Establishment of The Preclinical Model for Hematopoietic Stem Cell Gene Therapy Using Non-Human Primates

造血幹細胞による遺伝子治療のための サル類を用いた前臨床評価系の樹立

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Abbreviation

BM: bone marrow

BW: body weight

CRP: C-reactive protein

CyA: cyclosporin A

ECV: extracorporeal blood volume

EPO: erythropoietin

FL: fms-like tyrosine kinase-3 ligand

G-CSF: granulocyte colony-stimulating factor

HSCs: hematopoietic stem cells

Ht: hematocrit

Hb: hemoglobin

IL: interleukin

MNCs: mononuclear cells

RBC: red blood cell

SCF: stem cell factor

SPF: specificpathogen-free

TBV: total blood volume

TPO: thrombopoietin

WBC: white blood cell

General Introduction

After many years of the study on the identity of potential bloodforming stem cells so called hematopoietic stem cells (HSCs) [97], a lot of researchers have begun exploring their potentials for therapeutic use. HSCs have been pursued as highly desirable targets for gene therapy and regenerative medicine because of their selfrenewal and multilineage differentiation capabilities [37].

The successful HSC gene therapy for X-linked severe combined immunodeficiency has been reported [13, 34]. These encouraging results represented the first unequivocal demonstration of the clinical efficacy of gene therapy. However, elation was short-lived, as 3 out of 11 enrolled children in this clinical trial have developed T cell leukemia [35, 36, 94]. Thus, at present the risks and potential benefits of gene therapy are being reconsidered and US National Institutes of Health (NIH) recommended optimization using large animal models such as non-human primates, because it might be difficult to establish the HSCs gene therapy approach for clinical application [77].

In the mice HSCs marking study with retroviral vectors, the entire hematopoiesis can be reconstituted by a single or few HSCs [65]. However, no data about clonality of hematopoiesis in larger animals have been available. The hematopoietic demand of a mouse may be very small. A typical mouse (25 g) makes, in a two-year lifetime, the same amount of red blood cells (RBCs) as does a man in one day, raising the possibility that HSCs kinetics in large animals are more complex than small rodents [2]. As these results, we can easily and sharply imagine that the behaviors of human HSCs might be very different in mice models. Large animal transplantation models have proved to be essential for the assay of HSCs in human, because there are no reliable data in mice model for extrapolation to human HSCs assay [8]. Among large animals, non-human primates such as macaque monkeys may provide the best animal

models because of their close phylogenetic distance to humans [54, 102, 103]. It was essential to establish the non-human primate model to understand human hematopoiesis, to examin the efficacy and safety of hematopietic gene therapy. Therefore, I tried to establish the safe and efficient methods for collection of hematopietic stem cells and autologous transplantation of HSCs in non-human primates.

HSCs can be collected from the cytokine-mobilized peripheral blood, bone marrow (BM), and umbilical cord blood. HSCs in BM can be mobilized into peripheral blood by cytokine administration. Cytokine-mobilized peripheral blood stem cells are the most useful for being advantage in clinical applications. Therefore, it is indispensable to establish the massive collection of cytokine-mobilized peripheral blood cells from non-human primates by safe and effective leukapheresis procedure as a first step for preclinical study of HSCs gene therapy (Chapter 1). The second approach is to develop the transplantation protocol in non-human primates (Chapter 2). My non-human primate model will provide an important framework for preclinical study of HSCs gene therapy.

Chapter 1.

Collection of Cytokine-Mobilized Peripheral Blood Cells From Non-human primates.

Section 1:

Total Blood Volume in Cynomolgus Monkeys (*Macaca fascicularis*).

Introduction

It is essential to determine the correct total blood volume (TBV) for an index such as extracorporeal circulation in case of cytokine-mobilized peripheral blood stem cell collection and transfusion in case of hematopietic stem cell transplantation. Withdrawal of blood is the most common laboratory procedure in animal experiments. Recommendations regarding the frequency and maximum volume of blood to be withdrawn have been established for non-human primates by using the radiolabelling technique [28, 48, 101]. The TBV is influenced by age, sex, and body weight (BW); and the normal limits of TBV have been determined for humans and horses by using noninvasive methods such as Evans blue dye dilution technique [17, 46, 67]. In the light of animal welfare concerns, information about TBV is important for determining the amount of and frequency with which blood be drawn in non-human primates. The purpose of the present study is to clarify the normal value of TBV in non-human primates by using Evans blue dye dilution technique.

Materials and Methods

Breeding.

A large-scale cynomolgus monkey breeding colony derived from animals of the Philippines, Indonesia, and Malaysia was established in 1978 at Tsukuba Primate Center, National Institute of Infectious Diseases (Ibaraki, Japan) [43]. More than 4400 animals have been born over the past 22 years, with 201 normal births being obtained, on average, from 229 pregnancies (live birth rate; 87.8%) in a year. Approximately 180 cynomolgus monkeys, including juvenile monkeys and retired breeders, were supplied for research every year. Individual housing and timed mating systems are effective for prevention of horizontal infection of pathogens, resulting in establishment of a SPF colony that is free of blood parasites, intestinal parasites, herpes virus B and simian varicella virus. Our SPF definition does not include more ubiquitous or poorly defined viruses of non-human primates (foamy viruses, adenoviruses, other herpes viruses, or reoviruses) [11]. These animals are periodically screened to maintain their health status. These highly screened monkeys with detailed health records have been used in a variety of biomedical research experiments, including the study reported here.

Animals and husbandry.

To evaluate BW-dependent changes in the TBV value, I used 64 (34 male, 30 female) cynomolgus monkeys aged 15.5 ± 9.7 years (Table 1.1.1). All cynomolgus monkeys were bred and kept at the Tsukuba Primate Research Center (Ibaraki, Japan). All monkeys were housed indoors at 23-27°C and 50-70% humidity with 12 air changes per hour and a 12-hour/12-hour 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 for health control. This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [42] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [68]. 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).

Measuring the total blood volume.

TBV was measured using the Evans blue dye (Sigma Chemical Co., St. Louis, MO, USA) dilution technique [17, 33, 46] with slight modification. This method was reported as noninvasive because Evans blue dye showed no adverse effects including carcinogenic effect in the Ames test [32]. The concentration of Evans blue dye in the blood stabilized 5 to 10 min after the injection. From the saphenous vein, I collected 1 ml blood again into a tube containing 1.5 mg EDTA just before intravenous (i.v.) injection of 0.5 ml Evans blue solution (0.5% w/v in phosphate-buffered saline). At 10 min after injection of the Evans blue solution, 1 ml blood was collected again into a tube containing 1.5 mg EDTA. The plasma was separated from blood after centrifuging at 3,000 rpm for 10 min. The optical density (OD) at 610 nm of the Evans blue dye in a sample of plasma was determined by using a spectrophotometer (DU-640; Beckman Instruments, Inc., Fullerton, CA, USA). In addition, the OD of Evans blue dye serially diluted with normal monkey plasma was determined to obtain a standard curve of dye dilution. The correlation between dilution and OD in this standard curve always exceeded 0.9. The dilution factor for the Evans blue dye in the plasma sample was calculated by extrapolating the OD of the sample to the standard curve. The Hematocrit (Ht) was measured by using an automatic blood cell analyzer (Sysmex K-4500; Toa

iyoudenshi, Kobe, Japan). The TBV was calculated by using the following process.

1) Total plasma volume (ml) = dilution factor × 0.5 (ml ; Evans blue solution volume).

2) Plasmacrit (%) = 100-Ht

3) TBV (ml) = Total plasma volume / (Plasmacrit × 0.01)

Statistical analysis was achieved by using Student's t test. Linear regression analysis between BW and TBV was applied to determine their interrelationship. Statistical significance was accepted at $P < 0.05$. The data are presented as the mean ± 1 standard deviation (SD).

Results

Table 1.1.1 summarizes measured values in cynomolgus monkeys used to the TBV test. As shown in Figs. 1.1.1 and 1.1.2, significant correlation was observed between TBV and BW in all of the female monkeys ($r = 0.48$, $P < 0.05$), which all weighed less than 6 kg, and in males weighing 2 to 6 kg ($r = 0.85$, $P < 0.01$). However, there was no correlation between TBV and BW in male monkeys weighing more than 6 kg. In light of these results, the following formulae were established to estimate TBV by using BW.

For female cynomolgus monkeys weighing 2 to 6 kg, Y (TBV; ml) = 19.95X (BW; kg) + 167.24.

For male cynomolgus monkeys weighing 2 to 6 Kg, Y = 44.07X + 90.25.

The estimated TBV was approximately 6% of the BW and is within a range reported for mammals [69]. However, TBV was not correlated with BW and the range was 350 $\pm$ 50 in male monkeys that weighed more than 6 kg (Fig. 1.1.2).

Discussion

The average BW of adult (> 5 years) male cynomolgus monkeys was 5.51 ± 1.15 kg (my unpublished data), and the maximum fat mass of the TPC colony was 60% of the BW [90]. These results suggest that a BW of 2 to 6 kg is within the normal physiologic range but that male monkeys weighing more than 6 kg are obese. Because fat tissue has few blood vessels, the BW does not correlate with TBV in obese monkeys. Therefore, the range of TBV was 360 ± 50 in obese male monkeys.

The present data are important to establishing standard protocols for blood withdrawal from non-human primates in which the maximum volume of removable blood is based on the TBV of individual animals. In addition, efficient extracorporeal circulation such as cytokine-mobilized peripheral blood stem cell collection are possible to perform by assuming correct TBV as an index. The frequency of blood withdrawal and transfusion should be established by monitoring the Ht and calculating the TBV in the experiments using non-human primates.

Table 1.1.1. Characteristics of cynomolgus monkeys subjected in the TBV study.

	males	females
Number of Animals	34	30
Age (Years)	13.5±9.4	18.0±9.4
Body Weight (kg)	5.7±2.3	4.2±1.3
Hematocrit (%)	41.4±3.4	42.5±2.9

Each value is expressed by mean±SD.

Figure 1.1.1. Relationship between TBV and BW in female cynomolgus monkeys. There was correlation between TBV and BW in female monkeys.

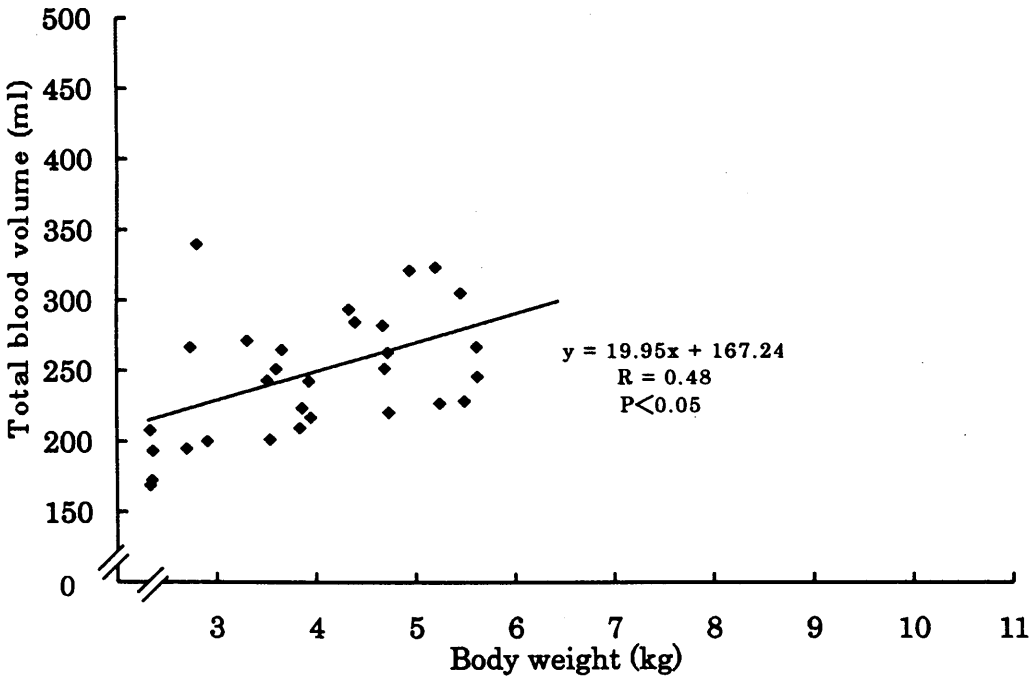
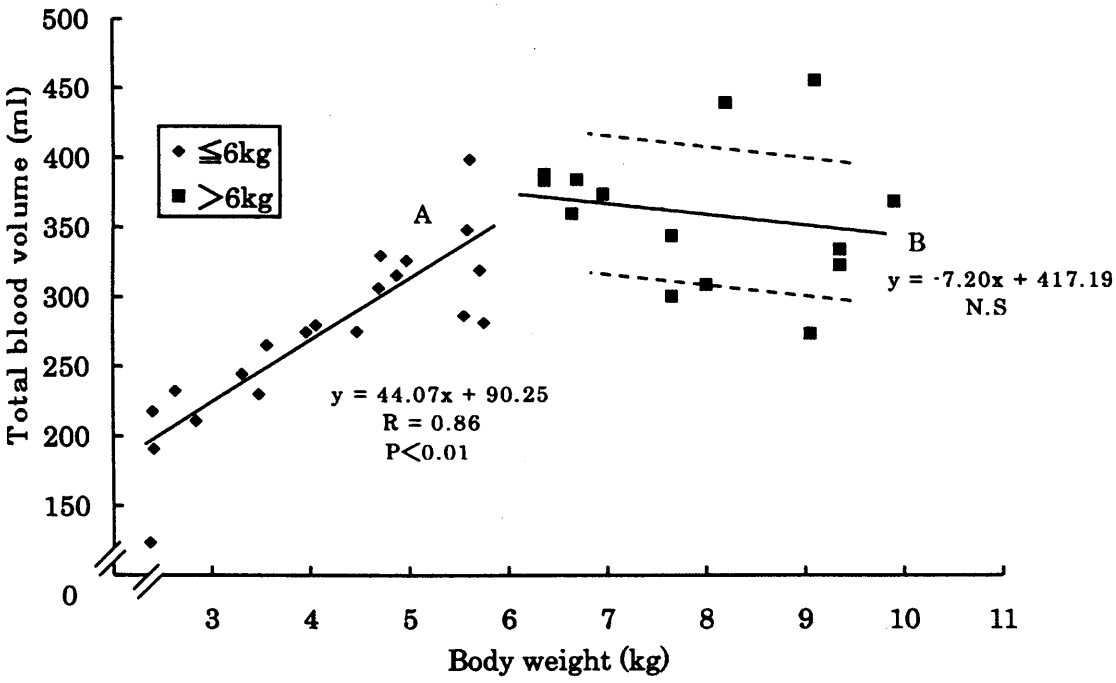

500
450
400
350
300
250
200
150
0
Total blood volume (ml)
y = 19.95x + 167.24
R = 0.48
P<0.05
3
4
5
6
7
8
9
10
11
Body weight (kg)

Figure 1.1.2. Relationship between total blood volume and BW in male cynomolgus monkeys. Line A comprises data from animals weighing 2 to 6 kg. Line B comprises data from animals weighing more than 6 kg. TBV did not correlate significantly with BW and the range of TBV was 360 $\pm$ 50 in obese monkeys that weighed more than 6 kg. The broken line shows the range of TBV in animals that weighning more than 6 kg.


500
450
400
350
300
250
200
150
0
Total blood volume (ml)
≦6kg
>6kg
A
B
y = 44.07x + 90.25
R = 0.86
P<0.01
y = -7.20x + 417.19
N.S
3
4
5
6
7
8
9
10
11
Body weight (kg)

Section 2:

Modification of the Leukapheresis Procedure: Massive Collection of Cytokine-mobilized Peripheral Blood Cells from Rhesus Monkeys (*Macaca Mulatta*).

Introduction

Cytokine-mobilized peripheral blood stem cell transplantation has been increasingly used clinically as an alternative to allogeneic BM transplantation [55, 78]. The cytokine-mobilized peripheral blood stem cells are prominent targets for stem cell gene therapy [20, 21, 39, 73] and recently have been used for induction of immune tolerance in organ transplantations [49, 53, 63, 81]. Although numerous clinical trials have demonstrated the safety and efficacy of leukapheresis for harvesting cytokine-mobilized peripheral blood cells from adults [29] and children [1, 50, 107], only one limited trial in human newborn babies has been reported [108]. Clinical trials are lacking partly because there is limited applicability of leukapheresis to the neonatal condition. One of the most serious problems when applying leukapheresis to a human newborn is the large amount of extracorporeal blood volume (ECV) that causes a substantial loss of platelets and RBCs.

The safety and therapeutic efficacy of hematopoietic stem cell (HSC) transplantation have been evaluated prior to human trials using monkey models. Since a large ECV frequently causes severe anemia, thrombocytopenia and a rapid reduction in Ht level during leukapheresis, which poses a risk for the patient, it is necessary to evaluate the safety and efficacy of leukapheresis using non-human primates as a model for human infants.

In this section, I modified a standard procedure for the collection of

cytokine-mobilized peripheral blood cells from rhesus monkeys (*Macaca mulatta*) using a blood cell separator (Fenwall CS3000+; Baxter, Deerfield, IL, USA) adapted for a small chamber and short apheresis kit as shown in Figure 1.2.1. In addition, ECV and processed blood volume were optimized by accurately TBV of each animals (Chapter 1, Section 1). This modified procedure made it possible to reduce ECV and perform safe and effective leukapheresis with monkeys whose BW is similar to that of human infants.

This study provides information on the modified leukapheresis process for the investigators who want to perform leukapheresis in human neonates. This non-human primate model will be useful to test new approaches for cytokine-mobilized peripheral blood stem cell transplantation, HSC gene therapy, and organ transplantation.

Materials and Methods

Animals

A total of 12 times of leukapheresis procedure was performed in nine male rhesus monkeys, aged 3 to 6 years, with a BW of 4.2 to 8.5 kg, which is a range that includes the mass of many human infants. Three of the monkeys (No. 099036, No. 000033, and No. 099030) underwent leukapheresis twice over two consecutive days. All of the rhesus monkeys were imported from China (Shin-nihon-kagaku, Tokyo, Japan) and were free of intestinal parasites, and herpes-B and simian varicella viruses. The animals were quarantined for 5 weeks and then kept in the Tsukuba Primate Center of the National Institute of Infectious Diseases. This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [42] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [68]. 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).

Preparative Regimen

Fifty μg/kg of recombinant human granulocyte colony-stimulating factor (G-CSF; lenograstim: Chugai, Tokyo, Japan) were subcutaneously administered to animals daily for 5 days prior to leukapheresis. For autologous blood donation, animals received 150 IU/kg recombinant human erythropoietin (EPO; EPOGIN: Chugai) subcutaneously three times a week during the two weeks preceding leukapheresis. Autologous blood (20-25 ml) was collected once a week during the three weeks and saline was infused for volume replacement. A total of 60-75 ml of peripheral blood was obtained from each animal and stored in a bag containing anticoagulant (acid-citrate

dextrose) at 4 °C prior to use for priming the apheresis kit [6]. To provide vascular access for leukapheresis, the right or left femoral artery was cannulated with a 5-Fr polyurethane catheter (Anthron PU; Toray, Tokyo, Japan). The saphenous vein was catheterized with a 19-gauge intracath (Terumo, Tokyo, Japan). This cannulation was performed under general anesthesia by administration of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Seraktar; Bayer, Leverkusen, Germany). Animals were treated a course of 0.5 mg/kg butorphanol tartrate intramuscularly for 3 days to alleviate any postoperative pain.

Leukapheresis Procedure

The leukapheresis protocol was a modification of the procedure originally developed by Donahue et al. [20]. All procedures were performed under general anesthesia (A.D.S.1000; Shin-ei, Tokyo, Japan) with isoflurane gas. Vital signs were monitored with electrocardiography, blood pressure, oxygen saturation and respiration. Collection of mononuclear cells (MNCs) was accomplished using a small S25A separation chamber and a shunt chamber in place of a standard collection chamber.

For the purpose of reducing ECV, the plasma line of a standard apheresis kit was cut and connected under sterile conditions to the RBC line using a polypropylene tubing connector (Iuchi, Osaka, Japan) (Fig. 1.2.1). The plasma flowed directly into the inlet line without passing through the shunt chamber. In addition, the inlet and draw lines were also exchanged with thin lines (extension tube: 70 cm length, 1.4 ml volume, 2.5 mm diameter; TOP, Tokyo, Japan) to reduce ECV as much as possible. A blood component inlet set with a 170-μm filter and drip chamber was sterilely connected to the packed RBC line using a polypropylene tubing connector (Iuchi). The inlet line was connected to a catheter (Terumo, Tokyo, Japan) that was placed in the saphenous vein

of the animal. Hemostats were placed on the unused return line and acid-citratedextrose (ACD) line. The apheresis kit was primed with autologous blood that had been collected for 3 weeks before leukapheresis.

The animal received a dose of 100 U/kg heparin (Aventis Pharma, Frankfurt, Germany) and the draw line was connected to the catheter in the femoral artery immediately before starting the procedure. Blood was processed at the rate of 10–12 ml/min for a total of two to three times of the TBV in each animal. When the processed blood volume reached 50 ml/kg, a 1-ml blood sample was collected via draw line and the Ht value and platelet count were monitored throughout the procedure for manual control of plasma pump speed [47]. The plasma pump speed was increased when a decrease in Ht value was observed. Conversely, plasma pump speed was decreased when Ht increased. In addition, when an increase in Ht value was observed, saline was infused into the inlet line for volume replacement to prevent blood pressure fluctuations. After the procedure was completed, the remaining cells in the apheresis kit were recovered and used either to prime the blood cell separator for future leukapheresis or to transfuse into the treated animal. Immediately after the leukapheresis, animals were given an appropriate dose of protamine sulfate (10 mg protamine sulfate per 1000 U heparin; Aventis Pharma). 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.

Analyses of Leukapheresis Contents

The content obtained during leukapheresis was collected in the S25A separation chamber. The content (40-45 ml) was mixed with 7 ml of ACD. The recovered RBCs, WBCs, MNCs and platelets were enumerated with a Sysmex K-4500

instrument (Toa-iyoudenshi, Kobe, Japan). Platelet counts and Ht value were also simultaneously examined with K-4500 instrument and i-STAT (Abbot, Illinois, U.S.A.) for real-time monitoring. Although the instrument was originally developed for human blood samples, I 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 [74, 83, 112]. The harvested $CD34^+$ cells were counted. The CD34 molecule 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, 64].

The total numbers of WBCs, MNCs and $CD34^+$ cells in the leukapheresis contents were calculated by multiplying the percentage of lymphocytes and $CD34^+$ cells by the total blood cell count in the leukapheresis contents.

Results

I administered G-CSF to monkeys for 5 days prior to leukapheresis. Administration of G-CSF increased peripheral WBC counts from 8,000 (6,000-10,000) to 42,000 (24,000–66,000) cells/μl on the average, and did not produce any adverse effects such as fever or anorexia. After the 5-day administration of G-CSF, leukapheresis was performed.

It is difficult to perform leukapheresis and autologous blood donation in small animals and human infants because of the large ECV involved [50, 62]. Modification of the leukapheresis procedure involved installing a small chamber and shortening the extracorporeal blood line in a standard apheresis kit, which made it possible to reduce ECV from 130 to 70 ml. The amount of autologous blood needed is only 60–75 ml, and can be collected safely without any adverse effects for the donor. Before using this modified apheresis kit in monkeys, I circulated the pooled blood to determine the correlation between tubing length and ECV in vitro, and demonstrated that ECV can be reduced safely when shortening the tube.

The presence of platelets and RBCs in the leukapheresis contents was observed when the equipment was operated in automatic mode. However, manual adjustment of plasma pump speed by monitoring Ht values and platelet counts during apheresis effectively prevented the overdraw of extracorporeal blood. Saline was infused into the inlet line for volume replacement when an increase in Ht value was observed. Leukapheresis was performed safely without any risks such as blood pressure reduction, and sufficient numbers of MNCs and $CD34^+$ cells could be collected. Leukapheresis was performed safely and efficiently on all nine monkeys weighing 4.2 to 8.5 kg, a range similar to that of human infants. As shown in Table 1.2.1, mean processed blood volume was 1,200 ml (180 ml/kg), which was accurately three times

the estimated TBV of an individual animal (Chapter 1, Section 1). Because the normal limit of BW for male rhesus monkeys are 9 kg [70], the TBV of rhesus monkeys that weighing less than 9 kg in BW might be not plateau. Therefore, I extrapolated the estimated TBV formula (Chapter 1, Section 1) for cynomolgus monkeys to rhesus monkeys in this experiment.

No complications, such as severe anemia or trombocytopenia, developed in the three monkeys that underwent leukapheresis on two consecutive days. Serial changes in circulating Ht levels and platelet counts were monitored throughout the leukapheresis. A transient drop in Ht occurred during leukapheresis, but a blood transfusion was not necessary. Platelet counts also fell briefly during the process but did not require treatment (Fig 1.2.2).

No viral or fungal organisms were isolated from culture of the leukapheresis contents after cutting and sterile docking of the tube, and no microbial contamination occurred in colony-forming progenitor assays.

Discussion

No problems were encountered in the animals or with the function of the blood cell separator throughout the process of leukapheresis in rhesus monkeys. I succeeded in reducing ECV, but still needed autologous blood for priming. However, reduction of the ECV allowed priming with autologous blood in these non-human primates. Therefore, the modified procedure enables safe and effective leukapheresis in non-human primates and other small animals. Sufficient numbers of $CD34^+$ cells and MNCs could be collected for stem cell transplantation as shown in Table 1.2.1. In addition, an adequate number of cells were also collected from monkeys (No. 099036, No. 000033, and No. 099030) that underwent leukapheresis on two consecutive days.

This is the first report of a leukapheresis procedure with a blood cell separator modified for use in non-human primates. The results clearly indicate that this modification enables the safe and effective application of the blood cell separator for leukapheresis in rhesus monkeys. TBV of each animal was exactly calculated by the formulae as described previously (Chapter 1, Section 1). Although the little blood volume (150~220 ml/kg) was processed in leukapheresis, sufficient numbers of MNCs and $CD34^+$ cells were harvested without any complications in the animals and any problems in the system. Since processing time was short, the procedure was less invasive. Decreases in platelet counts and Ht levels after leukapheresis were minimal in all animals (Fig 1.2.2), indicating that this procedure allows the collection of cytokine-mobilized peripheral blood cells with negligible contamination of platelets and RBCs in the leukapheresis contents. The decrease in Ht levels observed after leukapheresis was a complication of the cannulation operation, but it did not necessitate the treatment with a transfusion. Also, Ht levels and platelet counts fell transiently during leukapheresis but a blood transfusion was not necessary.

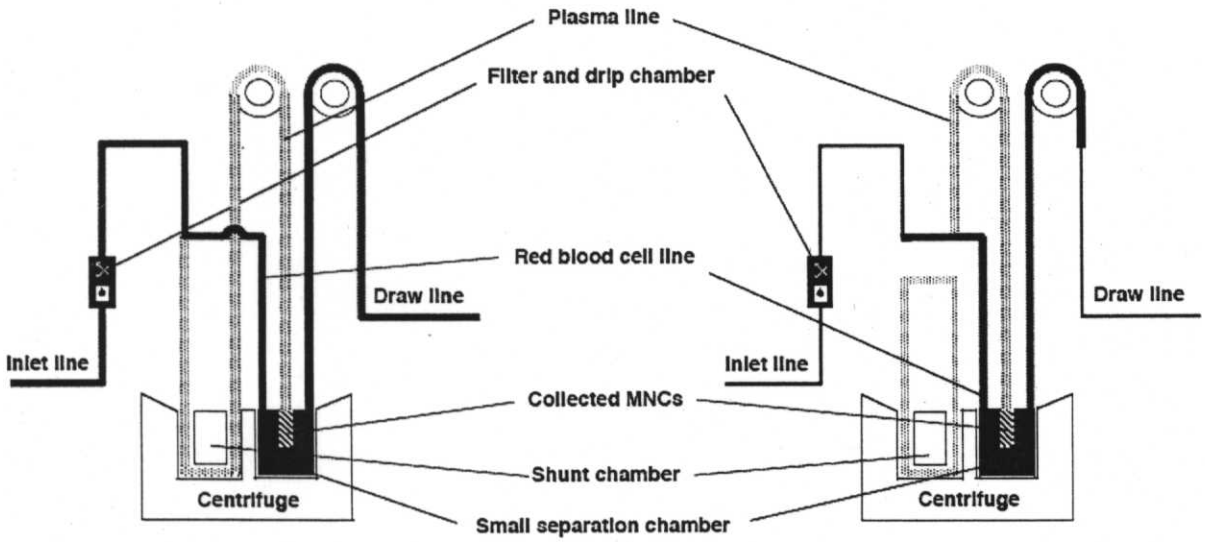
Although clinical trials in human infants using this modified procedure would be difficult, it is clear that my modified procedure makes it possible to perform safe and effective leukapheresis in non-human primates or other small animals. This animal model will be useful for testing new approaches to cytokine-mobilized peripheral blood cells transplantation, HSC gene therapy, and organ transplantation. Despite the need to modify the tube each time in a clinical setting, I believe this modification will be helpful to researchers who may need a noninvasive method of collecting cytokine-mobilized peripheral blood cells from human newborns [18, 71]. Next step is to apply this modified leukapheresis procedure to juvenile cynomolgus monkeys that weighed approximately 2-4 kg, a BW being equivalent to that of a human newborns. My non-human primate model will provide an important framework for such future clinical studies.

Table 1.2.1. Characteristics of Rhesus Monkeys Subjected in Leukapheresis

Animal ID (No.)	Body Weight (kg)	Estimated total blood volume[a] (ml)	Processed blood		Harvested cells		
			Total Volume (ml)	ml/kg	Total Nucleated Cells (x 10^9/kg)	MNCs (x 10^8/kg)	CD34+ cells (x 10^6/kg)
099033	4.9	310	1000	200	2.99	10.45	27.17
099038	4.9	300	1000	210	1.41	10.17	26.44
000026	7.5	420	1600	210	1.12	1.90	NT
000030	7.3	410	1600	220	0.94	1.69	NT
000031	8.5	470	1300	150	0.47	1.66	NT
000029	6.5	380	1000	150	2.21	1.46	NT
099036[b]	4.5	290	1000	220	1.68	0.55	1.44
099036[b]	4.2	270	900	210	1.29	6.22	16.16
000033[b]	7.4	420	1150	150	0.83	2.10	NT
000033[b]	7.4	420	1150	150	0.75	1.13	NT
099030[b]	4.3	400	1050	150	0.89	3.78	NT
099030[b]	4.3	400	1100	160	1.61	4.06	NT
Average	6.5	380	1200	180	1.38	3.67	17.80

[a]The total blood volume was estimated by the following formula: Y (total blood volume) = 44.07 X (Body weight) +90.25 [Chapter 1, Section 1]. [b]Leukapheresis were performed three animals in two consecutive days. NT=Not tested.

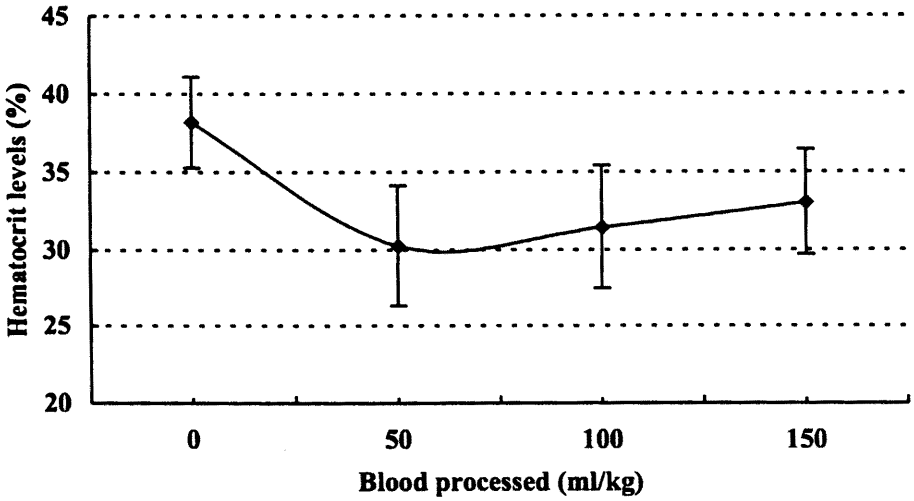
Figure 1.2.1. Diagram of apheresis kit before and after modification. Modifications included the use of a small separation chamber and the shortening of the standard apheresis kit. The plasma line (shadow line) was cut and connected under sterile conditions to the RBC line (solid line) using a polypropylene tubing connector. The plasma flowed directly into the inlet line without passing through the shunt chamber. Inlet and draw lines were exchanged for thin lines (2.5 mm in diameter) to reduce ECV as much as possible. This modification made it possible to reduce the volume of extracorporeal circulation from 130 to 70 ml. MNC=mononuclear cells.



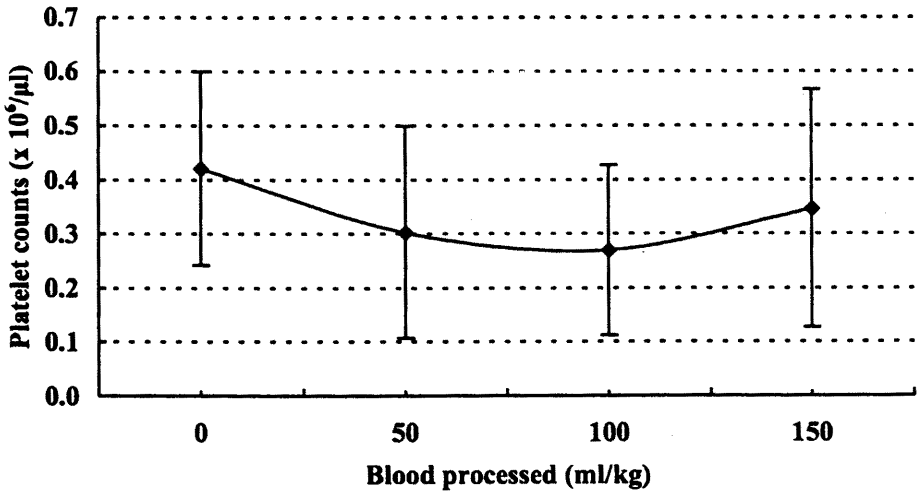
Before modification **After modification**

Figure 1.2.2. Monitoring of Ht levels (A) and platelet counts (B) in circulation. Though the transient drop in Ht and platelet during leukapheresis, treatment such as blood transfusion was not necessary.

A


45
40
35
30
25
20
Hematocrit levels (%)
0
50
100
150
Blood processed (ml/kg)

B


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Platelet counts (x 10⁵/µl)
0
50
100
150
Blood processed (ml/kg)

Section 3:

Safe and Efficient Collection of Cytokine-mobilized Peripheral Blood Cells from Cynomolgus Monkeys.

Introduction

Although HSCs usually reside in the BM, they can be mobilized into the peripheral blood by the administration of cytokines such as G-CSF [78]. Cytokine-mobilized peripheral blood stem cells are widely used for autologous and allogeneic transplantation therapies to treat hematological malignancies such as leukemia and lymphoma [51, 55]. The cells have also been intensively studied as a donor source of stem cells for gene therapy and regenerative medicine [38, 66, 100, 114]. 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 leukocytes 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 [75, 85]. The removed cells are then enriched for a stem cell fraction such as $CD34^+$ or $AC133^+$ cells for clinical applications [5, 89]. Although leukapheresis is widely conducted for human adults, it is difficult to apply to commonly used 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 BW [63].

I have established leukapheresis procedures for non-human primates with BW of less than 10 kg using rhesus monkeys (on average 7 kg) in Chapter 1, Section 2. In such small animals, acute cardiac failure due to the relatively large extracorporeal blood

flow is a critical adverse effect which can occur during leukapheresis. To avoid this, I modified the procedure by reducing the ECV as much as possible and adjusting the withdrawal speed frequently in response to the results of real-time monitoring of Ht levels and platelet counts in Chapter 1, Section 2. In this section, I examined the efficacy and safety of my procedure using even smaller non-human primates, i.e., cynomolgus monkeys (on average 3.3 kg), which have BW equivalent to human newborns.

Materials and Methods

Animals.

Twelve cynomolgus monkeys consisted of 7 males and 5 females (3-7 years old, 2.5-4.6 kg) bred at the Tsukuba Primate Center (Ibaraki, Japan) were enrolled in this study (Table 1.3.1). This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [42] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [68]. 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).

Apparatus.

A CS3000 blood separator (Baxter, Deerfield, IL, USA) was used as described previously (Chapter 1, Section 2). 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 ECV, the plasma line of the standard apheresis kit was cut away and the RBC line was directly connected to the inlet line using a polypropylene tube connector (Iuchi, Osaka, Japan) under sterile conditions, bypassing the shunt chamber. In addition, the regular inlet and draw lines were replaced with lines shorter in length, smaller in diameter and volume (extension tube: 70 cm length, 2.5 mm diameter, 1.4 ml volume; TOP, Tokyo, Japan) to further reduce the ECV (Chapter1, Section 2).

Preparative regimen, leukapheresis procedure and analysis of leukapheresis contents were described previously (Chapter 1, Section 2). Modified procedure included in reducing the ECV and real-time monitoring of Ht levels and platelet counts during leukapheresis. In brief, in this study, recombinant human stem cell factor (SCF: 50

μg/kg; Amgen, Thousand Oaks, CA, USA) and recombinant human G-CSF (50 μg/kg; Chugai, Tokyo, Japan) were administered to all animals subcutaneously daily during the 5 days preceding leukaphersis [20]. After the administration of G-CSF and SCF, I chose one monkey from the unmodified method-group and another monkey from the modified method-group at random to compare the safety and efficacy of leukapheresis in both methods.

Results

To compare the original and modified procedures, I examined the safety and efficacy of leukapheresis using the manufacturer's protocol (n = 6) and my modified version (n = 6) using cynomolgus monkeys (Fig. 1.3.1). In both groups, I 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 from 10,000 (5,000-17,000) to 66,000 (36,000-109,000) cells/μl on average (Table 1.3.1), and was not associated with any adverse effect such as fever or anorexia. 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 [20, 71]. As a result, the ECV was reduced from 130 to 70 ml. In both groups, blood was processed at a rate of 10-12 ml/min and the total processed volume was two to three times of the estimated TBV (Chapter 1, Section 1) in each animal (Table 1.3.2). In the modified protocol, every time the processed blood volume reached to 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 [47]. 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 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. 1.3.3). In contrast, none of the animals that underwent the modified procedure (n = 6) developed cardiac failure or severe anemia (Table 1.3.2). The Hb and Ht values were significantly better during the modified procedure (Fig. 1.3.2). In addition, the numbers of harvested nuclear cells, MNCs, and $CD34^+$ cells were significantly increased with the

modified procedure compared with those of the unmodified one (Fig. 1.3.3).

The leukapheresis contents 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, I performed manual adjustment of the plasma pump speed in response to the results of the real-time monitoring of Ht levels and platelet counts during the leukapheresis as described above, and successfully reduced the contamination. 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 contents from the unmodified or modified procedures.

Discussion

In this section, I reported leukapheresis in cynomolgus monkeys. My modified protocol significantly improved MNCs and $CD34^+$ cell harvest compared with the manufacturer's protocol. Under my modified procedure, I routinely collected 5×10^6 $CD34^+$ cells per kg, which is equivalent to numbers in human trials published in the literature [45, 80, 86, 107]. Thus, with my modified protocol, it is possible to collect sufficient numbers of $CD34^+$ stem cells for various applications including transplantation experiments in monkeys. In fact, I achieved successful hematopoietic reconstitution in myeloablated cynomolgus monkeys after the autologous transplantation of $CD34^+$ cells obtained with this procedure. 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 (Fig. 1.3.1). Although numerous clinical trials have demonstrated the safety and effectiveness of leukapheresis for adults [29, 45, 80, 86] and children [1, 18, 50, 107], only one very limited trial has been conducted on a human newborn baby [108]. To my knowledge, this paper is the first systematic documentation of leukapheresis for small primates.

One of the most serious complications with small animal subjects is cardiac failure due to the relatively large amount of ECV in leukapheresis [29, 108]. The main symptoms include hypotension and dyspnea, which sometimes result in death, *e.g.* animal No. 292079 (Table 1.3.2). To avoid this complication, the ECV should be reduced as much as possible. No monkeys underwent cardiac failure after my 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). Therefore,

age distribution in the modified and unmodified group was better than the high age monkey belonging to the modified procedure group.

TBV of each animal was exactly calculated by the formulae as described previously (Chapter 1, Section 1). Although the little blood volume i.e., less than 3 times of TBV was processed in leukapheresis (Table 1.3.1), sufficient numbers of MNCs and $CD34^+$ cells were harvested without any complications such as anemia in all animals. Therefore, calculated accurate TBV and optimization of the processed blood volume were able to reduce the time and stress of leukapheresis.

Non-human primate models would be useful for preclinical studies of cell and gene therapies. I 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 [114]. In other studies, I successfully transplanted gene-modified $CD34^+$ stem cells into cynomolgus monkeys as a preclinical gene therapy [38, 100]. My 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.

Table 1.3.1. Hematological analysis of cynomolgus monkeys before and after cytokine treatment

	Animal ID (No.)	Sex	Age (years)	Body weight (kg)	Before cytokine treatment					After cytokine treatment				
					White blood cells ($10^2/\mu l$)	Red blood cells ($10^4/\mu l$)	Hemoglobin (g/dl)	Hematocrit (%)	Platelets ($10^4/\mu l$)	White blood cells ($10^2/\mu l$)	Red blood cells ($10^4/\mu l$)	Hemoglobin (g/dl)	Hematocrit (%)	Platelets ($10^4/\mu l$)
Unmodified procedure	292049	Male	6	4.6	170	547	10.9	36.7	36.1	680	632	12.0	41.4	36.1
	293051	Female	6	2.5	73	503	10.3	36.8	44.8	1091	535	10.7	39.0	35.7
	292079	Female	7	3.2	101	472	11.0	36.4	50.7	514	480	11.6	36.5	35.8
	292238	Female	7	3.2	76	632	13.1	44.7	45.6	548	653	13.4	44.4	30.0
	394029	Female	5	3.2	72	542	11.2	39.0	46.0	355	590	11.7	42.3	42.4
	296116	Male	3	3.1	73	497	10.4	37.2	35.0	361	583	12.0	43.8	26.6
Average			5.7	3.3	94	532	11.2	38.5	43.0	592	579	11.9	41.2	34.4
Modified procedure	001046	Female	3	3.5	155	512	11.8	40.5	36.8	872	484	12.4	38.9	43.7
	001045	Male	3	3.3	73	433	10.3	34.6	49.0	519	415	9.7	33.4	44.2
	001049	Male	3	3.5	106	495	12.5	39.0	33.7	434	501	12.3	38.7	43.8
	001053	Male	3	2.6	49	525	11.2	39.4	54.0	802	456	10.3	35.1	57.7
	001047	Male	4	3.3	149	493	12.3	38.9	38.0	805	438	11.1	35.3	40.4
	398042	Male	5	3.8	124	624	13.9	44.3	43.4	887	521	11.9	36.8	38.4
Average			3.5	3.3	109	514	12.0	39.5	42.5	720	469	11.3	36.3	44.7

Table 1.3.2. Leukapheresis procedures

	Animal ID (No.)	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 [Chapter 1, Section 1]. For males, (Total blood volume, ml) = 44.07 X (Body weight, kg) + 90.25. For females, (Total blood volume, ml) = 19.95 X (Body weight, kg) + 167.24.

Figure 1.3.1. 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 1.3.1) were similar to those of human newborns.

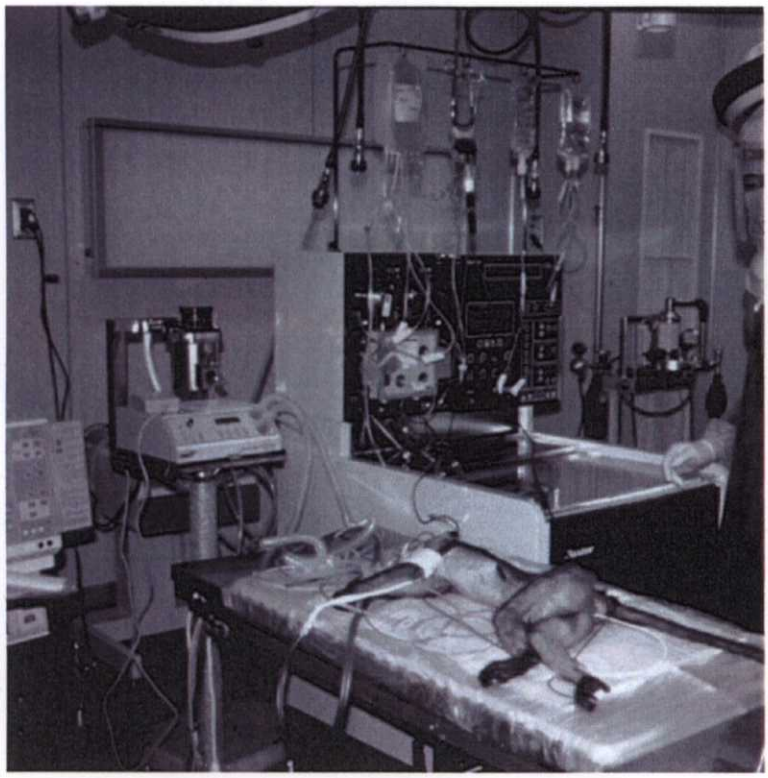
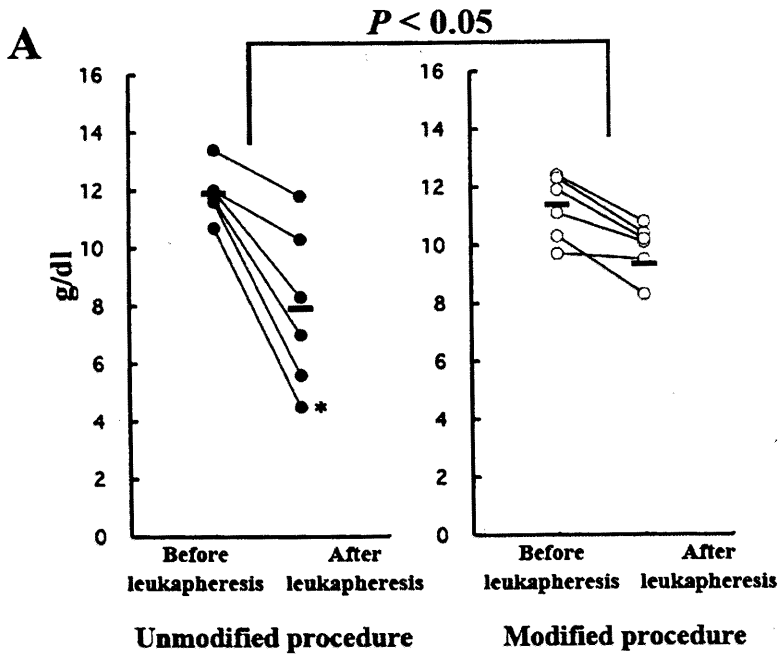


Figure 1.3.2. Avoidance of severe anemia with the modified procedure. The degree of anemia was significantly ameliorated with the modified procedure as compared to the unmodified one as assessed by the ratios of Hb (A) and Ht levels (B) after versus before the leukapheresis. One monkey died of cardiac failure (*, 292079) after the unmodified procedure. **A**: Hemoglobin levels. **B**: Hematocrit levels.


A
P < 0.05
g/dl
16
14
12
10
8
6
4
2
0
*
Before leukapheresis
After leukapheresis
Unmodified procedure
Modified procedure

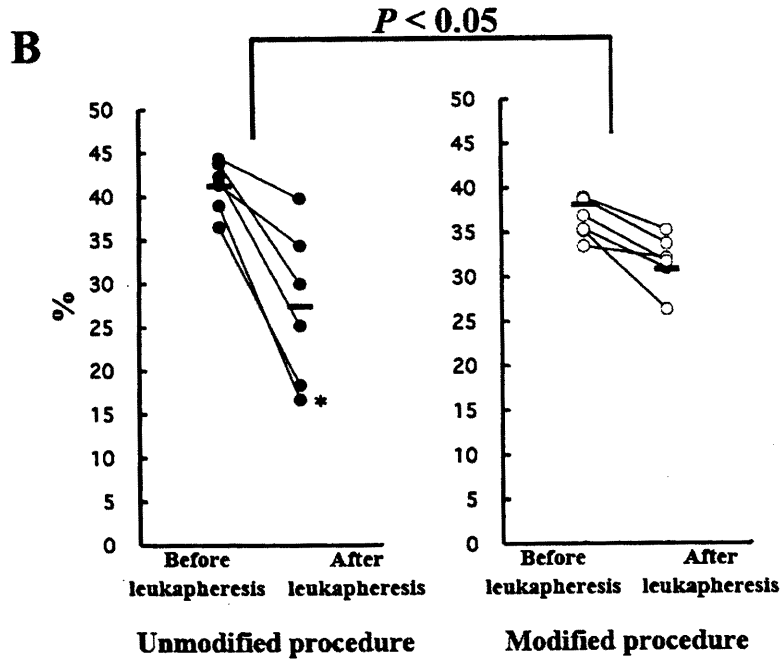
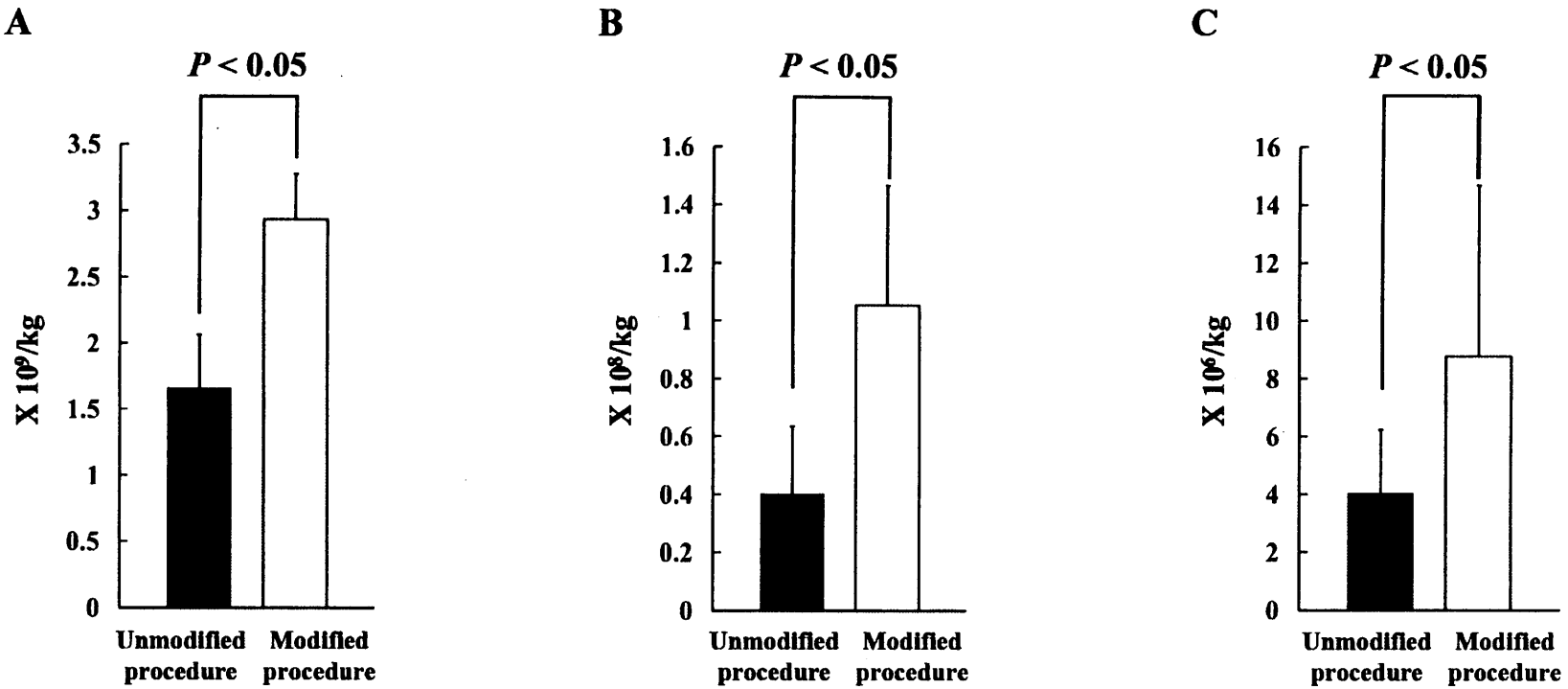

B
P < 0.05
%
50
45
40
35
30
25
20
15
10
5
0
*
Before leukapheresis
After leukapheresis
Unmodified procedure
Modified procedure

Figure 1.3.3. Larger harvest of cells with the modified procedure. The numbers of harvested nuclear cells (A), MNCs (B), and $CD34^+$ cells (C) were significantly increased in the modified procedure. **A**: Total nuclear cells. **B**: Mononuclear cells. **C**: $CD34^+$ cell.


A
P < 0.05
3.5
3
2.5
2
1.5
1
0.5
0
X 10⁹/kg
Unmodified procedure
Modified procedure
B
P < 0.05
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
X 10⁸/kg
Unmodified procedure
Modified procedure
C
P < 0.05
16
14
12
10
8
6
4
2
0
X 10⁶/kg
Unmodified procedure
Modified procedure

Chapter 2.

Establishment of Autologous Hematopoietic Stem Cell Transplantation Procedure.

Section 1:

Safe and Efficient Methods of Autologous hematopoietic Stem Cell Transplantation in Cynomolgus Monkeys.

Introduction

After many years of study on the identity of potential bloodforming stem cells called HSCs, researchers have begun exploring them for a therapeutic use. Currently, no other type (adult, fetal, or embryonic) of stem cell has attained such status. Transplantation of HSCs is now routinely used to treat patients with cancer and other disorders of the blood and immune systems [15, 96, 99]. Despite vast clinical experience with HSCs, researchers do not yet have an accurate in vitro method to completely distinguish HSCs from other cells harvested from BM, peripheral blood or cord blood. Transplantation of human HSCs to the immunocompromised mice such as SCID or nude mice has proved to be the only reliable method for assay of HSCs [8, 84, 87]. The cells capable of restoring multi-lineage hematopoiesis in recipient animals through self-renewal and differentiation can be called HSCs. If possible, autologous or allogeneic transplantation of presumable HSCs into humans would be the most reliable method to assess human HSCs, but it is impossible to design such experiments. Instead, xenograft models of human hematopoiesis have been used for the study of in vivo engraftment and proliferation potential of human HSCs. Until now, only two xenogeneic transplantation models have been available. One recipient is the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse and the other is the fetal sheep [3, 88]. These models take advantage of the animal's immunologically naive state.

Since NOD/SCID mice are severely immunodeficient and fetal sheep are immunologically immature, human HSCs can engraft and generate their progeny in these animals. The behavior of human HSCs, however, might be different in xenogenic recipients. The relevance of these xenograft models to natural human in vivo hematopoiesis remains unclear.

On the other hand, autograft or allograft transplantation models are ideal for evaluating engraftment, proliferation, and differentiation of HSCs, since natural hematopoiesis can be studied in these models. Although mouse transplantation models have been widely used for assay of murine HSCs, these models may not reliably predict the biology of HSCs in larger animals such as humans. The hematopoietic demand of a mouse may be very small. A typical mouse (25 g) makes, in a two-year lifetime, the same amount of red blood cells (RBCs) as does a man in one day, raising the possibility that HSCs kinetics in large animals are more complex than small rodents [2]. Large animal species may provide far more appropriate preclinical models that will more closely reflect human HSC characteristics and behavior [104]. Among large animals, non-human primates may provide the best models because of their close phylogenetic relationship to humans [54, 102]. In the study reported here, I used safe and efficient methods for autologous transplantation of immunoselected $CD34^+$ cells from BM and cytokine-mobilized peripheral blood in cynomolgus monkeys.

Materials and Methods

Animals.

Fifteen cynomolgus monkeys (3-5 years old, 2.1-3.8 kg) were enrolled in this study (Table 2.1.1). In addition, I prepared about 10 blood-donor monkeys (BW > 5 kg) for transfusion in this transplantation procedure. They were healthy, without signs of disease at annual health examinations. In addition, animals used in this study were SPF animals which were further certified to be free of simian type-D retrovirus as well as herpes virus B and simian varicella virus. Our SPF definition and animal housing were described previously (Refer to Capter 1, Section 1).

This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [42] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [68]. 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).

BM harvest (n= 11).

For autologous blood donation, animals received recombinant human EPO (Chugai, Tokyo, Japan; 150 IU/kg of BW) subcutaneously three times a week during the three weeks prior to BM harvest [76]. Autologous blood (20 to 30 ml) was drawn once a week for three times and saline was then infused for volume replacement. A total of 60 to 90 ml of peripheral blood was obtained from each animal and stored at 4°C in a bag containing the anticoagulant acid-citrate dextrose prior to use as an autologous blood transfusion at the time of BM harvest as same as leukapheresis procedure (Refer to Chapter 1, Section 2).

Eight animals (except No. 396042, No. 396051 and No. 396053) received recombinant human SCF (50 to 200 μg/kg) and recombinant human G-CSF (10 to 100 μg/kg;

Chugai) subcutaneously daily for five days [19, 22]. After cytokine administration, 50 ml of BM was aspirated from the iliac crest and/or tuber ischiae of monkeys under isoflurane (A.D.S.1000; Shin-ei, Tokyo, Japan)-induced general anesthesia into a syringe that had been rinsed with preservative-free heparin. Concurrently, the stored autologous blood mentioned above was transfused. After BM harvest, animals received butorphanol tartrate (0.5 mg/kg, intramuscularly) daily for 3 days to alleviate bone pain associated with the BM harvest.

Leukapheresis procedures for harvesting cytokine-mobilized peripheral blood cells (n=4).

Leukapheresis procedures were performed in four cynomolgus monkeys. Refer to Chapter 1, Section 3 about leukapheresis procedure.

Preparation of $CD34^+$ cells.

From the harvested BM and cytokine-mobilized peripheral blood cells, the nucleated cell fraction was obtained by RBC lysis by addition of ACK buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA; Wako, Osaka, Japan). Enrichment of $CD34^+$ cells was performed using magnet beads conjugated with a monoclonal anti-CD34 antibody (clone 561) (Dynal, Lake Success, N.Y.). The purity of $CD34^+$ cells was assessed by use of flow cytometry with another monoclonal anti-CD34 antibody (clone 563; PharMingen, San Diego, Calif.). The $CD34^+$ cells were cultured for four days until re-infusion in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS; Gibco), recombinant human interleukin 3 and 6 (IL-3, IL-6, 50 ng/ml; Ajinomoto, Osaka, Japan), recombinant human thrombopoietin (TPO, 100 ng/ml; Kirin, Tokyo, Japan), recombinant human SCF (100 ng/ml; Amgen), recombinant human fms-like tyrosine kinase-3 ligand (FL, 100 ng/ml; Research Diagnostics, Flanders, N.J.) as a cytokine and antibiotics (100 U of penicillin [Banyu, Tokyo, Japan] and 0.1 μg of streptomycin [Meiji,

Tokyo, Japan]/ml) (Table 2.1.1). I used the standard culture conditions for primate $CD34^+$ cells that included several cytokines (SCF, FL, and TPO) [52, 23]. Because the ex vivo culture of $CD34^+$ cells is required for vector transduction in future HSC gene therapy.

Total body irradiation.

Prior to irradiation, a non-absorbent antibiotic (polymyxin B sulfate, 5 x 10^4 U/kg; Pfizer, Brooklyn, N.Y. or kanamycin sulfate, 50 mg/kg; Meiji) was orally administered to animals for three days to sterilize the gastrointestinal tract. Microbial contamination on animals' body surface was decreased by immersion in an iodine bath before irradiation. Animals under general anesthesia by administration of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Seraktar, 0.5 mg/kg; Bayer, Leverkusen, Germany) received myeloablative total body x-ray irradiation (PANTAK HF-420, Shimazu, Tokyo, Japan). Irradiation was conducted at a dose of 500 cGy (all monkeys except No. 296113 and No. 296116) or 550 cGy (No. 296113 and No. 296116) daily for 2 days (total 1,000 or 1,100 cGy, dose rate: 10 to 15 cGy/min) just prior to transplantation [21, 52]. Since the x-ray device was originally intended for industrial use, the energy spectrum was altered to be similar to that of γ-radiation for medical use by addition of a specific filter (Al 0.5 mm + Pb 0.1 mm + Cu 0.3 mm + Al 1.0 mm; Shimazu).

Transplantation and supportive care.

Three weeks before BM harvest or leukapheresis, a central venous catheter, used with a tether and a jacket, was placed in each animal to allow administration of fluids, antibiotics, and transfusions. The catheter was tunneled subcutaneously and brought out through the skin of the back. After total body irradiation, the first animal (No. 396042) was transplanted intravenously with whole autologous BM nucleated cells. The subsequent 13

animals were transplanted intravenously with autologous $CD34^+$ cells. In this study, I have aimed to acquire minimum 1 x 10^6 $CD34^+$ cells/kg in monkey body weight for transplantation (Table 2.1.1). After transplantation of the cells, animals were kept in an intensive care unit with high efficiency particulate air (HEPA)-filtered airflow and were fed sterilized commercial monkey chow from the day of irradiation until the peripheral WBC count reached 5,000 cells/μl. Recombinant human G-CSF (Chugai) was administered intravenously to animals at a dosage of 5 to 10 μg/kg once a day from the day when the WBC count was < 1,000 cells/μl until the WBC count reached 5,000 cells/μl according to the transplantation protocol [20, 96]. Every day after irradiation, animals also received a histamine H2-receptor-blocking agent (famotidine, 4 mg, intravenously; Yamanouchi, Tokyo, Japan) to prevent gastric ulcer development. Three times a week during the period of myelosuppression, 1 ml of blood was drawn from animals under ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan) general anesthesia to obtain a serum biochemical profile and a complete blood count. The serum biochemical profile included sodium, potassium, chloride, total protein, albumin, globulin, blood urea nitrogen, creatinine, alanine transaminase, aspartate transaminase, and C-reactive protein (CRP) values.

Transfusion of irradiated (2,000 cGy) freshly obtained whole blood (approx. 30 ml/transfusion) from blood-donor monkeys was administered to treat anemia (Hb concentration < 7 g/dl). Transfusion of platelet-rich plasma from blood-donor monkeys was done to treat thrombocytopenia (platelet count < 40,000 cells/ μ l) according to the transplantation protocol [20, 96]. Blood-donor monkeys (BW > 5 kg) were grouped by blood type and were cross-matched before transfusion. Antibiotics were administered when the animals had high fever (> 38°C) or increased CRP value. Antibiotics were chosen from results of bacteriologic culture and antimicrobial susceptibility testing when possible. Diarrhea was a common adverse effect of total body irradiation. Lactobacillus and

Bifidobacterium spp. were administered when radiation-related diarrhea was prolonged.

Results

HSCs collection.

Autologous blood from each animal was stored before BM harvest or leukapheresis procedure. To avoid development of anemia, animals received recombinant human EPO subcutaneously prior to blood donation for BM harvest or leukapheresis procedures [76]. Removal of 20 ml of blood was performed once a week (total, 60 to 90 ml of blood) safely without adverse effect.

I administered SCF and G-CSF to twelve monkeys for five days just prior to BM harvest or leukapheresis procedures to expand hematopoietic stem/progenitor cells in the BM and mobilize into the peripheral blood [19, 22, 78]. Fig. 2.1.1A shows the mean and standard deviation of peripheral WBC counts from monkeys (n = 3) not administered SCF and G-CSF. On the other hand, administration of these cytokines resulted in a mean increases in the WBC count to 52,000 (range, 16,000 to 80,000) cells/μl (Fig. 2.1.1B).

Adverse effects associated with the cytokine administration, such as fever or anorexia, were not observed. After a five-day administration of SCF and G-CSF, HSCs were harvested while the stored autologous blood was transfused. The decrease in Hb values observed prior to transplantation (Fig. 2.1.2) was a complication of the HSCs harvest procedure. The transient increase in platelet count observed after transplantation (Fig. 2.1.3) may have been secondary responce to the decrease in Hb values.

Preparation of $CD34^+$ cells.

The CD34 molecule is a cell-surface marker of undifferentiated HSCs. Although recent reports suggest that all HSCs may not express CD34 and the $CD34^+$ cell may show the low population in HSCs [16], clinical trials of $CD34^+$ cell transplantation have been successfully conducted. In a variety of HSC transplantation and gene therapy studies

immunoselected $CD34^+$ cells are used [13, 64]. The $CD34^+$ cells were isolated from BM cells and cytokine-mobilized peripheral blood cells by use of commercially available magnet beads conjugated to a monoclonal antibody (clone 561) that recognizes human and cynomolgus monkey $CD34^+$ cells [83, 112]. On average, sorted $CD34^+$ cells accounted for 1.5% of total nucleated cells. The purity of $CD34^+$ cells ranged from 90 to 95%. The numbers of $CD34^+$ cells were generally higher in monkeys administered SCF and G-CSF, compared with monkeys without administration of these cytokines (Table 2.1.1). However, correlation between numbers of harvested $CD34^+$ cells and dose of SCF and G-CSF administration was not apparent, presumably because even the lowest administered dose of SCF and G-CSF seemed to reach biologically optimal serum values.

The $CD34^+$ cells were cultured ex vivo for four days prior to transplantation, since ex vivo culture of $CD34^+$ cells is required for many applications, such as ex vivo expansion of HSCs and genetic manipulation of the cells.

Transplantation and myelosuppression.

Just prior to transplantation, the animals received myeloablative total body irradiation (500 to 550 cGy) daily for two days [21, 52]. Animal No. 396042 was received initially all BM nucleated cells, and other 10 animals were received immunoselected autologous $CD34^+$ cells. From the day of irradiation to the day when the level of hematopoiesis being restored, all animals were kept in the intensive care unit with HEPA-filtered airflow. Although animal No. 396042 that received all BM nucleated cells developed only slight myelosuppression, the other 10 animals receiving $CD34^+$ cells showed moderate to severe myelosuppression (Table 2.1.2). After transplantation, all animals except two animals (No. 396042 and No. 099056) experienced neutropenia (WBC < 1,000 cells/μl) (Table. 2.1.2). On average, the WBC count nadir was 700 cells/μl (on average at 7 days), and

on average 13 days was required for the WBC count to reach a value > 1,000 cells/μl. Some animals experienced anemia (Hb concentration < 7g/dl) (Fig. 2.1.2), and eleven animals required blood transfusion (20 to 60 ml) one to three times. Four animals experienced thrombocytopenia (platelet count < 40,000 cells/μl) and required platelet-rich plasma transfusion (Fig. 2.1.3). On average sixteen days was required for the platelet count to reach > 50,000 cells/μl. Overall, eleven of fifteen animals required whole blood or platelet-rich plasma transfusion for treatment of anemia and/or thrombocytopenia (Table 2.1.2). Numbers of harvested $CD34^+$ cells and days required for hematopoietic recovery were not correlated. Moreover, difference in source of HSCs (BM and cytokine-mobilized peripheral blood cells) and days required for hematopoietic recovery were not correlated. When the harvested $CD34^+$ cell numbers reached 1 to 2 × 10^6 cells/kg, further increase did not appear to accelerate recovery in both sources of HSCs (BM and cytokine-mobilized peripheral blood cells).

Complications.

Eight animals had high fever (body temperature > 38°C) and increase in CRP values during myelosuppression. Fever subsided after administration of antibiotics (Table 2.1.2). Enrofloxacin, ceftazidime, aminoglycoside, or other antibiotics were chosen according to antimicrobial susceptibility test results. Enterococcus faecalis, Enterobacter cloacae or Staphylococcus aureus was isolated from culture of the blood from three animals. Some animals developed nausea and anorexia during myelosuppression. Intravenous hyperalimentation, including amino acids, fat emulsion, and vitamins, was conducted to supplement intake of food and water in those animals. Radiation-associated diarrhea ceased within three weeks after irradiation in all animals.

Animal No. 396051 had high serum alanine transaminase and aspartate transaminase activities, which may have been associated with antibiotic treatments, because

the ceftazidime has a hepatotoxicity. The values completely normalized after change of antibiotics. Animals No. 396053 and No. 396060 had high blood urea nitrogen and creatinine values, and it was presumably due to mild renal failure. Diarrhea, anorexia and dehydration induced electrolyte disturbance, and it lead to the mild renal failure. However, these symptoms were not progressed to severe renal failure, because administration of fluids and cardiac diuretic was effective and the values were normalized. Other abnormal serum biochemical values were not observed.

Three weeks after transplantation, animal No. 296113 died due to accidental catheter fracture. Otherwise all procedures were safely conducted. Up to two years after transplantation, complications such as radiation pneumonitis or other radiation-related disorders, were not observed.

Discussion

I have established protocols of autologous $CD34^+$ cell transplantation in young cynomolgus monkeys. Hematopoietic reconstitution was observed around two weeks after transplantation in all animals. Although some animals required antibiotic administration, blood transfusion, and intravenous hyperalimentation, these procedures were performed safely with few adverse effects. The focus of this study was on the use of BM and cytokine-mobilized peripheral blood cells as a source of $CD34^+$ cells.

Results of this study documented a fourth large animal model for CD34-selected hematopoietic cell transplantation in addition to that of baboons [4], rhesus macaques [9], and dogs [10]. Compared with the dog, the other animals mentioned are closer phylogenetically to humans, and more reagents, including cytokines and antibodies, are available that were originally developed for humans [112]. Although baboons may provide the best animal model among them because of its phylogenetic relatedness to humans, there is no clear evidence that the hematopoietic system of baboons would resemble that of humans more than those of macaques. In addition, the macaque model has the advantages of animal availability, cost, and down size, compared with the baboon model. On the other hand, among macaque monkeys, the advantages of using cynomolgus monkeys over rhesus monkeys are as follows; they are full seasonal breeders and smaller body size. Full seasonal breeding capacity is important in maintaining a self-sustaining breeding colony without need for introduction of new breeders. The self-sustaining breeding system has made it possible to establish the SPF colony and to accumulate background data and set normal limits according to the physiological data [93, 113].

There are a few disadvantages to using monkeys, compared with other smaller animals such as mice, for biomedical research. Although it is not a true disadvantage, monkeys, like humans, are outbred, allowing the potential of substantial individual

differences between animals. Other disadvantages are fewer monkey models for human diseases, compared with mice [61, 105], and that primate research costs are substantially higher [27].

The study reported here included 1) administration of a five-day course of SCF and G-CSF, 2) harvest of BM and cytokine-mobilized peripheral blood cells, 3) expanding immature hematopoietic cells in BM and peripheral blood [19, 22, 78], 4) ex vivo culture of $CD34^+$ cells for four days [23], and 5) transplantation of the cultured $CD34^+$ cells and reconstitution of the hematopoiesis. The $CD34^+$ cells were stimulated overall for nine days (in vivo for five and ex vivo for four days) prior to transplantation. One may claim that these stimulated cells lost their engraftment ability [23, 30] and that hematopoietic restoration was attributable to endogenous recovery. To address the issue, I genetically modified $CD34^+$ cells with retroviral vectors under the same protocol, followed by transplantation of the cells into each monkey. In the setting of same transplantation protocol, levels of 30–50% of gene-marked progenitor cells were still detected in monkeys one year after transplantation [38]. This result clearly indicates that the transplanted cells engrafted and generated their progeny, resulting in the hematopoietic restoration in vivo.

Animals were irradiated with 1,000 to 1,100 cGy for myeloablation. The point at issue is to know whether this dose of irradiation can really cause "full" myeloablation. If not, endogenous recovery could occur. To answer the question, it would be necessary to test whether animals, after total body irradiation with 1,000 to 1,100 cGy, could survive without infusion of cells. I, however, could not design such experiment because of considerations of the animal welfare. It has been reported that 1,300 cGy may be required for "full" ablation (100% lethal) but that this dose would cause radiation-induced pneumonitis at much higher frequency, compared with the dose of 1,000 cGy [21]. Since myeloablative irradiation is associated with high systemic toxicity or potential damage to BM stroma, projects of BM and

microenvironment transplantation (e.g. intra-BM transplantation, ref. [57]) are needed to assess how much the dose of radiation can be reduced without losing engraftment efficiency of transplanted HSCs, or whether alternative methods for myeloablation, such as cyclophosphamide administration, can be used instead of total body irradiation. As described above, other genetic marking study [39, 100] has shown hematopoietic reconstitution from transplanted cells after this dose of irradiation, suggesting the dose was sufficient enough at least to allow engraftment of transplanted cells without complication such as radiation-induced pneumonitis.

Recently, researchers have observed, principally in murine models, that hematopoietic cells appear to be able to form other kinds of cells, such as liver, muscle, and blood vessels [31], although cell fusions may in part account for such change of phenotype [92, 111]. If plasticity of hematopoietic cells can be applied to human cells, it may eventually be possible to use hematopoietic cells to replace a wider array of cells and tissues than was initially thought. My non-human primate model will provide an important framework for such future clinical studies.

Table 2.1.1. Bone marrow harvest and cytokine-mobilized peripheral blood cells collection from cynomolgus monkeys

Source of HSCs	Animal ID (No.)	Sex	Age (yr)	BW (kg)	Priming		Total nucleated cells (x 10^9/kg)	CD34+ Selection		Culture conditions
					G-CSF (μg/kg)	SCF (μg/kg)		Cell numbers (x 10^6/kg)	Proportion in the total nucleated cells (%)	
Bone Marrow (not administered)	396042	female	2	2.3	0	0	NA	NA	NA	-
	396051	female	3	2.4	0	0	0.29	1.4	0.5	-
	396053	female	3	2.3	0	0	0.29	4.2	1.5	IL3/IL6/SCF/FL
Bone Marrow (administered with G-CSF and SCF)	396058	female	3	2.3	100	200	1.17	16.3	1.4	IL3/IL6/SCF/FL
	396060	female	3	2.1	10	50	0.34	8.1	2.4	IL6/SCF/FL
	296102	female	3	2.3	50	50	0.71	7.3	1.0	IL6/SCF/FL/TPO
	099053	male	3	2.5	50	50	0.59	4.2	0.7	IL6/SCF/FL/TPO
	099061	male	4	3.3	50	50	0.47	13.6	2.9	IL6/SCF/FL/TPO
	099056	male	2	2.7	50	50	0.77	14.2	1.8	IL6/SCF/FL/TPO
	296113	female	4	3.2	50	50	0.23	3.5	1.5	IL6/SCF/FL/TPO
	296116	female	4	3.3	50	50	0.37	3.5	1.0	IL6/SCF/FL/TPO
Cytokine-mobilized peripheral blood cells	001045	male	3	3.3	50	50	2.58	10.0	0.4	IL6/SCF/FL
	001049	male	3	3.5	50	50	2.94	19.5	0.7	IL6/SCF/FL/TPO
	001053	male	3	2.6	50	50	3.48	3.5	0.1	IL6/SCF/FL/TPO
	398042	male	5	3.8	50	50	2.95	3.4	0.1	IL6/SCF/FL/TPO
Average	-	-	3	2.8	-	-	1.90	8.1	1.2	-

BW=body weight; G-CSF=granulocyte colony-stimulating factor; SCF=stem cell factor; IL3=interleukin-3; IL6=interleukin-6; FL= fms-like tyrosine kinase-3 ligand; TPO=thrombopoietin; and NA=not applicable.

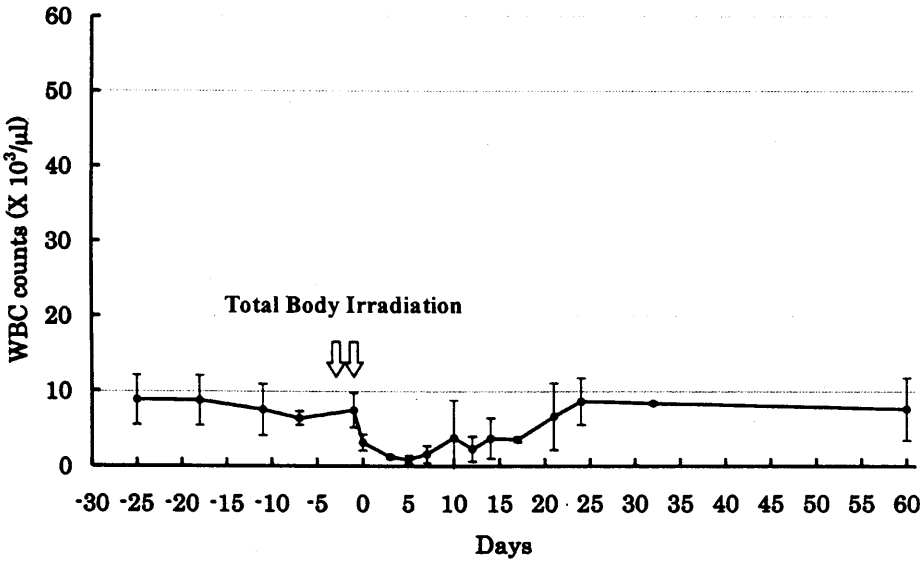
Table 2.1.2. Clinical course of autologous hematopoietic stem cell transplantation in cynomolgus monkeys

Source of HSCs	Animal ID (No.)	White blood cells		Platelet		Blood transfusion	Body temperature > 38 °C	Peak CRP value (mg/dl)
		Nadir (cells/μl)	>1,000/μl	Nadir (cells/μl)	>50,000/μl			
Bone Marrow (not administered)	396042	1,300	NA	83,000	NA	-	0 day	4.69
	396051	400	Day 13	23,000	Day 15	3 times (total 80ml)	4 days	10.26
	396053	500	Day 10	67,000	NA	2 times (total 50ml)	2 days	8.56
Bone Marrow (administered with G-CSF and SCF)	396058	700	Day 12	26,000	Day 17	4 times (total 75ml)	2 days	0.6
	396060	300	Day 14	40,000	Day 14	3 times (total 45ml)	0 day	1.02
	296102	700	Day 6	145,000	NA	-	2 days	0.18
	099053	700	Day 14	80,000	NA	1 time (total 25ml)	0 day	1.77
	099061	700	Day 10	69,000	NA	1 time (total 30ml)	0 day	3.81
	099056	1200	NA	109,000	NA	-	2 days	1.02
	296113†	200	-	16,000	-	3 times (total 60ml)	2 days	10.01
	296116	500	Day 14	45,000	Day 17	1 time (total 60ml)	0 day	10.39
Cytokine-mobilized peripheral blood cells	001045	200	Day 19	21,000	Day 19	2 times (total 50ml)	2 days	20.5
	001049	700	Day 19	87,000	NA	1 time (total 30ml)	0 day	8.22
	001053	500	Day 7	93,000	NA	-	0 day	4.19
	398042	500	Day 13	49,000	Day 13	2 times (total 50ml)	3 days	4.69
Average	-	700	Day 13	64,000	Day 16	2 times (total 50ml)	1.3 days	5.99

†Died due to accidental catheter fracture at 3 weeks after transplantation. CRP=C-reactive protein. Nadir=lowest point. NA=not applicable.

Figure 2.1.1. WBC counts before and after transplantation, which was conducted on day 0. Data are expressed as mean and standard deviation of WBC counts from animals administered with G-CSF and SCF (B, n = 12) or not administered (A, n = 3). Administration of these cytokines (shaded arrows in B) resulted in an increase in the WBC count between 16,000 and 80,000 (mean, 52,000) cells/ μ l. After total body irradiation (500 to 550 cGy two times, open arrows in A and B), the WBC count decreased to < 1,000 cells/ μ l. To facilitate granulocyte recovery, the G-CSF was administered intravenously to animals at a dosage of 10 μ g/kg once a day from the day whenever the WBC count became < 1,000 cells/ μ l until the WBC count reached 5,000 cells/ μ l according to the transplantation protocol [20, 96].

A


WBC counts (X 10³/μl)
60
50
40
30
20
10
0
Total Body Irradiation
-30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60
Days

B

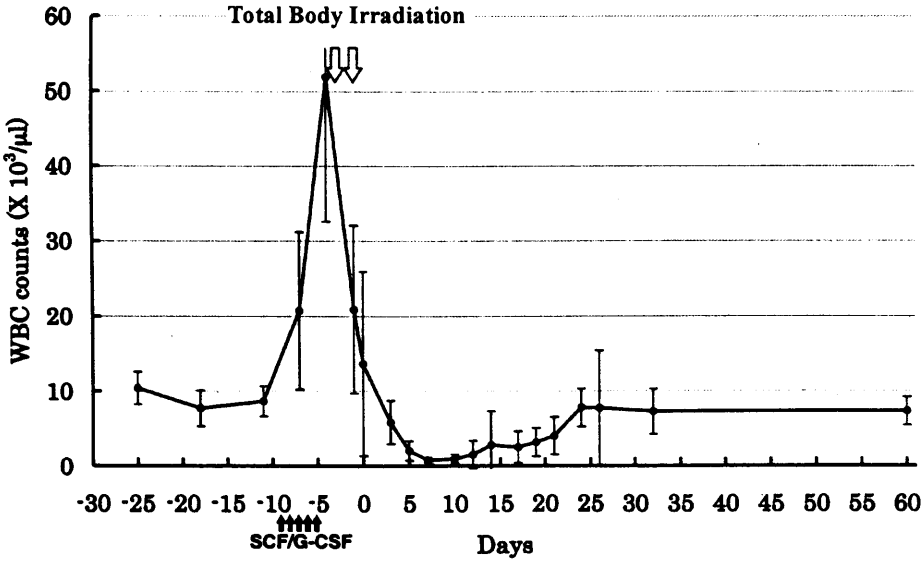

WBC counts (X 10³/μl)
60
50
40
30
20
10
0
Total Body Irradiation
-30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60
SCF/G-CSF
Days

Figure 2.1.2. Blood Hb concentrations (g/dl) before and after transplantation, which was conducted on day 0. Mean values for all monkeys (n = 15), with or without cytokine administration, are shown, since administration of SCF and G-CSF did not affect Hb concentration. Bars indicate the standard deviation. Freshly obtained, irradiated (2,000 cGy), whole blood from blood-donor monekys were transfused to anemic animals whenever the blood Hb value was < 7 g/dl according to the transplantation protocol [20, 96].

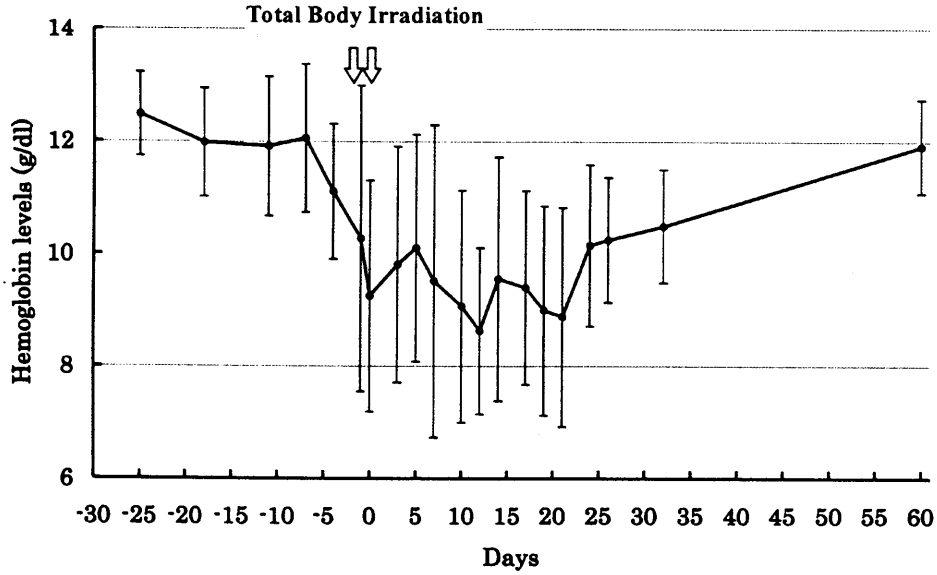
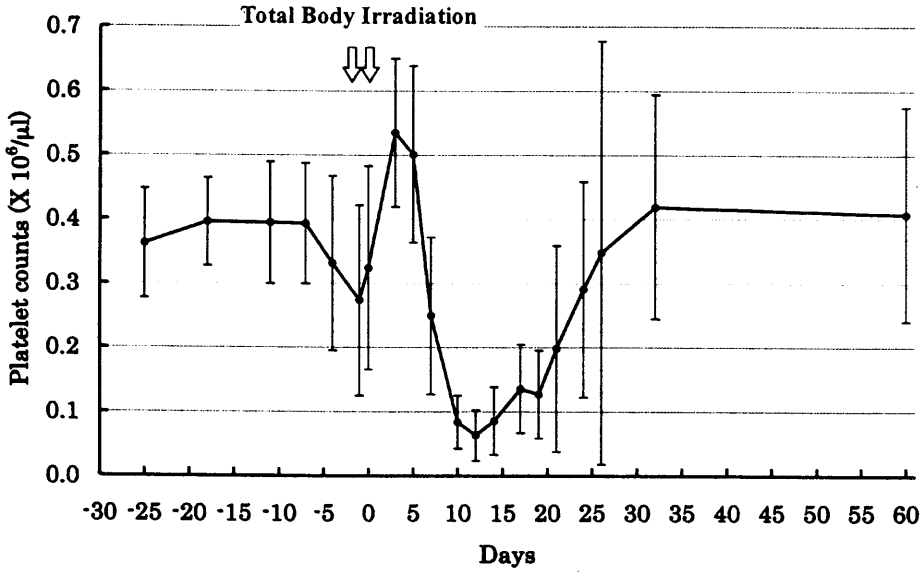

Total Body Irradiation
Hemoglobin levels (g/dl)
14
12
10
8
6
-30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60
Days

Figure 2.1.3. Blood platelet counts before and after transplantation, which was conducted on day 0. Mean values for all monkeys (n = 11) with or without cytokine administration, are shown, since administration of SCF and G-CSF did not affect platelet count. Bars indicate the standard deviation. Freshly obtained, irradiated (2,000 cGy), whole blood or platelet-rich plasma was administered to thrombocytopenic animals whenever the platelet count was < 70,000 cells/μl according to the transplantation protocol [20, 96].


Total Body Irradiation
Platelet counts (X 10⁶/μl)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60
Days

Section 2 :

A Major Problem being Related in Preclinical Study of HSCs Transplantation: Inhibition of Antibodies Production against Recombinant Human Protein in Cynomolgus Monkeys.

Introduction

There is a major problem which might occur in HSCs transplantation for preclinical study of HSCs gene therapy. Macaque monkeys are widely used for preclinical testing of genes and proteins of human origin, taking advantage of their close phylogenetic relationship to humans [39, 58, 109]. Despite the genetic similarity between the two species, human gene products or proteins are often immunogenic to monkeys [38, 72]. An example is EPO. EPO is a hematopoietic growth factor that stimulates the proliferation and differentiation of erythroid progenitor cells [56]. Recombinant human EPO (hEPO) has a variety of clinical uses [26, 40, 91, 110]. Although 92% of amino acid residues (142/166) are shared between human and macaque EPO [59, 106], I show here that hEPO induces potent immune responses in macaque monkeys, precluding its administration to monkeys. On the other hand, recombinant human G-CSF presumably induce weak or no immune response in macaque monkeys because the WBC counts increased in response to the repeated administration of G-CSF (Refer to Chapter 2, Section 1 and Figure 2.1.1).

Therefore, it is necessary to develop a method to prevent such immune responses following the administration of hEPO. Among many immunosupressants available, CyA is widely used to suppress detrimental immune reactions associated with allogenic BM and organ transplantation [12, 14, 25, 95]. CyA is a calcineurin inhibitor and inhibits nuclear factor of activated T cells (NFAT) activity and induces immunosuppression [44, 60]. In this

study, I showed that hEPO can be successfully administered to cynomolgus monkeys to stop immunological clearance by using CyA.

In addition, there is only a case that hEPO induced immune responses and produced the pure red cell aplasia in renal failure patient in human [98]. Although treatment with EPO is well tolerated in the majority of renal failure patients, only this case generate antibody responses to hEPO that can effectively neutralize both circulating endogenous EPO and the recombinant protein. Therefore, using this CyA administration method may effectively prevent immune responses to hEPO in this human case.

Materials and Methods

Animals

Four cynomolgus monkeys (4-6 years old, 2.5-5.5 kg) bred in the Tsukuba Primate Research Center (Ibaraki, Japan) were enrolled in this study (Table 2.2.1). Animals were free of intestinal parasites, herpes-B, simian type-D retrovirus, and simian varicella virus (Refer to Chapter 1, Section 1). This study was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [42] and the Guiding Principles for Animal Experiments Using Non-human Primates formulated by the Primate Society of Japan [68]. 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).

EPO administration

First, I administered hEPO (Chugai, Tokyo, Japan) to a cynomolgus monkey (No. 099054) at a dose of 3000 IU/kg three times a week subcutaneously for inducing strong immune response. The dose was determined by referred to the maximum dose in human clinical use [91].

A second cynomolgus monkey (No. 001051) was intravenously (instead of subcutaneously) given a much lower dose of hEPO (200 IU/kg, three times a week) for inducing the weak immune response.

Next, two cynomolgus monkeys (No. 396053 and No. 396058) were given 6 mg/kg of cyclosporin A (CyA: Sandimmun; Novartis Pharma, Basel, Switzerland) intramuscularly every other day in combination with the subcutaneous hEPO administration (200 IU/kg, three times a week) (Table 2.2.1).

EPO, anti EPO antibody and CyA assays

The hEPO concentrations in the serum were assessed by standard enzyme-linked immunosorbent assay (ELISA; Roche Applied Science, Mannheim, Germany). ELISA was performed according to the manufacturer's instruction. In the first step, hEPO is bound to anti-hEPO-coated surface of the microplate and the second to the peroxidase-conjugated detection antibody. Following the washing step, the peroxidase bound in the complex is developed by the substrate tetramethylbenzidine, and determined photometrically. The color intensity is proportional to the concentration of EPO.

The presence of anti-hEPO antibodies in the plasma was evaluated using previously reported assay [41]. The assay was designed to measure the binding of the antibody to EPO in serial dilutions of the animal plasma. Such binding resulted in interference (competitive ELISA of antibodies) with the detection of known concentrations of EPO in a standard ELISA (mentioned above).

CyA concentrations in the plasma were assessed by radioimmunoassay [79]. The method was performed in accordance with the instructions provided by the manufacturer (Immunotech, Beckman Coulter, UK). Briefly, duplicated 100 μl samples exposed to 1 μg/ml CyA and 200 μg/ml cilastatin, and then incubated for 30 min with ^{125}I-labelled CyA in polystyrene tubes coated with anti-CyA monoclonal antibody. Thereafter, the supernatants of the tubes were discarded and the bound radioactivity of calibrators, controls and samples was measured using a gamma scintillation counter.

The blood biochemical profile and a complete blood count were monitored throughout the administration of hEPO and CyA in all monkey.

Results

Low levels (< 0.1 IU/ml) of hEPO were detected for the first 3 weeks but thereafter the levels decreased to the lowest limit of detection (0.001 IU/ml) despite the continued administration of hEPO in first monkey (No. 099054) (Fig. 2.2.1A). It turned out that anti-hEPO antibody was generated (Fig. 2.2.1A), and hEPO was cleared from the serum. Thus, it was difficult to obtain sufficient levels of the effect of hEPO *in vivo* due to immunological clearance. The administered hEPO was consequently eliminated from the animals. During the administration of hEPO in second monkey (No. 001051), very low levels (< 0.01 IU/ml) of hEPO were detected with the exception of one time point (0.1 IU/ml at day 28) and the levels eventually decreased to zero (Fig. 2.2.1B). Despite the lower dose administration of hEPO, it turned out again that anti-hEPO antibody was generated (Fig. 1B), leading to the clearance of hEPO from the serum. The Hb levels did not increase in either animal (Table 2.2.1). Despite the genetic similarity of EPO between humans and macaques [59, 106], hEPO is a potent immunogen to macaque monkeys. This is the first report on the immune responses in monkeys following the administration of hEPO.

On the other hand, two cynomolgus monkeys (No. 396053 and No. 396058) were given CyA intramuscularly every other day in combination with the subcutaneous hEPO administration. As a result, in both monkeys, anti-hEPO antibody was not generated and high serum levels (around 0.1 IU/ml) of hEPO were obtained during the administration of hEPO (Figs. 2.2.2A and B). A second trial of hEPO also resulted in a similar elevation of the serum levels of hEPO (Figs. 2.2.2A and B). The Hb levels apparently increased in response to the administration of hEPO and CyA compared with administration EPO alone (Table 2.2.1), suggesting that hEPO trial was effective when CyA was administered together. Blood biochemical profile revealed no adverse effects associated with the CyA and hEPO treatment. CyA concentrations in the plasma were maintained within an effective range between 200 and

600 ng/ml. Using this CyA administration method, I successfully maintained long-term (around 1 year) hEPO and CyA concentration as shown in Fig. 2.2.3.

Discussion

I have thus established a method to prevent immune responses to the xenogenic hEPO in cynomolgus monkeys using CyA. This CyA administration method was possible to maintain the CyA concentration with in the effective range between 200-600 ng/ml (Fig. 2.2.3). Although I did not try subcutaneous administration of 200 IU/kg hEPO in the present study (Table 2.2.1), it is assumed that subcutaneous administration of 200 IU/kg hEPO would also result in the anti-hEPO antibody generation, because intravenous administration of the same dose of hEPO resulted in the anti-hEPO antibody generation. There was a report that subcutaneous administration induced stronger immune responses than intravenous administration [82]. The subcutaneous route is known to be the most immunogenic, because the antigens were trapped effectively by the presence of antigen-presenting cells such as dendritic cells in the skin [24].

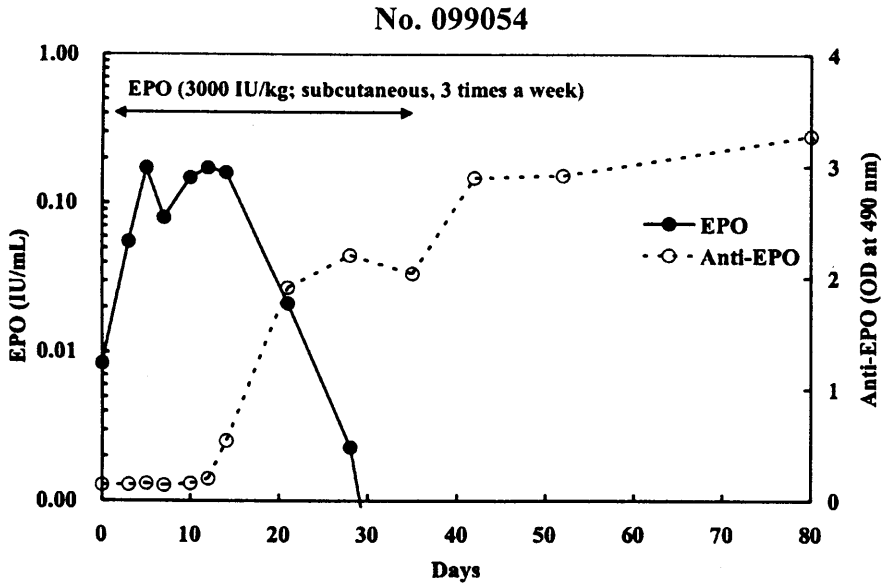
In fact, this method was successfully applied to monkey HSCs transplantation cases, and the long-term EPO and CyA concentration was maintained effectively and safely in administered monkeys (Fig.2.2.3. and ref. [100]). CyA administration will be useful to prevent immune responses when human proteins are administered to monkeys for research purposes.

Table 2.2.1. Characteristics of cynomolgus monkeys subjected to the hEPO administration

	Animal No. (Sex)	Age (years)	Body Weight (kg)	hEPO		CyA		Hemoglobin levels (g/dl)		Complication
				Dose (IU/kg)	Administration Route and Frequency	Dose (*ng*/kg)	Administration Route and Frequency	Day 0	Day 35	
hEPO Alone	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	-	-	6	-	11.0	11.8	-

Figure 2.2.1. Administration of only hEPO in cynomolgus monkeys. After the subcutaneous administration of hEPO (3,000 IU/kg) to a monkey (No. 099054), anti-hEPO antibody was generated and serum hEPO levels decreased to almost zero level(A). The hEPO levels can not reached to sufficient levels due to immunological clearance. The administered hEPO was thus eliminated immediately from the animals. Anti-hEPO antibody was also generated in another monkey (No. 001051) receiving hEPO at a lower dose (200 IU/kg) intravenously, leading to the clearance of hEPO from the serum (B).

A


No. 099054
EPO (3000 IU/kg; subcutaneous, 3 times a week)
EPO
Anti-EPO
EPO (IU/mL)
Anti-EPO (OD at 490 nm)
Days
1.00
0.10
0.01
0.00
4
3
2
1
0
0
10
20
30
40
50
60
70
80

B

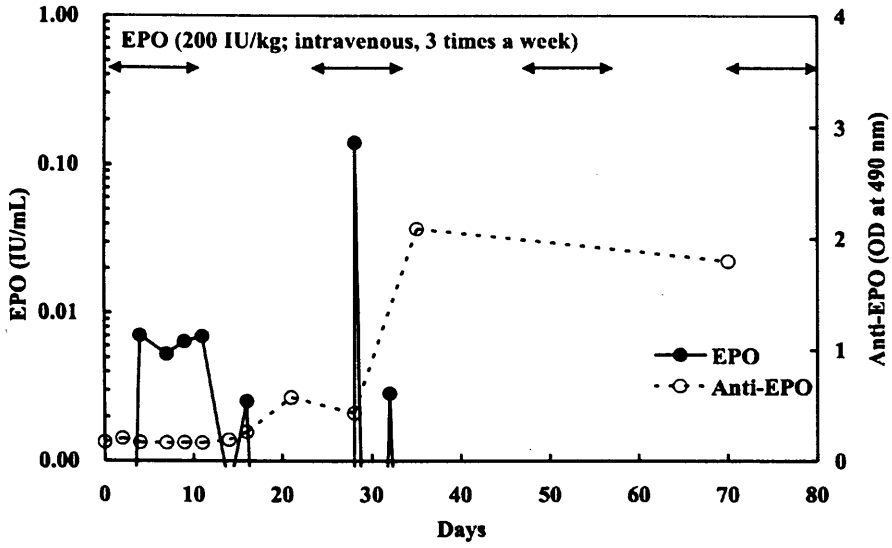
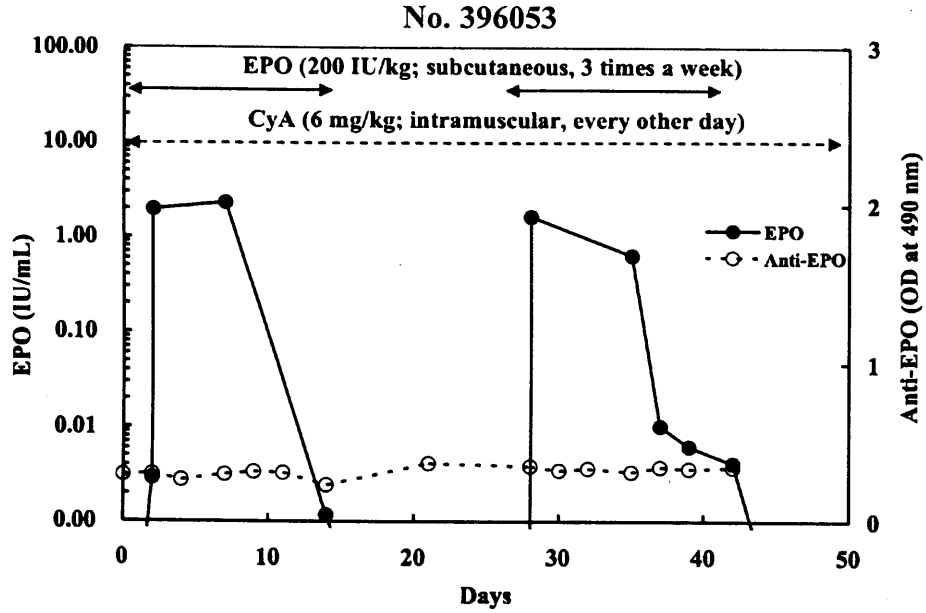

No. 001051
EPO (200 IU/kg; intravenous, 3 times a week)
EPO
Anti-EPO
EPO (IU/mL)
Anti-EPO (OD at 490 nm)
Days
1.00
0.10
0.01
0.00
4
3
2
1
0
0
10
20
30
40
50
60
70
80

Figure 2.2.2. Administration of hEPO in combination with CyA in cynomolgus monkeys. Generation of anti-hEPO antibody was prevented by the treatment with CyA in 2 cynomolgus monkeys (No. 396053 and No. 396058) receiving hEPO (200 IU/kg) subcutaneously (A, B). The plasma CyA concentrations were within an effective range of 200 to 400 *n*g/m*l*. 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 two monkeys.

A


No. 396053
EPO (200 IU/kg; subcutaneous, 3 times a week)
CyA (6 mg/kg; intramuscular, every other day)
EPO
Anti-EPO
EPO (IU/mL)
Anti-EPO (OD at 490 nm)
Days
100.00
10.00
1.00
0.10
0.01
0.00
0
10
20
30
40
50
3
2
1
0

B

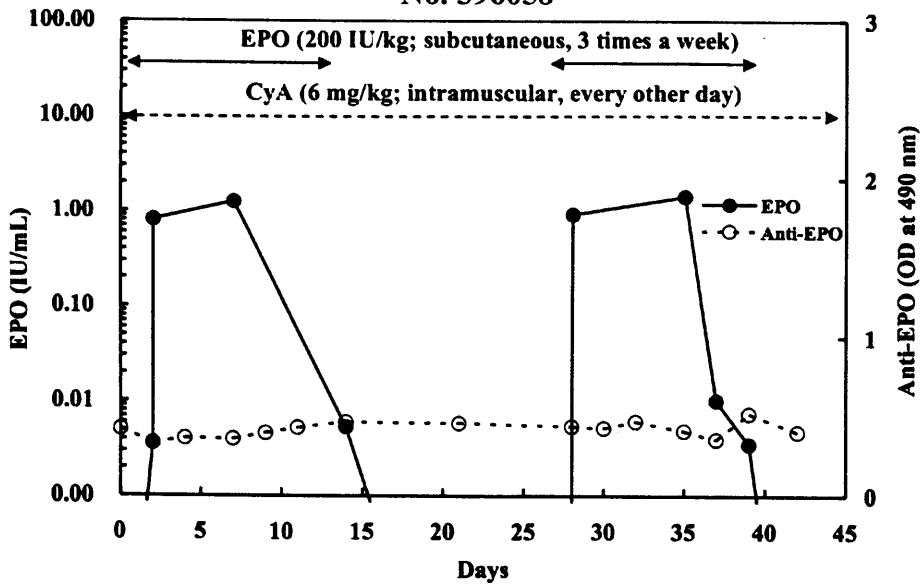
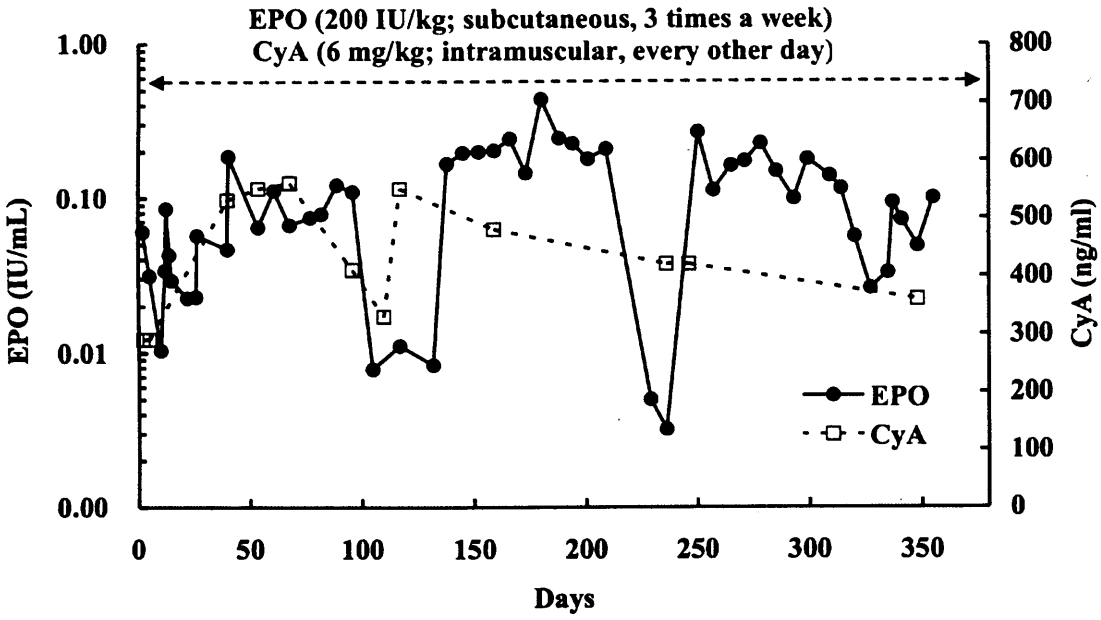

No. 396058
EPO (200 IU/kg; subcutaneous, 3 times a week)
CyA (6 mg/kg; intramuscular, every other day)
EPO
Anti-EPO
EPO (IU/mL)
Anti-EPO (OD at 490 nm)
Days
100.00
10.00
1.00
0.10
0.01
0.00
0
5
10
15
20
25
30
35
40
45
3
2
1
0

Figure 2.2.3. EPO and CyA concentration was maintained effectively and safely in administered a monkey. There were no adverse effects through the administration of CyA and EPO in this animal.


EPO (200 IU/kg; subcutaneous, 3 times a week)
CyA (6 mg/kg; intramuscular, every other day)
EPO (IU/mL)
1.00
0.10
0.01
0.00
CyA (ng/ml)
800
700
600
500
400
300
200
100
0
0
50
100
150
200
250
300
350
Days
EPO
CyA

General conclusion

Studies using non-human primate have provided an important framework for human clinical studies. In a real sense, non-human primates as animal models represent a "step to human" i.e., the translational study.

In chapter 1, I have established safe and effective leukapheresis procedure for collection of cytokine-mobilized peripheral blood cells from non-human primates. First step is to establish baseline data for extracorporeal circulation, such as TBV of non-human primates. To determine the TBV, blood was collected after intravenous injection of Evans blue solution. The TBV was obtained after correcting for the Ht and the dilution factor of the Evans blue solution. 1) Significant correlation was observed between TBV and BW in male and female monkeys. The formulae were established to estimate TBV by referring to BW. 2) There was no significance, however, between TBV and BW in male monkeys weighing more than 6 kg.

Next, I developed a safe and effective modified procedure to collect cytokine-mobilized peripheral blood cells from rhesus monkeys using a Baxter CS3000+ Blood Cell Separator (Baxter, Deerfield, IL) with several devices that were reduced in chamber size and shortened in tube length of the standard apheresis kit to decrease ECV from 130 to 70 ml. Pump speed was controlled by monitoring Ht values and platelet counts during leukapheresis. This system makes it possible to perform safe and effective leukapheresis in rhesus monkeys whose BW is similar to that of human infants. A total of 12 leukapheresis procedures were performed in nine monkeys and resulted in the collection of sufficient numbers of WBCs (mean, 1.38×10^9 cells/kg), MNCs (mean, 3.67×10^8 cells/kg) and $CD34^+$ cells (mean, 17.80×10^6 cells/kg) in all cases. In addition, no complications such as anemia or trombocytopenia, occurred after leukapheresis.

Then, I examined the efficacy and safety of this method with even smaller macaques, cynomolgus monkeys, which are equivalent to human newborns in BW (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 my modified procedure (n = 6), no such complication was observed in any animal. In addition, the harvested nuclear cell, MNC 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. HSCs in BM can be mobilized into peripheral blood by cytokine administration. Cytokine-mobilized peripheral blood stem cells are of great use in clinical applications such as transplantation and regenerative medicine.

In chapter 2, I have established safe and efficient methods for autologous HSCs transplantation in cynomolgus monkeys. The methods include regimens of supportive care to ensure subjected monkey's survival during hematopoietic reconstitution following to lethal dose of the total body irradiation. Fifteen young adult cynomolgus monkeys were studied. BM was aspirated from the ilium and/or tuber ischiae (n=11) and cytokine-mobilized peripheral blood cells was collected by leukapheresis (n=4) after administration of recombinant human SCF and G-CSF. Using the immunomagnetic selection method, $CD34^+$ cells were then isolated (90 to 95% pure) as a fraction containing HSCs. Just prior to transplantation, the animals were received myeloablative total body irradiation-500 to 550 cGy daily for two days (Total 1000-1100 cGy). The monkeys transplanted with $CD34^+$ cells developed moderate to severe myelosuppression, with some animals requiring intravenous hyperalimentation,

antibiotic administration, and blood transfusion. Hematopoiesis was restored in all animals after transplantation. It took 13 days, on average, until the peripheral WBC count reached more than 1,000 cells/μl. Up to two years after transplantation, signs of radiation-induced pneumonitis or other radiation-related disorders were not evident at the aforementioned dose of irradiation. This transplantation model will be useful for testing new approaches using HSCs for therapy of many diseases and will offer unique insights into the biology of these cells.

Finally, I overcame a major problem which might be limiting HSCs transplantation for preclinical study of HSCs gene therapy. 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 effect *in vivo* due to immunological clearance. In this study, I show that hEPO induces the generation of anti-hEPO antibody in cynomolgus monkeys (n = 2), although 92% of amino acid residues are shared between the human and macaque EPO. The administered hEPO was thus eliminated from the animals. On the other hand, when an immunosuppressant, CyA, was administered (6 mg/kg) intramuscularly every other day in combination with hEPO (n = 2), anti-hEPO antibody was not generated and high serum levels of hEPO were obtained during the administration of hEPO, resulting in an increase in Hb levels. No adverse effects associated with CyA were observed. Thus, CyA administration method might be useful for the prevention of immune responses in monkeys associated with the administration of human proteins.

I established preclinical model for hematopoietic stem cell gene therapy in non-human primates. Consequently, this model was successfully applied to preclinical gene therapy, and has brought about precious information in that study [38, 100].

Well-designed non-human primate study will continue to offer unique insights into the biology of HSCs, the immune system, transplantation, and therapies for many human diseases. This non-human primate model will provide an important framework for such future clinical studies as well as HSCs gene therapy.

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References

1. Abe T, Makimoto A, Kawano Y, Okamoto Y, Kajiume T, Nakagawa R, Watanabe H, Sato J, Watanabe T, Kuroda Y, Takaue Y. 1998. Intra-apheresis recruitment of blood progenitor cells in children. Transfusion. 38:944-950.

2. Abkowitz JL, Persik MT, Shelton GH, Ott RL, Kiklevich JV, Catlin SN, Guttorp P. 1995. Behavior of hematopoietic stem cells in a large animal. Proc Natl Acad Sci U S A 92: 2031-2035.

3. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. 1998. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat. Med. 4:1038-1045.

4. Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID. 1988. Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. J. Clin. Invest. 81:951-955.

5. Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID, Thomas ED. 1991. Engraftment after infusion of $CD34^+$ marrow cells in patients with breast cancer or nueroblastoma. Blood 77:1717-1722.

6. Biesma DH, Kraaijenhagen RJ, Dalmulder J, Marx JJ, Van De Wiel A. 1994. Recombinant human erythropoietin in autologous blood donors: a dose-finding study. Br J Haematol. 86: 30-35.

7. Blanchette VS, McCombie NE, Rock G. 1985. Factors that influence lymphocyte yields in lymphocytapheresis. Transfusion. 25:242-245.

8. Bock TA. 1997. Assay systems for hematopoietic stem and progenitor cells. Stem Cells 15(Suppl. 1):185-195.

9. Bodine DM, Moritz T, Donahue RE, Luskey BD, Kessler SW, Martin DI, Orkin SH, Nienhuis AW, Williams DA. 1993. Long-term in vivo expression of a murine

adenosine deaminase gene in rhesus monkey hematopoietic cells of multiple lineages after retroviral mediated gene transfer into $CD34^+$ bone marrow cells. Blood 82:1975-1980.

10. Bruno B, Nash RA, Wallace PM, Gass MJ, Thompson J, Storb R, McSweeney PA. 1999. $CD34^+$ selected bone marrow grafts are radioprotective and establish mixed chimerism in dogs given high dose total body irradiation. Transplantation 68:338-344.

11. Buchl SJ, Keeling ME, Voss WR. 1997. Establishing specific pathogen-free (SPF) nonhuman primate colonies. ILAR J. 38:22-27.

12. Burke JF Jr, Pirsch JD, Ramos EL, Salomon DR, Stablein DM, Van Buren DH, West JC. 1994. Long-term efficacy and safety of cyclosporine in renal-transplant recipients. N. Engl. J. Med. 331:358-363.

13. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. 2000. Gene therapy of human severe combined immunodeficiency (SCID)X1 disease. Science 288:669-672.

14. Chao NJ, Schmidt GM, Niland JC, Amylon MD, Dagis AC, Long GD, Nademanee AP, Negrin RS, O'Donnell MR, Parker PM, Smith EP, Snyder DS, Stein AS, Wong RM, Blume KG. Forman SJ. 1993. Cyclosporine, methotrexate, and prednisone compared with cyclosporine and prednisone for prophylaxis of acute graft-versus-host disease. N. Engl. J. Med. 329:1225-1230.

15. Childs R, Chernoff A, Contentin N, Bahceci E, Schrump D, Leitman S, Read EJ, Tisdale J, Dunbar CE, Linehan WM, YoungNS, Barrett AJ. 2000. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. N. Engl. J. Med. 343:750-758.

16. Dao MA, Nolta JA. 2000. CD34: to select or not to select? That is the question. Leukemia 14:773-776.

17. Davy, K. P., and D. R. Seals. 1994. Total blood volume in healthy young and older

men. J. Appl. Physiol. 76:2059-2062.

18. Diaz MA, Alegre A, Benito A, Villa M, Madero L. 1998. Peripheral blood progenitor cell collection by large-volume leukapheresis in low-weight children. J Hematother. 7:63-68.

19. Dicke KA, Hood DL, Arneson M, Fulbright L, DiStefano A, Firstenberg B, Adams JG, Blumenschein R. 1997. Effects of short-term in vivo administration of G-CSF on bone marrow prior to harvesting. Exp. Hematol. 25:34-38

20. Donahue RE, Kirby MR, Metzger ME, Agricola BA, Sellers SE, Cullis HM. 1996. Peripheral blood $CD34^+$ cells differ from bone marrow $CD34^+$ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. Blood. 87:1644-1653.

21. Donahue RE, Wersto RP, Allay JA, Agricola BA, Metzger ME, Nienhuis AW, Persons DA, Sorrentino BP. 2000. High levels of lymphoid expression of enhanced green fluorescent protein in nonhuman primates transplanted with cytokine-mobilized peripheral blood CD34 1 cells. Blood. 95:445–452.

22. Dunbar CE, Seidel NE, Doren S, Sellers S, Cline AP, Metzger ME, Agricola BA, Donahue RE, Bodine DM. 1996. Improved retroviral gene transfer into murine and rhesus peripheral blood or bone marrow repopulating cells primed in vivo with stem cell factor and granulocyte colony-stimulating factor. Proc. Natl. Acad. Sci. USA 93:11871-11876.

23. Dunbar CE, Takatoku M, Donahue RE. 2001. The impact of ex vivo cytokine stimulation on engraftment of primitive hematopoietic cells in a non-human primate model. Ann. N. Y. Acad. Sci. 938:236-245.

24. Eckardt KU, Casadevall N. 2003. Pure red-cell aplasia due to anti-erythropoietin antibodies. Nephrol Dial Transplant. 18:865-9.

25. Eisen HJ, Hobbs RE, Davis SF, Laufer G, Mancini DM, Renlund DG, Valantine H, Ventura H, Vachiery JL, Bourge RC, Canver CC, Carrier M, Costanzo MR,

Copeland J, Dureau G, Frazier OH, Dorent R, Hauptman PJ, Kells C, Master R, Michaud JL, Paradis I, Smith A, Vanhaecke J, Feutren G, Turkin D, Mellein B, Mueller EA. 1999. Safety, tolerability and efficacy of cyclosporine microemulsion in heart transplant recipients: a randomized, multicenter, double-blind comparison with the oil based formulation of cyclosporine--results at six months after transplantation. Transplantation 68:663-671.

26. Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW. 1987. Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N. Engl. J. Med. 316:73-78.

27. Fitzgerald TA. 1983. Comparison of research cost: man-primate animal-other animal models. J. Med. Primatol. 12:138-145.

28. Garner MG, Phippard AF, Horvath JS, Duggin GG, Tiller DJ. 1985. Blood volume measurement in baboons: simultaneous estimation of red cell volume and plasma volume. J. Med. Primatol. 14:345-356.

29. Goldberg SL, Mangan KF, Klumpp TR, Macdonald JS, Thomas C, Mullaney MT, Au FC. 1995. Complications of peripheral blood stem cell harvesting: review of 554 PBSC leukapheresis. J Hematother. 4:85-90.

30. Gothot A, van der Loo JC, Clapp DW, Srour EF. 1998. Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood $CD34^+$ cells in non-obese diabetic/ severe combined immune-deficient mice. Blood 92:2641-2649.

31. Graf T. 2002. Differentiation plasticity of hematopoietc cells. Blood 99:3089-3101.

32. Greenleaf J, Hinhofer-Szalkay H. 1985. Plasma volume methodology: Evans blue, hemoglobin-hematocrit, and mass-density transformations. NASA technical memo 86834. Ames Research Center, Moffett Field, Calif.

33. Gregersen M. 1944. A practical method for the determination of blood volume with dye T-1824. A survey of the present basis of the dye-method and its clinical

applications. J. Lab. Clin. Med. 29:1266-1286.

34. Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M. 2002. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med. 2002. 346:1185-93.

35. Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A. 2003. A Serious Adverse Event after Successful Gene Therapy for X-Linked Severe Combined Immunodeficiency. N Engl J Med. 348:255-256

36. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JI, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M. 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 302:415-9.

37. Halene S, Kohn DB. 2000. Gene therapy using hematopoietic stem cells: sisyphus approaches the crest. Hum Gene Ther. 11:1259-1267.

38. Hanazono Y, Nagashima T, Takatoku M, Shibata H, Ageyama N, Asano T, Ueda Y, Dunbar CE, Kume A, Terao K, Hasegawa M, Ozawa K. 2002. In vivo selective expansion of gene-modified hematopoietic cells in a nonhuman primate model. Gene Ther. 9:1055-1064.

39. Hanazono Y, Terao K, Ozawa K. 2001. Gene transfer into nonhuman primate hematopoietic stem cells: implications for gene therapy. Stem Cells. 19:12–23.

40. Henke M, Laszig R, Rube C, Schafer U, Haase KD, Schilcher B, Mose S, Beer KT, Burger U, Dougherty C, Frommhold H. 2003. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised,

double-blind, placebo-controlled trial. Lancet 362:1255-1260.

41. Hoesel W, Gross J, Moller R, Kanne B, Wessner A, Muller G, Muller A, Gromnica-Ihle E, Fromme M, Bischoff S, Haselbeck A. 2004. Development and evaluation of a new ELISA for the detection and quantification of antierythropoietin antibodies in human sera. J. Immunol. Methods. 294:101-110.

42. Honjo, S. 1985. The Japanese Tsukuba Primate Center for Medical Science (TPC): an outline. J. Med. Primatol. 14:75-89.

43. Honjo S, Cho F, Terao K. 1984. Establishing the cynomolgus monkey as a laboratory animal. Adv. Vet. Sci. Comp. Med. 28:51-80.

44. Jain J, McCaffrey PG, Miner Z, Kerppola TK, Lambert JN, Verdine GL, Curran T, Rao A. 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature 365:352-355.

45. Jeanne M, Bouzgarrou R, Lafarge X, Fizet D, Dazey B, Vezon G, Ivanovic Z. 2003. Comparison of $CD34^+$ cell collection on the CS-3000+ and Amicus blood cell separators. Transfusion. 43:1423-1427.

46. Jones PP, Davy KP, DeSouza CA, et al. 1997. Absence of age related decline in total blood volume in physically active females. Am. J. Physiol. 272 (Heart Circ. Physiol. 41):H2534-H2540.

47. Kajiume T, Kawano Y, Takaue Y, Abe T, Okamoto Y, Makimoto A, Watanabe T, Sato J, Yokobayashi A, Kuroda Y. 1998. Continuous monitoring of hematocrit values during apheresis for allogeneic peripheral blood stem cell collection. J Hematother. 7:493-497.

48. Kamis, A. B., and N. M. Noor. 1981. Blood volume in Macaca fascicularis. Primates 22:281-282.

49. Kawai T, Cosimi AB, Colvin RB, Powelson J, Eason J, Kozlowski T, Sykes M, Monroy R, Tanaka M, Sachs DH. 1995. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. Transplantation. 59:256-262.

50. Kanold J, Halle P, Rapatel C, Berger M, Gembara P, de Lumley L, Vannier JP, Lutz P, Plantaz D, Malpuech G, Demeocq F. 1998. Safe and efficient peripheral blood stem cell collection in the smallest of children. Ther Apher. 2:49-57.

51. Kessinger A, Armitage JO, Smith DM, Landmark JD, Bierman PJ, Weisenburger DD. 1989. High-dose therapy and autologous peripheral blood stem cell transplantation for patients with lymphoma. Blood. 74:1260-1265.

52. Kiem HP, Andrews RG, Morris J, Peterson, L. Heyward S, Allen JM,Rasko JE, Potter J, Miller AD. 1998. Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. Blood 92:1878-1886.

53. Kimikawa M, Sachs DH, Colvin RB, Bartholomew A, Kawai T, Cosimi AB. 1997. Modifications of the conditioning regimen for achieving mixed chimerism and donor-specific tolerance in cynomolgus monkeys. Transplantation. 64:709–716.

54. King FA, Yarbrough CJ, Anderson DC, Gordon TP, Gould KG. 1988. Primates. Science 240:1475-1482.

55. Korbling M, Przepiorka D, Huh YO, Engel H, van Besien K, Giralt S, Andersson B, Kleine HD, Seong D, Deisseroth AB. 1995. Allogeneic blood stem cell transplantation for refractory leukemia and lymphoma: potential advantage of blood over marrow allografts. Blood. 85:1659-1665.

56. Krantz SB. 1999. Erythropoietin. Blood 77:419-434.

57. Kushida T, Inaba M, Ikebukuro K, Nagahama T, Oyaizu H, Lee S, Ito T, Ichioka N, Hisha H, Sugiura K, Miyashima S, Ageyama N, Ono F, Iida H, Ogawa R, Ikehara S. 2000. A new method for bone marrow cell harvesting. Stem Cells. 18: 453-456.

58. Kuramoto K, Follmann DA, Hematti P, Sellers S, Agricola BA, Metzger ME, Donahue RE, von Kalle C, Dunbar CE. 2004. Effect of chronic cytokine therapy on clonal dynamics in nonhuman primates. Blood 103:4070-4077.

59. Lin FK, Lin CH, Lai PH, Browne JK, Egrie JC, Smalling R, Fox GM, Chen KK, Castro M. Suggs S. 1986. Monkey erythropoietin gene: cloning, expression and comparison with the human erythropoietin gene. Gene 44:201-209.

60. McCaffrey PG, Luo C, Kerppola TK, Jain J, Badalian TM, Ho AM, Burgeon E, Lane WS, Lambert JN, Curran T, Verdine GL, Rao A, Hogan PG. 1993. Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science 262:750-754.

61. McClure HM. 1984. Nonhuman primate models for human disease. Adv. Vet. Sci. Comp. Med. 28:267-304.

62. Moog R, Muller N. 1998. Technical aspects and performance in collecting peripheral blood progenitor cells. Ann Hematol. 77:143–147.

63. Nash K, Chang Q, Watts A, Treter S, Oravec G, Ferrara V, Buhler L, Basker M, Gojo S, Sachs DH, White-Scharf M, Down JD, Cooper DK. 1999. Peripheral blood progenitor cell mobilization and leukapheresis in pigs. Lab Anim Sci. 49:645-649.

64. Negrin RS, Atkinson K, Leemhuis T, Hanania E, Juttner C, Tierney K, Hu WW, Johnston LJ, Shizurn JA, Stockerl-Goldstein KE, Blume KG, Weisman IL, Bower S, Baynes R, Dansey R, Karanes C, Peters W, Klein J. 2000. Transplantation of highly purified $CD34^+$ $Thy\text{-}1^+$ hematopoietic stem cells in patients with metastatic breast cancer. Biol Blood Marrow Transplant. 6:262-271.

65. Nolta JA, Dao MA, Wells S, Smogorzewska EM, Kohn DB. 1996. Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice. Proc Natl Acad Sci U S A 93: 2414-2419.

66. Norol F, Merlet P, Isnard R, Sebillon P, Bonnet N, Cailliot C, Carrion C, Ribeiro M, Charlotte F, Pradeau P, Mayol JF, Peinnequin A, Drouet M, Safsafi K, Vernant JP, Herodin F. 2003. Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model. Blood. 102:4361-4368.

67. Persson SG, Funkquist BP, G. Nyman. 1996. Total blood volume in the normally performing standardbred trotter: age and sex variations. J. Vet. Med. Assoc. 43:57-64.

68. Primate Society of Japan. 1986. Guiding principles for animal experiments using nonhuman primates. Primate Res. 2:111-113.

69. Prosser CL, Brown FA. 1961. Comparative animal physiology. W. B. Saunders Co., Philadelphia.

70. Raman A, Colman RJ, Cheng Y, Kemnitz JW, Baum ST, Weindruch R, Schoeller DA. 2005. Reference body composition in adult rhesus monkeys: glucoregulatory and anthropometric indices. J Gerontol A Biol Sci Med Sci. 60:1518-1524

71. Rosenfeld CS. 1993. A strategy for processing of peripheral blood stem cells utilizing the small volume collection chamber and cryopreservation without a rate controller using pentastarch. J Hematother. 2:525-528.

72. Rosenzweig M, Connole M, Glickman R, Yue SPS, Noren B, Demaria M, Johnson RP. 2001. Induction of cytotoxic T lymphocyte and antibody responses to enhanced green fluorescent protein following transplantation of transduced $CD34^+$ hematopoietic cells. Blood. 97:1951–1959.

73. Rosenzweig M, MacVittie TJ, Harper D, Hempel D, Glickman RL, Johnson RP, Farese AM, Whiting-Theobald N, Linton GF, Yamasaki G, Jordan CT, Malech HL. 1999. Efficient and durable gene marking of hematopoietic progenitor cells in nonhuman primates after nonablative conditioning. Blood. 94:2271–2286.

74. Rosenzweig M, Marks DF, DeMaria MA, Connole M, Johnson RP. 2001. Identification of primitive hematopoietic progenitor cells in the rhesus macaque. J Med Primatol. 30:36–45.

75. Rowley SD, Prather K, Bui KT, Appel M, Felt T, Bensinger WI. 1999. Collection of peripheral blood progenitor cells with an automated leukapheresis system. Transfusion. 39:1200-1206.

76. Sans T, Bofil C, Joven J, Cliville X, Simo JM, Llobet X, Pero A, Galbany J. 1996. Effectiveness of very low doses of subcutaneous recombinant human erythropoietin in facilitating autologous blood donation before orthopedic surgery. Transfusion 36:822-826.

77. Seggewiss R, Dunbar CE. A new direction for gene therapy: intrathymic T cell-specific lentiviral gene transfer. J Clin Invest. 2005 ;115:2064-2067.

78. Schmitz N, Dreger P, Suttorp M, Rohwedder EB, Haferlach T, Loffler H, Hunter A, Russell NH. 1995. Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor). Blood. 85:1666-1672.

79. Schuurman HJ, Slingerland W, Mennninger K, Ossevoort M, Hengy JC, Dorobek B, Vonderscher J, Ringers J, Odeh M, Jonker M. 2001. Pharmacokinetics of cyclosporine in monkeys after oral and intramuscular administration: relation to efficiency in kidney allografting. Transpl. Int. 14:320-328.

80. Schwella N, Movassaghi K, Scheding S, Ahrens N, Salama A. 2003. Comparison of two leukapheresis programs for computerized collection of blood progenitor cells on a new cell separator. Transfusion. 43:58-64.

81. Sharabi Y, Sachs DH. 1989. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. J Exp Med. 169:493–502.

82. Shepard CC, Walker LL, Van Landingham RM, Ye SZ. 1982. Sensitization or tolerance to Mycobacterium leprae antigen by route of injection. Infect. Immun. 38:673-680.

83. Shibata H, Hanazono Y, Ageyama N, Nagashima T, Ueda Y, Hasegawa M, Ozawa K, Yoshikawa Y, Terao K. 2003. Collection and analysis of hematopoietic progenitor cells from cynomolgus macaques (*Macaca fascicularis*): assessment of cross-reacting monoclonal antibodies. Am J Primatol. 61:3-12.

84. Smith LG, Weissman IL, Heimfeld S. 1991. Clonal analysis of hematopoietic

stem-cell differentiation in vivo. Proc. Natl. Acad. Sci. USA. 88:2788-2792.

85. Snyder EL, Baril L, Cooper DL, Min K, Mechanic S, Stoddart L, Burtness B, Seagraves P, Debelak J, Gudino M, McCullough J. 2000. In vitro collection and posttransfusion engraftment characteristics of MNCs obtained by using a new separator for autologous PBPC transplantation. Transfusion. 40:961-967.

86. Sohn SK, Kim JG, Chae YS, Kim DH, Lee NY, Suh JS, Lee KB. 2003. Large-volume leukapheresis using femoral venous access for harvesting peripheral blood stem cells with the Fenwal CS 3000 Plus from normal healthy donors: predictors of $CD34^+$ cell yield and collection efficiency. J Clin Apheresis. 18:10-15.

87. Spangrude GJ, Heimfeld S, Weissman IL. 1988. Purification and characterization of mouse hematopoietic stem cells. Science 241:58-62.

88. Srour EF, Zanjani ED, Cornetta K, Traycoff CM, Flake AW, Hedrick M, Brandt JE, Leemhuis T, Hoffman R. 1993. Persistence of human multilineage, self-renewing lymphohematopoietic stem cells in chimeric sheep. Blood 82:3333-3342.

89. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G. 2003. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet. 361:45-46.

90. Tanaka S, Narita H, Ohtoh K, Yoshida T, Cho H, Yoshikawa Y. 1995. Obesity and scrawniness in female cynomolgus monkeys (*Macaca facicularis*): their relation to hematological and serum biochemical properties. J. Growth 34:43-48. (Japanese)

91. Tasaki T, Ohto H, Hashimoto C, Abe R, Saitoh A. Kikuchi S. 1992. Recombinant human erythropoietin for autologous blood donation: effects on perioperative red-blood-cell and serum erythropoietin production. Lancet 339:773-775.

92. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542-545.

93. Terao K, Kobayashi R, Tokairin M, Fujisaki M, Honjo S. 1991. Immune function in aged cynomolgus monkeys. [I] Hemolytic complement activity, natural antibody level, blastogenesis, natural killer activity and phagocytosis, p. 639-640. In E. Akiyoshi (ed.), Primatology today. Elsevier Science Publishers, New York.

94. Thrasher AJ, Hacein-Bey-Abina S, Gaspar HB, Blanche S, Davies EG, Parsley K, Gilmour K, King D, Howe S, Sinclair J, Hue C, Carlier F, von Kalle C, de Saint Basile G, le Deist F, Fischer A, Cavazzana-Calvo M. 2005. Failure of SCID-X1 gene therapy in older patients. Blood. 105:4255-4257.

95. The U. S. Multicenter FK506 Liver Study Group. 1994. A comparison of tacrolimus (FK 506) and cyclosporine for immunosuppression in liver transplantation. N. Engl. J. Med. 331:1110-1115.

96. Thomas ED, Clift RA. 1999. Allogeneic transplantation for chronic myeloid leukemia, p. 807-816. In E. D. Thomas, K. G. Blume, and S. J. Forman (ed.), Hematopoietic cell transplantation. Blackwell Scientific, Malden, Mass.

97. Thomas ED, Storb R, Clift RA, Fefer A, Johnson L, Neiman PE, Lerner KG, Glucksberg H, Buckner CD. 1975 Bone-marrow transplantation. N Engl J Med 292: 832-843.

98. Thorpe R, Swanson SJ. 2005. Assays for detecting and diagnosing antibody-mediated pure red cell aplasia (PRCA): an assessment of available procedures. Nephrol Dial Transplant. Suppl 4:iv16-22. Review.

99. Traynor AE, Schroeder J, Rosa RM, Cheng D, Stefka J, Mujais S, Baker S, Burt RK. 2000. Treatment of severe systemic lupus erythematosus with high-dose chemotherapy and haematopoietic stem-cell transplantation: a phase I study. Lancet 356:701-707.

100. Ueda K, Hanazono Y, Shibata H, Ageyama N, Ueda Y, Ogata S, Tabata T, Nagashima T, Takatoku M, Kume A, Ikehara S, Taniwaki M, Terao K, Hasegawa M, Ozawa K. 2004. High-level in vivo gene marking after gene-modified autologous hematopoietic stem cell transplantation without marrow conditioning in

nonhuman primates. Mol Ther. 10:469-477.

101. Valeri CR, Lindberg JR, Contreras TJ, Pivacek LE, Austin RM, Valeri DA, Gray A, Emerson CP. 1981. Measurement of red blood cell volume, plasma volume, and total blood volume in baboons. Am. J. Vet. Res. 42:1025-1029.

102. VandeBerg JL, Williams-Blangero S. 1997. Advantages and limitations of nonhuman primates as animal models in genetic research on complex diseases. J. Med. Primatol. 26:113-119.

103. Van Beusechem VW, Valerio D. 1996. Gene transfer into hematopoietic stem cells of nonhuman primates. Hum Gene Ther. 7:1649-1668.

104. Wagner JL, Storb R. 1996. Preclinical large animal models for hematopoietic stem cell transplantation. Curr. Opin. Hematol. 3:410-415.

105. Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, Cellona RV, Nazareno JB, Horwitz MA. 1996. The Philippine cynomolgus monkey (*Macaca fascicularis*) provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat. Med. 2:430-436.

106. Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, Goodman M, Bunn HF. 1993. Erythropoietin structure-function relationships: high degree of sequence homology among mammals. Blood 82:1507-1516.

107. Witt V, Fischmeister G, Scharner D, Printz D, Pottschger U, Fritsch G, Gadner H. 2001. Collection efficiencies of MNC subpopulations during autologous $CD34^+$ peripheral blood progenitor cell (PBPC) harvests in small children and adolescents. J Clin Apheresis. 16:161-168.

108. Woloskie S, Armelagos H, Meade JM, Haas D. 2001. Leukodepletion for acute lymphocytic leukemia in a three-week-old infant. J Clin Apheresis. 16:31-32.

109. Xu LC, Karlsson S, Byrne ER, Kluepfel-Stahl S, Kessler SW, Agricola BA, Sellers S, Kirby M, Dunbar CE, Brady RO, Nienhuis AW, Donahue RE. 1995. Long-term in vivo expression of the human glucocerebrosidase gene in nonhuman primates

after $CD34^+$ hematopoietic cell transduction with cell-free retroviral vector preparations. Proc. Natl. Acad. Sci. U. S. A. 92:4372-4376.

110. Yamaguchi-Yamada M, Manabe N, Goto Y, Anan S, Miyamoto K, Miyamoto Y, Nagao M, Yamamoto Y, Ogura A. 2004. Improvement of anemia associated with chronic renal failure by recombinant human erythropoietin treatment in ICR-derived glomerulonephritis (ICGN) mice. J. Vet. Med. Sci. 66:883-886.

111. Ying Q-L, Nichols J, Evans EP, Smith AG. 2002. Changing potency by spontaneous fusion. Nature 416:545-548.

112. Yoshino N, Ami Y, Terao K, Tashiro F, Honda M. 2000. Upgrading of flow cytometric analysis for absolute counts, cytokines and other antigenic molecules of cynomolgus monkeys (*Macaca fascicularis*) by using anti-human cross-reactive antibodies. Exp Anim. 49:97-110.

113. Yoshida T, Cho F, Goto N. 1992. Change of relationship between body measurements and serum biochemical values in growing cynomolgus monkeys (*Macaca fascicularis*); acanonical correlation analysis. J. Growth 31:23-24.

114. Yoshioka T, Ageyama N, Shibata H, Yasu T, Misawa Y, Takeuchi K, Matsui K, Yamamoto K, Terao K, Shimada K, Ikeda U, Ozawa K, Hanazono Y. 2005. Repair of infarcted myocardium mediated by transplanted bone marrow-derived $CD34^+$ stem cells in a nonhuman primate model. Stem Cells. 23:355-364.