEPO Dose (IU/kg)	hEPO Administration Route and Frequency	CyA Dose (ng/kg)	CyA Administration Route and Frequency	Hemoglobin Levels (g/dl) Day 0	Hemoglobin Levels (g/dl) Day 35	Complication
hEPO Only	099054 (Male)	5	5.5	3000	Subcutaneous (3 times a week)	–	–	12.7	12.6	Antibody production
	001051 (Female)	4	2.5	200	Intravenous (3 times a week)	–	–	12.4	12.4	Antibody production
	Average	4.5	4.0	–	–	–	–	12.6	12.5	–
hEPO and CyA	396053 (Female)	6	3.2	200	Subcutaneous (3 times a week)	6	Intramuscular (every other day)	10.9	11.6	None
	396058 (Female)	6	4.0	200	Subcutaneous (3 times a week)	6	Intramuscular (every other day)	11.1	12.0	None
	Average	5.5	3.6	–	–	–	–	11.0	11.8	–

given that intravenous administration of the same dose of hEPO produced this result. The reason for this was subcutaneous administration is known to induce a stronger immune response than intravenous administration [16]. The hemoglobin levels did not increase in either animal (Table 1). Despite the genetic similarity of EPO between humans and macaques [12, 20], hEPO is a potent immunogen in macaque monkeys. This is the first report on the immune responses in monkeys following administration of hEPO.

On the other hand, two cynomolgus monkeys (396053, 396058) were given 6 mg/kg of CyA (Sandimmun; Novartis Pharma, Basel, Switzerland) intramuscularly every other day in combination with subcutaneous hEPO administration (200 IU/kg, three times a week) (Table 1). CyA concentrations in the plasma were assessed by radioimmunoassay according to a previously reported method [15], and it was found that the concentrations were maintained within an effective range of 200 to 400 *ng*/m*l*. As a result, no anti-hEPO antibody was generated in either monkey and high serum levels (around 10 *ng*/m*l*) of hEPO were obtained during administration of hEPO (Figs. 2A and 2B). A second trial of hEPO resulted in a similar elevation of the serum levels of hEPO (Figs. 2A and 2B). The hemoglobin levels apparently increased in response to administration of hEPO (Table 1), suggesting that the hEPO trial was effective when CyA was administered together. Blood biochemistry tests revealed no adverse effects associated with the CyA and hEPO treatment.

We have thus established a method to prevent immune responses to hEPO in cynomolgus monkeys using CyA. In fact, this method has successfully been applied to our preclinical monkey testing, and the long-term (around 1 year) efficacy and safety of CyA administration has been well demonstrated [18]. CyA administration will be useful in preventing immune responses when human proteins are administered to monkeys for research purposes.

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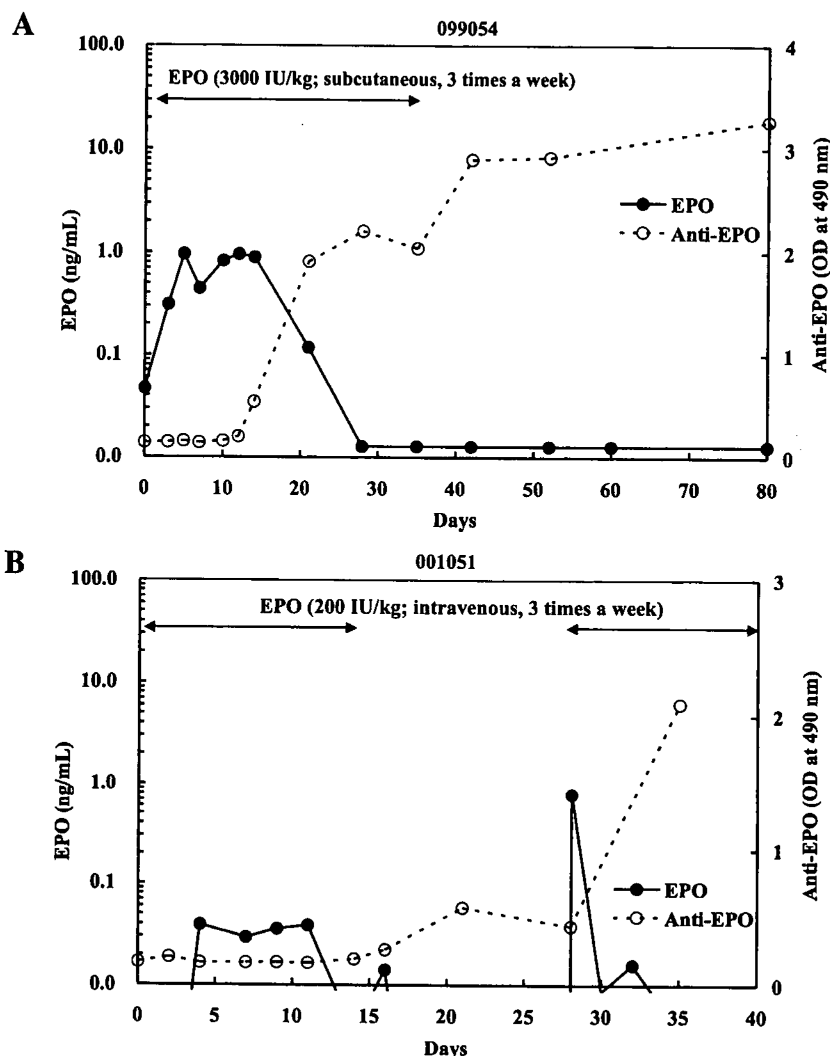

Fig. 1. Administration of only hEPO in cynomolgus monkeys. After subcutaneous administration of hEPO (3,000 IU/kg) to a monkey (099054), anti-hEPO antibody was generated and serum hEPO levels decreased to almost zero (A). Anti-hEPO antibody was also generated in another monkey (001051) receiving hEPO intravenously at a lower dose (200 IU/kg), leading to clearance of hEPO from the serum (B).

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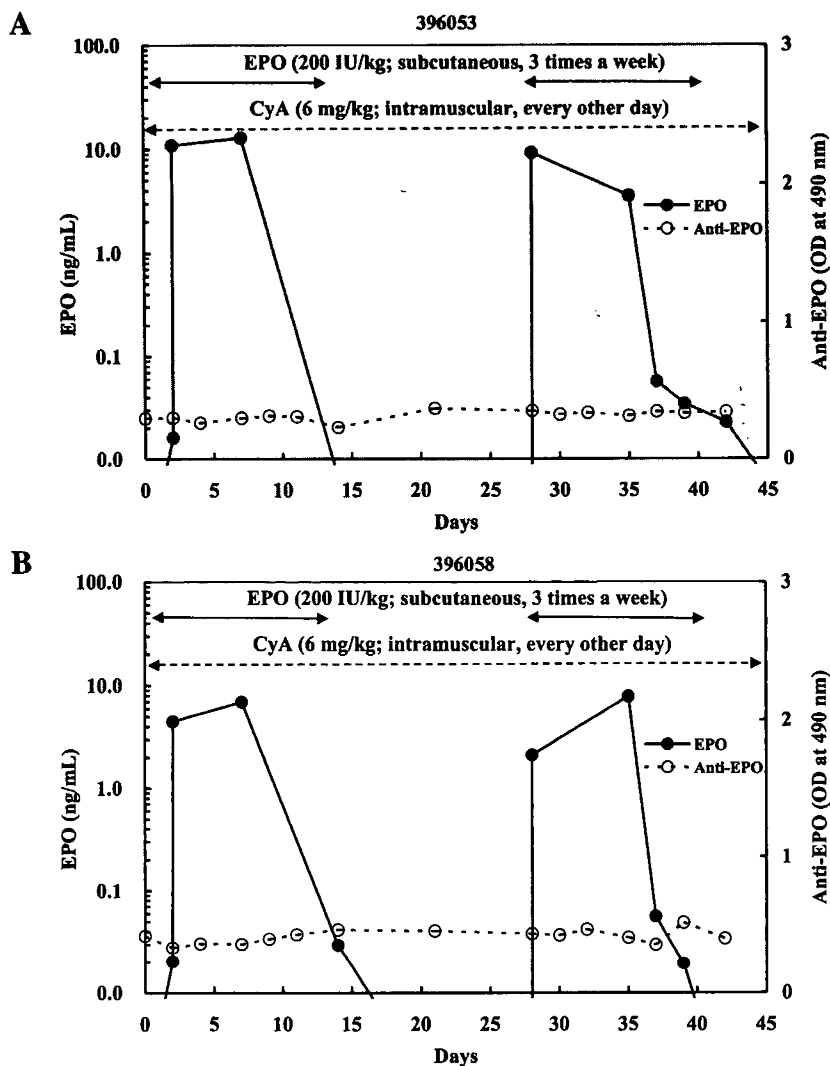

Fig. 2. Administration of hEPO in combination with CyA in cynomolgus monkeys. Generation of anti-hEPO antibody was prevented by treatment with CyA in 2 cynomolgus monkeys (396053, 396058) receiving hEPO (200 IU/kg) subcutaneously (A, B). The plasma CyA concentrations were within an effective range of 200 to 400 *ng/ml*. Under the treatment with CyA, high serum levels of hEPO were obtained during hEPO administration. A second trial of hEPO administration resulted in a similar elevation of serum hEPO levels in 2 monkeys.

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Safe And Efficient Collection of Cytokine-Mobilized Peripheral Blood Cells From Cynomolgus Monkeys (*Macaca fascicularis*) with Human Newborn-Equivalent Body Weights

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Abstract: *Hematopoietic stem cells in bone marrow can be mobilized into peripheral blood by cytokine administration. Cytokine-mobilized peripheral blood stem cells are of great use in clinical applications. We previously established a modified procedure for the collection of cytokine-mobilized peripheral blood cells from rhesus monkeys (*Macaca mulata*) using a commercially available apparatus originally developed for human subjects. In this study, we examined the efficacy and safety of this method with even smaller macaques, cynomolgus monkeys (*Macaca fascicularis*), which are equivalent to human newborns in body weight (mean = 3.3 kg). Using the manufacturer's unmodified protocol (n=6), one monkey died of cardiac failure and three developed severe anemia. In contrast, using our modified procedure (n=6), no such complication was observed in any animal. In addition, the harvested nuclear cell, mononuclear cell and $CD34^+$ cell counts were significantly higher with the modified method. The modified method should allow safe and efficient collection of cytokine-mobilized peripheral blood cells from non-human primates as small as human newborns in a non-invasive manner.*

Key words: *cynomolgus monkey, cytokine mobilization, leukapheresis, peripheral blood stem cell*

Introduction

Although hematopoietic stem cells (HSCs) usually reside in the bone marrow, they can be mobilized into the peripheral blood by the administration of cytokines such as granulocyte colony-stimulating factor (G-CSF) [24]. Cytokine-mobilized peripheral blood stem cells are widely used for autologous and allogeneic trans-

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Table 1. Hematological analysis of cynomolgus monkeys after cytokine treatment

Animals	Sex	Age (years)	Body weight (kg)	After cytokine treatment				
				White blood cells (10^2/µl)	Red blood cells (10^4/µl)	Hemoglobin (g/dl)	Hematocrit (%)	Platelets (10^4/µl)
Unmodified procedure								
292049	Male	6	4.6	680	632	12.0	41.4	36.1
293051	Female	6	2.5	1091	535	10.7	39.0	35.7
292079	Female	7	3.2	514	480	11.6	36.5	35.8
292238	Female	7	3.2	548	653	13.4	44.4	30.0
394029	Female	5	3.2	355	590	11.7	42.3	42.4
296116	Male	3	3.1	361	583	12.0	43.8	26.6
Average		5.7	3.3	592	579	11.9	41.2	34.4
Modified procedure								
001046	Female	3	3.5	872	484	12.4	38.9	43.7
001045	Male	3	3.3	519	415	9.7	33.4	44.2
001049	Male	3	3.5	434	501	12.3	38.7	43.8
001053	Male	3	2.6	802	456	10.3	35.1	57.7
001047	Male	4	3.3	805	438	11.1	35.3	40.4
398042	Male	5	3.8	887	521	11.9	36.8	38.4
Average		3.5	3.3	720	469	11.3	36.3	44.7

plantation therapies to treat hematological malignancies such as leukemia and lymphoma [16, 17]. The cells have also been intensively studied as a donor source of stem cells for gene and cell therapies [11, 20, 30]. An efficient method for collecting cytokine-mobilized peripheral blood cells in monkeys would facilitate such studies in a clinically relevant manner.

The procedure for collecting peripheral blood cells from living animals is referred to as leukapheresis: peripheral blood is withdrawn, nuclear cells are removed, and the rest of the blood is returned [7]. Automatic instruments for this procedure are commercially available [23, 27]. The removed cells are then enriched for a stem cell fraction such as CD34$^+$ or AC133$^+$ cells for clinical applications [5, 29]. Although leukapheresis is widely conducted for human adults, it is difficult to apply to regular experimental macaque monkeys because of their small size. Generally speaking, the procedures involved preclude the application of leukapheresis to animals weighing less than 10 kg in body weight [18].

We and others have previously reported leukapheresis procedures for non-human primates with body weights of less than 10 kg using rhesus monkeys (*Macaca mulata*, average 7 kg) [3, 9]. In such small animals, acute cardiac failure due to the relatively large extracorporeal blood flow is a critical adverse event which can occur during leukapheresis. To avoid this, we modified the procedure by reducing the extracorporeal blood volume as much as possible and adjusting the withdrawal speed frequently in response to the results of real-time monitoring of hemoglobin (Hb) and hematocrit (Ht) values [3]. In this study, we examined the efficacy and safety of our procedure using even smaller non-human primates, cynomolgus monkeys (*Macaca fascicularis*, average 3.3 kg), which have body weights equivalent to human newborns.

Materials and Methods

Animals

Twelve cynomolgus monkeys (*Macaca fascicularis*) (3–7 years old, 2.5–4.6 kg) bred at the Tsukuba Primate Research Center (Ibaraki, Japan) were enrolled in this study (Table 1). Animals were free of intestinal parasites, herpes-B, simian type-D retrovirus and simian varicella virus. All monkeys were housed indoors at 23–27°C and 50–70% humidity with 12 air changes per hour and a 12-h/12-h light/dark cycle. Animals were individually housed in stainless steel cages and fed 70 g of commercial monkey chow (Type AS; Oriental Yeast, Chiba, Japan) and 200 g of fruit daily. All monkeys were healthy as assessed by annual examinations. This

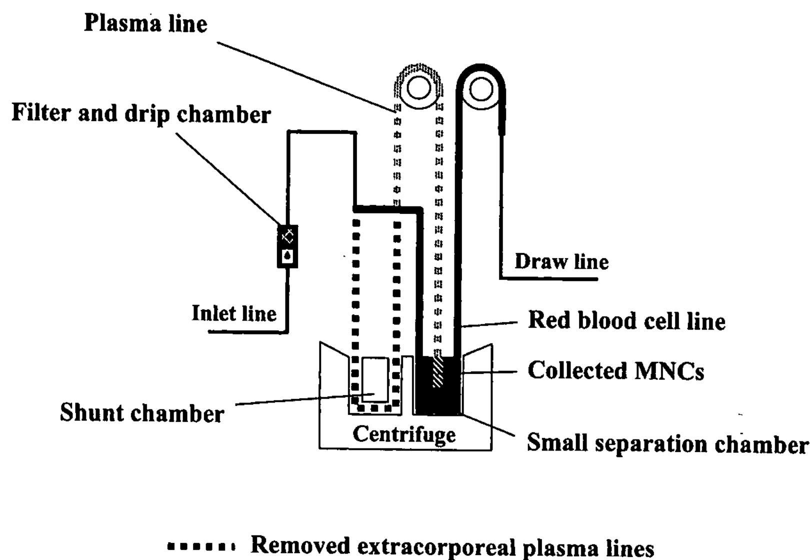

Fig. 1. Diagram of the apheresis kit after the modifications. In the modified system, a small separation chamber (S25A) was installed in the standard apheresis kit and the extracorporeal blood lines were shortened to reduce the extracorporeal blood volume from 130 to 70 ml. MNCs, mononuclear cells.

study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [12] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [21]. This study was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases.

Apparatus

A CS3000 blood separator (Baxter, Deerfield, IL, USA) was used as described previously [3]. Briefly, a standard apheresis kit was installed in the CS3000 blood separator. The smallest separation chamber (S25A) in the kit was used. To reduce the extracorporeal blood volume, the plasma line of the standard apheresis kit was cut away and the red blood cell (RBC) line was directly connected to the inlet line using a polypropylene tube connector (Iuchi, Osaka, Japan) under sterile conditions, bypassing the shunt chamber (Fig. 1). In addition, the regular inlet and draw lines were replaced with lines shorter in length and smaller in diameter (extension tube, 70 cm, 1.4 ml, 2.5 mm diameter; TOP, Tokyo, Japan) to further reduce the extracorporeal blood volume.

Preparative regimen

During the three weeks preceding leukapheresis, a total of 60–90 ml of autologous blood was obtained from each monkey as described previously [6]. The collected autologous blood was supplemented with acid citrate dextrose (ACD) and stored at 4°C prior to use. Recombinant human (rh) stem cell factor (SCF, 50 μg/kg; Amgen, Thousand Oaks, CA, USA) and rhG-CSF (50 μg/kg; Chugai, Tokyo, Japan) were administered to animals subcutaneously daily during the 5 days preceding leukaphersis [9]. On the day of leukapheresis, the right or left femoral artery was cannulated using a 5-Fr polyurethane catheter (Anthron PU; Toray, Tokyo, Japan). The saphenous vein was also catheterized with a 19-gauge intracatheter (Terumo, Tokyo, Japan). This cannulation was performed under general anesthesia with the administration of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan).

Leukapheresis

All leukapheresis procedures were performed under general anesthesia with endotracheal intubation (Fig. 2). Vital signs were monitored with electrocardiography, blood pressure, oxygen saturation and respiration. Animals received a dose of 100 U/kg heparin (Aventis Pharma, Frankfurt, Germany) just before the initiation of leukapheresis. The plasma flowed directly into the inlet line bypassing the shunt chamber (Fig. 1). The inlet line

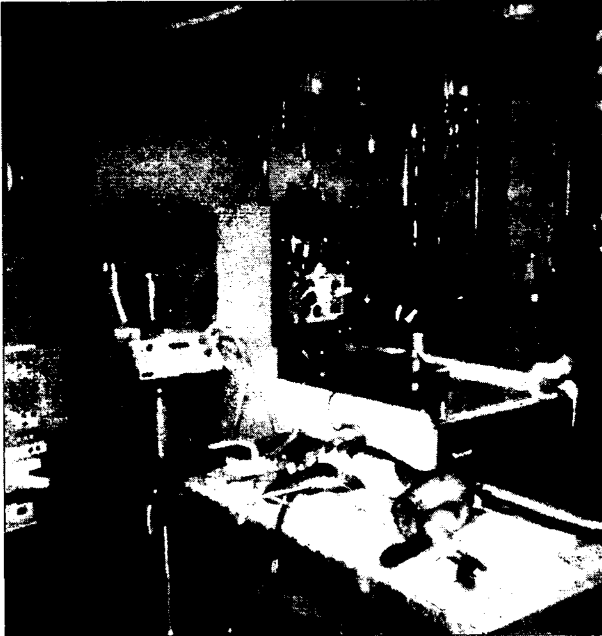

Fig. 2. Leukapheresis procedure. Cynomolgus monkeys were intubated and all procedures were performed under general anesthesia with monitoring of vital signs. The body weights (3.3 kg on average, see Table 1) were similar to those of human newborns.

was connected to an intracatheter placed in the saphenous vein of the animal. The draw line was connected to a catheter in the femoral artery just before starting the procedure. The apheresis kit was filled with the autologous blood collected as described above.

After the leukapheresis was completed, the blood remaining in the apheresis kit was recovered and either used to fill another apheresis kit or returned into the animal. Immediately after the leukapheresis, animals were given an appropriate dose (10 mg per 1,000 U heparin) of protamine sulfate (Aventis Pharma) to neutralize heparin. Animals also received a course of 0.5 mg/kg butorphanol tartrate (Bristol-Myers Squibb, New York, NY, USA) intramuscularly for 3 days to alleviate any post-operative pain.

Analysis of leukapheresis products

The product obtained during leukapheresis was collected in the S25A separation chamber. The product (40–45 ml) was mixed with 7 ml of ACD. The recovered RBCs, white blood cells (WBCs), mononuclear cells and platelets were enumerated with a Sysmex K-4500 instrument (Toa-iyoudenshi, Kobe, Japan). Hb and Ht values were also examined with this instrument. Although the instrument was originally developed for human blood samples, we have confirmed that it works properly for monkey blood samples. Blood cells were collected after centrifugation at 1,200 rpm for 10 min and suspended in the ACK buffer (Biosource, Camarillo, CA, USA) for the lysis of RBCs. $CD34^+$ cells were isolated with immunomagnetic beads conjugated to a monoclonal antibody clone 561 (Dynal, Lake Success, NY, USA) that reacts to both human and cynomolgus CD34 [26, 33]. The harvested $CD34^+$ cells were counted. CD34 is a clinically-relevant cell-surface marker of HSCs, and $CD34^+$ cell transplantation is widely performed as HSC transplantation in patients with cancer or other disorders [5, 19].

Results

We examined the safety and efficacy of leukapheresis using the manufacturer's protocol (n=6) and our modified version (n=6) in cynomolgus monkeys (Fig. 2). In both groups, we administered G-CSF and SCF to monkeys for 5 days to mobilize HSCs into the peripheral blood. The administration resulted in an increase in peripheral WBC counts to 66,000 cells/μl on average (Table 1), and was not associated with any adverse effect such as fever or anorexia. There was no significant difference in the increase in the peripheral WBC counts after cytokine treatment between the unmodified and modified procedure groups. In the modified protocol, a small separation chamber (S25A) was installed instead of the regular one in the blood separator, and the extracorporeal blood lines in the standard apheresis kit were shortened [3, 9, 22]. As a result, the extracorporeal blood volume was reduced from 130 to 70 ml (Fig. 1). In both groups, blood was processed at a rate of 10–12 ml/min and the total processed volume was two to three times the estimated total blood volume [4] of each animal (Table 2). In the modified protocol, every time the processed blood volume increased by 50 ml/kg, a 1-ml blood sample was collected *via* the draw line, and Hb and Ht values were examined throughout the procedure to adjust the plasma pump speed [14]. The plasma pump speed was increased when Hb and Ht values decreased. Conversely, it was decreased when Hb and Ht values increased. In addition, when Hb and Ht values increased, normal saline

Table 2. Leukapheresis procedures

Animals	Estimated total blood volume (ml)*	Processed blood		Complications
		Total volume (ml)	ml/kg	
Unmodified procedure				
292049	293	600	130	None
293051	217	600	240	Severe anemia
292079	231	400	125	Severe anemia Died of cardiac failure
292238	231	600	188	None
394029	231	700	219	Severe anemia
296116	227	600	194	None
Average	238	583	183	
Modified procedure				
001046	237	750	214	None
001045	236	600	182	None
001049	244	800	229	None
001053	205	500	192	None
001047	236	600	182	None
398042	258	700	183	None
Average	236	658	197	

*The total blood volume was estimated with the following formula [4]. For males, (Total blood volume, ml) = 44.07 × (Body weight, kg) + 90.25. For females, (Total blood volume, ml) = 19.95 × (Body weight, kg) + 167.24.

was infused *via* the inlet line for volume replacement.

After the completion of the unmodified procedure (n=6), one animal died of acute cardiac failure and three animals developed severe anemia (Hb<8.0 g/dl, Fig. 3). In contrast, none of the animals that underwent the modified procedure (n=6) developed cardiac failure or severe anemia (Table 2). The Hb and Ht values were significantly better preserved during the modified procedure (Fig. 3). In addition, the numbers of harvested nuclear cells, mononuclear cells, and $CD34^+$ cells were significantly increased with the modified procedure compared to the unmodified one (Fig. 4).

The leukapheresis products were contaminated with considerable amounts of RBCs and platelets, when the apparatus was operated in automatic mode under the unmodified protocol. In the modified version, we performed manual adjustment of the plasma pump speed in response to the results of the real-time monitoring of Hb and Ht values during the leukapheresis as described above, and successfully reduced the contamination (data not shown). The reduction in contaminated RBCs also contributed to the amelioration of anemia after leukapheresis in the modified procedure group. No microbial contamination was detected in cultures of the leukapheresis products from the unmodified or modified procedures.

Discussion

In this paper, we reported leukapheresis in cynomolgus monkeys. Our modified protocol significantly improved mononuclear and $CD34^+$ cell harvest compared to the manufacturer's protocol. Under our modified procedure, we routinely collected 5×10^6 $CD34^+$ cells per kg, which is equivalent to numbers in human trials published in the literature [13, 25, 28, 31]. Thus, with our modified protocol, it is possible to collect sufficient numbers of $CD34^+$ stem cells for various applications including transplantation experiments in monkeys. In fact, we achieved successful hematopoietic reconstitution in myeloablated cynomolgus monkeys after the autologous transplantation of $CD34^+$ cells obtained with this procedure [2]. Of note, this procedure can be safely and effectively applied to monkeys with small body weights (2.6–3.8 kg), equivalent to those of human newborns. Although numerous clinical trials have demonstrated the safety and effectiveness

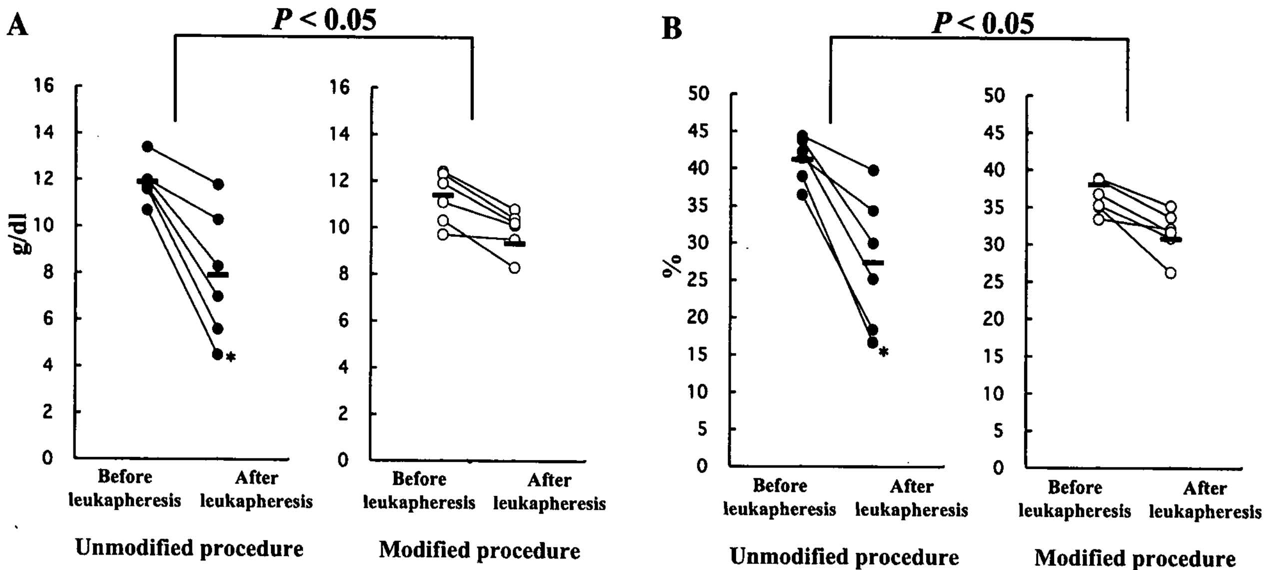

Fig. 3. Avoidance of severe anemia with the modified procedure. The degree of anemia was significantly ameliorated with the modified procedure as compared to the unmodified one as assessed by the ratios of hemoglobin (A) and hematocrit levels (B) after versus before the leukapheresis. One monkey died of cardiac failure (*, 292079) after the unmodified procedure. A: Hemoglobin levels. B: Hematocrit levels.

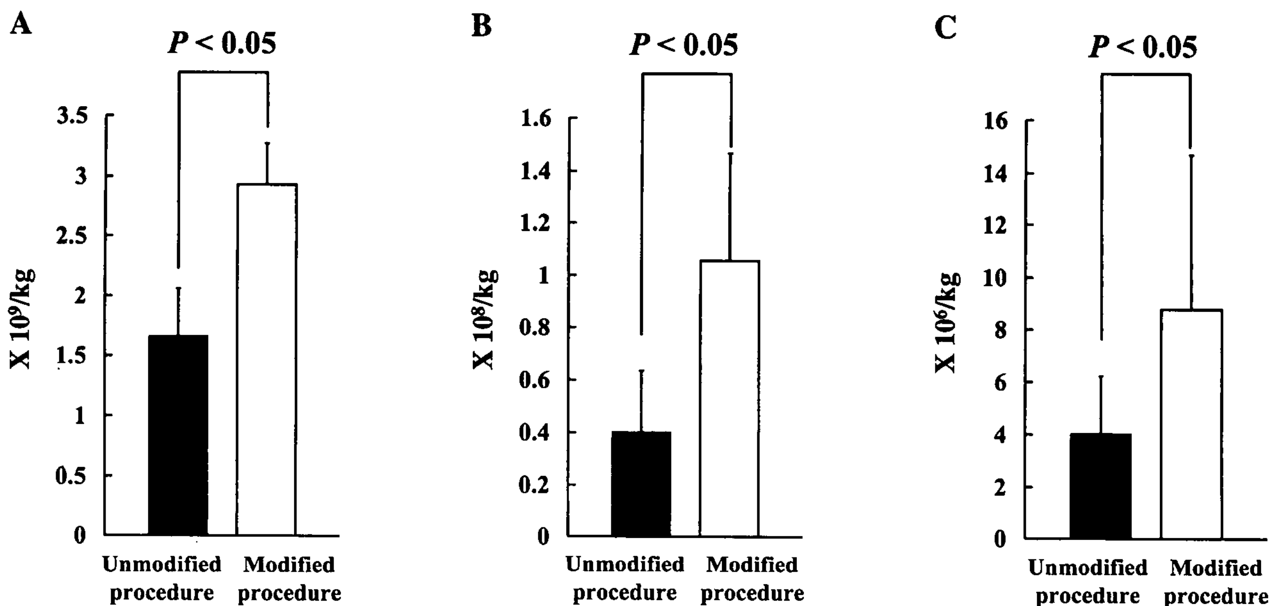

Fig. 4. Larger harvest of cells with the modified procedure. The numbers of harvested nuclear cells (A), mononuclear cells (B), and CD34+ cells (C) were significantly increased with the modified procedure. A: Total nuclear cells. B: Mononuclear cells. C: CD34+ cells.

of leukapheresis for adults [10, 13, 25, 28] and children [1, 8, 15, 31], only one very limited trial has been conducted on a human newborn baby [32]. To our knowledge, this paper is the first systematic documentation of leukapheresis for small primates.

One of the most serious complications in leukapheresis with small animal subjects is cardiac failure due to the relatively large amount of extracorporeal blood volume [10, 32]. The main symptoms include hypotension and dyspnea, which sometimes result in

death, *e.g.* animal 292079 (Table 2). To avoid this complication, the extracorporeal blood volume should be reduced as much as possible. No monkeys underwent cardiac failure after our modified procedure. There was, however, an age variation in the unmodified and modified procedure groups. The unmodified procedure group included higher age animals (6 and 7 years). Cynomolgus monkeys of these ages are young adults, and presumably they are more resistant to stress or invasion than monkeys of a juvenile age (3 years). Clearly, the age distribution of the groups, the higher age monkeys belonging to the modified procedure group, was not better than the present way.

Non-human primate models would be useful for preclinical studies of cell and gene therapies. We have previously reported the transplant of CD34+ stem cells into the ischemic myocardium in cynomolgus monkeys and found that the cardiac function was improved, indicating that further investigation is warranted for clinical application of CD34+ stem cell transplant to such disorder [34]. In other studies, we successfully transplanted gene-modified CD34+ stem cells into cynomolgus monkeys as a preclinical gene therapy [11, 30]. In this way, our safe and efficient method for collecting peripheral blood stem cells should allow investigators to develop and test new therapies using stem cells in small non-human primates.

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STEM CELLS®

Repair of Infarcted Myocardium Mediated by Transplanted Bone Marrow–Derived CD34+ Stem Cells in a Nonhuman Primate Model

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Key Words. Nonhuman primate • Acute myocardial infarction • Stem cell transplantation Genetic marking • Lentivirus vector • Plasticity • Neoangiogenesis

ABSTRACT

Rodent and human clinical studies have shown that transplantation of bone marrow stem cells to the ischemic myocardium results in improved cardiac function. In this study, cynomolgus monkey acute myocardial infarction was generated by ligating the left anterior descending artery, and autologous CD34+ cells were transplanted to the peri-ischemic zone. To track the in vivo fate of transplanted cells, CD34+ cells were genetically marked with green fluorescent protein (GFP) using a lentivirus vector before transplantation (marking efficiency, 41% on average). The group receiving cells (*n* = 4) demonstrated improved regional blood flow and cardiac function compared with the saline-treated group (*n* = 4) at 2 weeks after transplant. However, very few transplanted cell–derived, GFP-positive cells were found incorporated into the vascular structure, and GFP-positive cardiomyocytes were not detected in the repaired tissue. On the other hand, cultured CD34+ cells were found to secrete vascular endothelial growth factor (VEGF), and the in vivo regional VEGF levels showed a significant increase after the transplantation. These results suggest that the improvement is not the result of generation of transplanted cell–derived endothelial cells or cardiomyocytes; and raise the possibility that angiogenic cytokines secreted from transplanted cells potentiate angiogenic activity of endogenous cells. STEM CELLS *2005;23:355–364*

INTRODUCTION

Recent clinical studies have shown that the introduction of bone marrow cells can restore blood flow in ischemic myocardium and ameliorate cardiac function [1–6]. Despite enthusiasm for these studies, it is unclear how transplanted bone marrow cells contribute to the clinical improvement. Because endothelial progenitor cells are identified in bone marrow cells [7], these cells might participate in the repair

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of vascular tissue. On the other hand, it has been reported that hematopoietic stem cells differentiate into endothelial cells and cardiomyocytes when transplanted into the ischemic myocardium in mice [8]. More recently, however, it has been reported that hematopoietic stem cells do not give rise to nonhematopoietic cells in the ischemic myocardium in murine models [9–11].

In vivo tracking and plastic properties of hematopoietic stem or progenitor cells have not been examined in primate cardiac ischemia. We have transplanted genetically marked autologous CD34$^+$ cells to the ischemic myocardium in a nonhuman primate (cynomolgus macaque) model and tracked the in vivo fate of the cells. We have used CD34$^+$ cells because the cells are widely used as a fraction of hematopoietic stem cells in clinical and nonhuman primate studies [12]. In addition, CD34$^+$ cells contain vascular endothelial progenitor cells [7]. Thus, the present study can address the question of whether transplanted CD34$^+$ cells really give rise to endothelial cells and cardiomyocytes in ischemic myocardium in primates.

Materials and Methods

Animals

Eight cynomolgus macaques bred in the Tsukuba Primate Center (Ibaraki, Japan) were enrolled in the present study. This study strictly adhered to the rules for animal care and management of the Tsukuba Primate Center, as well as the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The protocols of animal experiments were approved by the animal welfare and animal care committee of the National Institute of Infectious Diseases (Tokyo).

Preparation of CD34$^+$ Cells

Cynomolgus bone marrow (50 ml) was aspirated from the iliac crest under an isoflurane-induced general anesthesia. From the bone marrow, a nucleated cell fraction was obtained after red blood cell lysis with addition of ACK buffer (Biosource, Camarillo, CA). CD34$^+$ cells were isolated using magnetic beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY), which cross-reacts with cynomolgus CD34 [13]. The purity of CD34$^+$ cells at harvest ranged from 90% to 95%, as assessed with another anti-human CD34 (clone 563; PharMingen, San Diego) that cross-reacts with cynomolgus CD34 [13]. The purity remained at the same levels after the 1-day transduction culture, which is discussed next.

Lentiviral Transduction

A simian immunodeficiency virus (SIV)-based lentivirus vector carrying enhanced jellyfish green fluorescent protein (GFP) (Clontech, Palo Alto, CA) was used for transduction. The vector was prepared as previously reported [14, 15]. All recombinant DNA experiments were approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

CD34$^+$ cells (1×10^6) were seeded in six-well plates in 2 ml of StemSpan serum-free expansion medium (Stem Cell Technologies, Vancouver) supplemented with recombinant human thrombopoietin (100 ng/ml; Kirin, Tokyo), recombinant human stem cell factor (100 ng/ml; Biosource, Camarillo, CA), recombinant human Flt-3 ligand (100 ng/ml; Research Diagnostics, Flanders, NJ), and antibiotics (100 U/ml of penicillin and 0.1 μg/ml of streptomycin; Meiji, Tokyo). The cells were transduced twice each for 12 hours (total, 24 hours) with the SIV vector at 50 transducing units per target cell. After transduction, cells were cryopreserved with 10% dimethylsulfoxide (Wako, Osaka, Japan) and 1% Dextran 40 (Yoshitomi, Osaka, Japan) in a controlled-rate programmable freezer (Kryo 10; Planer Biomed, Middlesex, UK) until transplantation. The viability of cells after thawing was $53.0 \pm 6.5\%$, as assessed by trypan blue staining. An aliquot of transduced cells was assessed for GFP expression at 48 hours after transduction by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ) with excitation at 488 nm and fluorescence detection at 530 ± 30 nm.

In Vitro Endothelial Differentiation

CD34$^+$ cells were seeded on fibronectin-coated plates (Becton Dickinson) in M199 medium (Invitrogen, Carlsbad, CA) with 20% fetal calf serum and bovine pituitary extracts (Invitrogen) as previously described [7]. After 7 days in culture, cells were examined for the uptake of DiI-acetylated low-density lipoprotein (LDL) and for the expression of CD31, von Willebrand factor (vWF), vascular endothelial (VE)-cadherin, and vascular endothelial growth factor receptor (VEGFR)-2. Briefly, adherent cells were incubated with 1 μg/ml of DiI-acetylated LDL (Molecular Probes, Eugene, OR) for 4 hours at 37°C. For immunofluorescence staining, after fixation in ice-cold 4% paraformaldehyde for 10 minutes and blocking in 1% bovine serum albumin (BSA) for 15 minutes, cells were incubated with a primary antibody: mouse anti-human CD31 (VM-59; Becton Dickinson), rabbit anti-human vWF (DakoCytomation, Glostrup, Denmark), mouse anti-human VE-cadherin (55-7H1; Becton Dickinson), or rabbit anti-mouse VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room

temperature. Cells were then incubated with a secondary antibody, Texas red–conjugated horse anti-mouse immunoglobulin G (IgG) (Vector, Burlingame, CA) or goat anti-rabbit IgG (Vector) for 30 minutes at room temperature.

Myocardial Infarction and Transplantation

All operations on cynomolgus monkeys were performed under an isoflurane-induced general anesthesia. Thoracotomy was conducted, the pericardium was opened, and the left anterior descending coronary artery was ligated with 5-0 prolene sutures. One to 2 hours after the ligation, GFP-transduced, autologous $CD34^+$ cells in normal saline were injected with a microsyringe through a 27-gauge needle into 10 sites (5 μl/site) in the peri-ischemic zone. In the control group, saline alone was injected in the same way. The pericardium and chest were closed. The animals then received butorphanol tartrate (0.5 mg/kg, intramuscularly) daily for 5 days to alleviate the pain associated with the operation and myocardial infarction.

Echocardiography

Echocardiographic imaging was obtained using a Sonos 5500 system (Philips Medical Systems, Andover, MA) before transplantation and at 2 weeks after transplant. The echocardiography was conducted by independent technicians irrelevant to our study group. In one animal (BM97080), it was additionally performed at 12 weeks. Short-axis two-dimensional images at the midpapillary level of the left ventricle were stored, and percent fractional shortening (%FS) was calculated to assess cardiac function.

Myocardial contrast echocardiography (MCE) was performed at day 0 (just before transplantation) and at 2 weeks after transplant to assess regional blood flow and blood flow defect size. In one animal (BM97080), chronic assessment was performed at 12 weeks after transplant. The electrocardiograph-triggered end-systolic intermittent imaging was conducted in short-axis views at incremental pulsing intervals (triggering intervals of 1, 2, 3, 4, and 8 beats) using an S12 probe. Once optimized, the settings of depth (4 cm), mechanical index (0.9), and focus (3 cm) were fixed. The contrast agent (perflutren; Yamanouchi, Tokyo) consisted of lipid-coated microbubbles of perfluorocarbon [16]. Perflutren diluted with saline (1:10) was administered intravenously at a constant rate (0.01 ml/kg per min). For the assessment of regional blood flow, MCE images were analyzed using ORIGIN 6.0J (Lightstone, Tokyo), and the blood flow was calculated as previously described [17]. Data are presented as a blood flow ratio (the peri-infarct versus nonischemic control region or the infarct versus nonischemic control region). For the assessment of blood flow defect, MCE images obtained at triggering interval of four beats were analyzed using National Institutes of Health Image software (version 1.61). Data are presented as percent defect compared with the total blood flow.

Microspheres

Colored microspheres (15 μm ± 2% diameter; E-Z Trac, Los Angeles) were used to evaluate regional blood flow 2 weeks after transplant [18], with the exception of one animal (BM97080), in which evaluation was performed 12 weeks after transplant. A set of microspheres (2×10^6) was diluted in 2 ml of saline and injected into the left ventricle over 30 seconds. A reference blood sample was withdrawn at a constant rate of 5 ml/min through the femoral artery. After the collection of blood samples, monkeys were irrigated with saline for mercy killing and blood was completely washed out. The heart was excised from each monkey. Tissue samples from the infarct, peri-infarct, and nonischemic regions (one sample per region) were digested, microspheres were collected, and the blood flow was calculated according to the manufacturer's instructions. Data are presented as blood flow ratio (the peri-infarct versus nonischemic control region or the infarct versus nonischemic control region).

Immunohistochemistry

Tissue samples from the infarct, peri-infarct, and nonischemic regions at 2 weeks after transplant were embedded in optimal cutting temperature compound (Sakura, Zoeterwoude, Netherlands) and frozen in liquid nitrogen. Sections were prepared (6 μm), fixed for 10 minutes at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS), and blocked with 1% BSA in PBS. The sections were incubated at room temperature with a primary antibody, monoclonal mouse anti-human CD31 (1:200; Becton Dickinson), followed by a secondary antibody, biotin-conjugated horse anti-mouse IgG (1:500; Vector). The sections were then treated with avidin-alkaline phosphatase (ABC AP kit; Vector) for 30 minutes. The reaction was developed with a Vector Red substrate kit (SK-5100; Vector). In the case of double staining of CD31 and GFP, the above sections were further incubated with polyclonal rabbit anti-GFP (1:200; Clontech) followed by biotin-conjugated anti-rabbit IgG (1:500; Vector) and treated with avidin-peroxidase (ABC Elite kit; Vector). The reaction was developed with a Vector SG substrate kit (SK-4700; Vector). The sections were counterstained with hematoxylin, mounted in glycerol, and examined under a light microscope.

In Situ Polymerase Chain Reaction

In situ detection of transduced cell progeny was performed by amplifying proviral sequences as previously reported [19]. The following primer set for the GFP gene was used:

5'-CGT CCA GGA GCG CAC CAT CTT C-3' and 5'-GGT CTT TGC TCA GGG CGG ACT-3'. The polymerase chain reaction (PCR) mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche, Mannheim, Germany), 0.8 μM of each GFP primer, 4.5 mM $MgCl_2$, 1 × PCR buffer (Mg^{2+} free), and 4 U of Takara Taq DNA polymerase (Takara, Kyote). Sections were prepared with a Takara slide frame (Takara) from the infarct, peri-infarct, and nonischemic regions at 2 weeks after transplant. PCR was performed using a PTC100 thermal cycler (MJ Research, Watertown, MA) with the following conditions: 94°C for 1 minute and 57°C for 1 minute with 10 cycles. The digoxigenin-incorporated DNA fragments were detected using horseradish peroxidase (HRP)-conjugated rabbit F(ab') anti-digoxigenin antibody (DakoCytomation). The sections were then stained for HRP using a Vector SG substrate kit (Vector). Finally, the sections were counterstained with a Kernechtrot solution (Muto, Tokyo) that stains nucleotides, mounted in glycerol, and examined under a light microscope.

ELISA

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) levels in tissue lysate or medium were assessed by ELISA (R&D Systems, Minneapolis) according to the manufacturer's instructions. Tissue lysate was obtained from the peri-infarct region (three samples from each monkey) at 2 weeks after transplant. Briefly, tissue was homogenized and suspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, and protease inhibitor cocktail tablets (Complete Mini, Roche). The suspension was rocked at 4°C for 20 minutes and centrifuged at 16,000g and 4°C for 30 minutes. The supernatant was used for ELISA. The protein concentration of lysate was determined with DC Protein Assay (Bio-Rad, Hercules, CA).

RESULTS

Lentiviral Marking

The $CD34^+$ fraction of autologous bone marrow cells was used for transplantation in our study (Table 1). Before transplantation, $CD34^+$ cells were genetically marked with GFP using an SIV-based lentivirus vector. The ex vivo transduction results are summarized in Table 1. The transduced cells were frozen until transplantation. An aliquot of the transduced cells was examined in vitro for the endothelial differentiation ability. After the differentiation culture, a vessel-like structure was observed (Fig. 1A). The ability of cells to take up DiI-acetylated LDL and the expression of CD31, vWF, VE-cadherin, and VEGFR-2 suggested the endothelial lineage (Fig. 1B). We and others have already confirmed the ability of hematopoietic differentiation of the cells [20, 21]. Taken together, the SIV-mediated GFP gene transfer does not spoil the differentiation abilities of $CD34^+$ cells. In addition, on average, 41% of cells fluoresced 48 hours after transduction, and 56% of

Table 1. Summary of ex vivo transduction and transplantation

	Sex	Age (y)	Body weight (kg)	Harvested bone marrow cell number	Isolated $CD34^+$ cell number	Transplanted cell number	% GFP expression Before[a]	After[b]
Saline group								
CTR01061[c]	M	3	4.1		NA			
CTR99056	M	3	3.4					
CTR96116	F	5	3.2					
CTR99051	M	5	5.9					
$CD34^+$ cell group								
BM01052	M	3	3.9	213×10^6	1.00×10^6	0.47×10^6	49	87
BM01051[d]	M	3	4.1	396×10^6	5.14×10^6	2.20×10^6	51	54
BM97080[e]	M	5	3.9	330×10^6	2.35×10^6	1.04×10^6	49	67
BM90047	M	13	5.8	343×10^6	3.10×10^6	1.07×10^6	16	14
Average		5	4.3	321×10^6	2.90×10^6	1.20×10^6	41	56

[a]Before endothelial differentiation of GFP-transduced $CD34^+$ cells.
[b]After the in vitro endothelial differentiation.
[c]CTR01061 died of heart failure 5 days after myocardial infarction.
[d]BM01051 developed a ventricular aneurysm after myocardial infarction.
[e]BM97080 was killed 12 weeks after the treatment. All other animals were killed 2 weeks after the treatment.
Abbreviations: GFP, green fluorescent protein; NA, not applicable.

endothelial cells still fluoresced after in vitro differentiation (Table 1), showing that the GFP expression is stable during the in vitro differentiation to endothelial cells. Thus, GFP was expected to serve as a good genetic tag after transplantation.

Acute Myocardial Infarction and Autologous Transplantation

Cynomolgus acute myocardial infarction was generated by ligating the left anterior descending artery. One to two hours after the ligation, GFP-transduced, autologous $CD34^+$ cells were injected in the peri-ischemic zone at 10 sites (total, $1.20 \pm 0.73 \times 10^6$ cells; $n = 4$). In the control group, saline was injected in the same way ($n = 4$). We conducted contrast echocardiography immediately after the coronary ligation and found no significant differences in the blood flow defect size (percent blood flow defect compared with the total) between the cell-treated and saline-treated groups ($13.0 \pm 2.1\%$ versus $12.3 \pm 3.5\%$, $p = .75$), suggesting that the initial risk of infarction did not differ between the two groups. In addition, we tried to assess the cardiac isozyme of serum creatine kinase (CK) to evaluate the infarct size; however, either the immuno-inhibition assay or chemical luminescence immunoassay did not work well for cynomolgus monkey samples. We were at least able to show that total CK values at 24 hours after the ligation did not significantly differ between the two groups ($p = .83$).

One of the control monkeys (CTR01061) died of heart failure 5 days after myocardial infarction, and the other control monkeys showed a decrease in %FS at 2 weeks after infarction (Fig. 2). Thus, all four control animals showed the deteriorated cardiac function. In the cell-treated group, one monkey (*, BM01051) underwent ventricular fibrillation immediately after the ligation and survived after cardiopulmonary resuscitation but eventually developed a ventricular aneurysm. Only this animal showed a decrease in %FS despite $CD34^+$ cell treatment; the other animals receiving $CD34^+$ cells showed an increase in %FS (Fig. 2). $CD34^+$ cell treatment may not be able to rescue such a heavily impaired heart but otherwise had a significant effect on cardiac function. Even an old monkey (BM90047, Table 1) showed improved %FS.

The relative blood flow in the peri-infarct to nonischemic control region was also significantly ameliorated in the $CD34^+$ cell–treated monkeys compared with the saline-treated ones, as assessed using contrast echocardiography (Fig. 3A) and colored microspheres (Fig. 3B). An excellent correlation was found between the two methods (Fig. 3C; correlation coefficient = 0.93). Two groups ($CD34^+$ cell-treated and saline-treated) were well separated on the panel, showing an obvious positive effect of $CD34^+$ cell injection on the blood flow in the peri-infarct zone after acute myocardial infarction. In fact, the average myocardial blood flow in the peri-infarct region in the absolute value was 0.988 ml/g per minute and 0.383 ml/g per minute for the cell-treated and saline-treated groups, respectively. Of note, the blood flow in the peri-infarct zone was ameliorated even in the animal with a ventricular aneurysm. On the other hand, the relative blood flow in the infarct to nonischemic region did not show

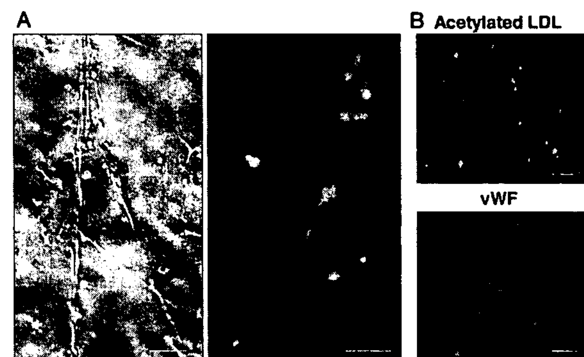

Figure 1. In vitro endothelial differentiation of cynomolgus $CD34^+$ cells lentivirally transduced with GFP. The transduced $CD34^+$ cells were differentiated to endothelial cells after 7 days in culture. **(A)**: Representative vessel-like structure derived from $CD34^+$ cells observed under a phase-contrast microscope (left) and a fluorescent microscope (right). **(B)**: The transduced $CD34^+$ cells differentiated into fluorescent cells (green) positive for the cellular intake of acetylated LDL and immunostaining for von Willebrand factor (vWF) (stained in red). Bar = 100 μm. Abbreviations: GFP, green fluorescent protein; LDL, low-density lipoprotein.

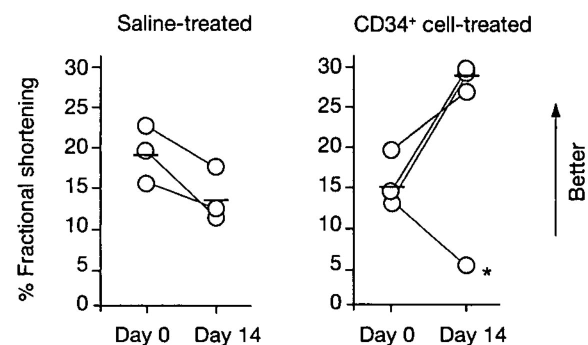

Figure 2. Improved cardiac function after $CD34^+$ cell transplantation. Cardiac function was assessed by echocardiography in terms of percent fractional shortening (%FS) before and 2 weeks after treatment. One monkey in the saline-treated group (CTR01061) died of heart failure 5 days after myocardial infarction and is not included in the figure. One monkey in the $CD34^+$ cell–treated group (*, BM01051) developed a left ventricular aneurysm after myocardial infarction. If this animal was excluded from the statistical analysis, the cardiac function was significantly improved in the $CD34^+$ cell–treated compared with the saline-treated group in terms of the ratio of %FS at day 14 versus day 0 after transplant ($p = .017$).