stimulated animals. The most active expansion was likely to occur at the progenitor level since an overt response was observed in a few weeks and subsequently subsided. However, a single Tm stimulation resulted in a prolonged elevation of transduced cells. This fact suggests that SAG transduction occurred at more immature stages as well, which later gave rise to more EGFP$^+$ cells following the administration of Tm.

Lineage analysis

As expected from the relatively short-term increase in the frequency of EGFP$^+$ leukocytes upon administration of Tm, the cells that responded were mostly derived from myeloid progenitors. To determine how each hematopoietic lineage responded to Tm, the secondary transplants were subjected to a detailed FACS analysis 4 months post-BMT (i.e. 3 months after Tm stimulation). Peripheral blood leukocytes were gated for granulocytes (Gr1^{high}/Mac1$^+$), monocytes (Gr1$^{low-mid}$/Mac1$^+$), CD4$^+$ T cells (CD4$^+$/CD8$^-$), CD8$^+$ T cells (CD4$^-$/CD8$^+$), B cells (IgM$^+$/B220$^+$) and NK cells (NK1.1$^+$/TCRβ^-), and EGFP$^+$ cells were enumerated in each subset (Figure 4). In the control animals, granulocytes contained slightly higher percentages of EGFP$^+$ cells ($7 \pm 5\%$) than other leukocyte subsets (monocytes $2 \pm 1\%$, CD4$^+$ T cells $1 \pm 1\%$, CD8$^+$ T cells $1 \pm 1\%$, B cells $1 \pm 1\%$, and NK cells $4 \pm 2\%$). This predisposition was eminent in the Tm-challenged mice. These animals had significantly higher percentages of EGFP$^+$ granulocytes ($51 \pm 31\%$) than other cell types (monocytes $7 \pm 4\%$, CD4$^+$ T cells $1 \pm 1\%$, CD8$^+$ T cells $2 \pm 1\%$, B cells $1 \pm 0\%$, and NK cells $6 \pm 2\%$; $p < 0.05$ by Student's t-test), suggesting

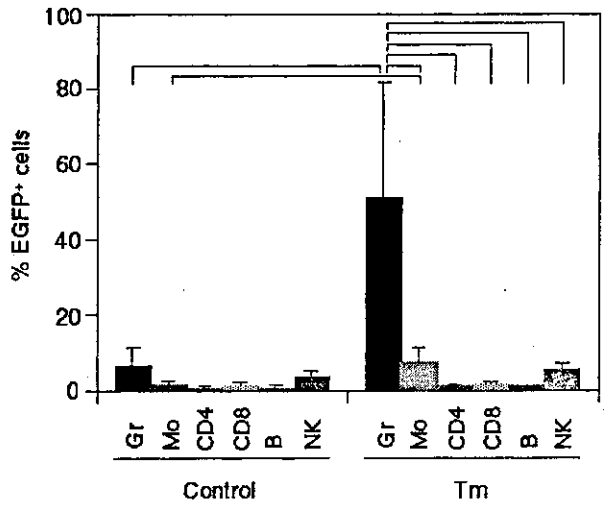

Figure 4. EGFP$^+$ cells in different leukocyte subsets. Three months after the stimulation of secondary transplants with Tm, EGFP$^+$ cells were enumerated in granulocytes (Gr: Gr1^{high}/Mac1$^+$), monocytes (Mo: Gr1$^{low-mid}$/Mac1$^+$), CD4$^+$ T cells (CD4: CD4$^+$/CD8$^-$), CD8$^+$ T cells (CD8: CD4$^-$/CD8$^+$), B cells (B: IgM$^+$/B220$^+$) and NK cells (NK: NK1.1$^+$/TCRβ^-). Tied columns represent statistically different values ($p < 0.05$ by Student's t-test)

that Tm stimulation appeared to induce expansion of SAG-transduced cells in granulocytes most effectively. Moreover, the Tm-challenged mice had more EGFP$^+$ granulocytes and monocytes than the control animals. EGFP$^+$ granulocytes in the challenged mice were $51 \pm 31\%$ (absolute number 1024 ± 274 cells/μl) while that in the controls were $7 \pm 5\%$ (absolute number 183 ± 226 cells/μl), and the difference was significant ($p = 0.03$ for percentage and $p = 0.004$ for absolute number, by Student's t-test). EGFP$^+$ monocytes in the treated group were $7 \pm 4\%$ (48 ± 22 cells/μl) while that in the untreated group were $2 \pm 1\%$ (10 ± 10 cells/μl), and the difference was significant, too ($p = 0.03$ for percentage and $p = 0.02$ for absolute number, by Student's t-test). Thus, Tm administration led to an increase of SAG-transduced granulocytes and monocytes by fivefold in the challenged animals. On the other hand, EGFP$^+$ lymphoid cells (i.e. CD4$^+$ T, CD8$^+$ T, B and NK cells) were not significantly different between these groups, either in percentage (Figure 4) or in absolute number (data not shown). These results showed that the ΔY703FGCRTmR-mediated growth signal was most effective in expanding granulocyte/monocyte precursors in our murine transplant model.

Discussion

In this study, we harnessed mouse BM cells with a modified SAG and examined whether they had an *in vivo* growth advantage under specific stimulation. The SAG was designed for a GCR-based chimeric molecule to generate a growth signal in response to Tm. After hematopoietic reconstitution with the SAG-transduced BM, Tm administration resulted in an increase of transduced cells. Moreover, a Tm administration led to an increase of transduced cells in the secondary transplants, indicating that long-term reconstituting cells were transduced with the SAG and that the transduced cells were readily responsive to the specific stimulus. However, the proliferation of gene-modified cells was relatively short-lived and transduced cell numbers returned to prestimulatory levels. This result suggested that a majority of expanded cells upon Tm stimulation were in the progenitor compartment with a definite proliferative capacity. Still, some SAG-transduced long-term repopulating cells appeared to maintain EGFP$^+$ cells at a higher level than that in the control animals. Recently, we demonstrated a successful expansion of SAG-transduced hematopoietic cells in a nonhuman primate autotransplant model [19]. Although the nature of this kind of experiment allowed us to analyze only a limited number of monkeys without secondary transplantation, a multiclonal expansion was observed following a drug stimulation at 9 months post-transplantation. Taken together, we consider that the feasibility of the SAG system in expanding genetically modified cells *in vivo* was further supported.

The idea of *in vivo* cell expansion by conferring a direct growth advantage has been reported by others, too. Blau and colleagues have developed another chimeric receptor system in which FKBP-based fusion proteins are cross-linked with synthetic dimerizer compounds. They discussed the signal specificity of three different growth factor receptors (wild-type GCR, Mpl and Flt3) and found that only the Mpl-mediated signal enhanced the self-renewal of murine multipotential hematopoietic progenitors [20]. With a fusion of the Mpl cytoplasmic domain and FKBP, they expanded murine hematopoietic cells *in vivo* [21]. It is of note that this expansion was also transient, suggesting that the major responders were progenitors, too. In addition, the Mpl-mediated signal preferentially led to erythroid, and slightly less efficiently megakaryocytic, expansion, similar to our finding that ΔY703FGCRTmR-mediated expansion preferentially occurred in granulocytes.

These results may suggest that hematopoietic growth factor receptor signaling is not free from lineage specificity and biased expansion. On the other hand, many studies on transgenic animals and primary culture systems have shown that the cytokine receptor-mediated signals are permissive (i.e. not confined to a single lineage), and supported a stochastic model of hematopoiesis [22]. The reason for the apparent discrepancy between these reports and the lineage-specific expansion observed in our study is currently unknown. We are now constructing another switching system to activate the GCR-mediated signal to see whether a granulocyte-specific expansion occurs. If this is indeed the case, incorporation of the GCR-based SAGs may be suitable for the treatment of disorders involving this lineage, such as chronic granulomatous disease.

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Modification of the Leukapheresis Procedure for Use in Rhesus Monkeys (*Macaca mulata*)

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One of the most serious problems in applying leukapheresis to human infants is the large extracorporeal blood volume (ECV), resulting in substantial loss of platelets and red blood cells (RBCs). In this study, we developed a safe and effective modified procedure to collect peripheral blood stem cells (PBSCs) from rhesus monkeys (*Macaca mulata*) using a Baxter CS3000+ Blood Cell Separator (Baxter, Deerfield, IL) with several devices that reduced chamber size and shortened the standard apheresis kit to decrease ECV from 130 to 70 ml. Pump speed was controlled by monitoring hematocrit values and platelet counts during leukapheresis. This system makes it possible to perform safe and effective leukapheresis in rhesus monkeys whose body weight 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 white blood cells (mean, 1.38×10^9 cells/kg), $CD34^+$ cells (mean, 17.80×10^6 cells/kg), mononuclear cells (mean, 3.67×10^8 cells/kg), and colony forming units (mean, 75.02×10^6 cells/kg) in all cases. In addition, no complications, such as anemia or trombocytopenia, occurred after leukapheresis. This modified leukapheresis procedure will be useful to test new approaches in gene therapy, perform organ transplantation using nonhuman primates, and collect PBSCs from human infants in a noninvasive manner. Our nonhuman primate model provides an important framework for such future clinical studies. J. Clin. Apheresis 18:26–31, 2003.

Key words: PBSCs; extracorporeal blood volume; nonhuman primates

INTRODUCTION

Peripheral blood stem cell (PBSC) transplantation has been increasingly used clinically as an alternative to allogeneic bone marrow transplantation [1,2]. The PBSCs and bone marrow (BM) stem cells are prominent targets for stem cell gene therapy [3–6] and recently have been used for induction of immune tolerance in organ transplantations [7–10]. Although numerous clinical trials have demonstrated the safety and effectiveness of leukapheresis for harvesting PBSCs from adults [11] and children [12–14], only one limited trial in human newborn babies has been reported [15]. 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 red blood cells (RBCs).

The close phylogenetic relationship of macaque monkeys to humans has resulted in their widespread use as a preclinical model for BM transplantation and hematopoietic stem cell (HSC) gene therapy [3,5,6]. The safety and therapeutic efficacy of PBSCs and BM transplantation have been evaluated prior to human trials using monkey models. Since a large ECV frequently causes severe anemia and trombocytopenia and a rapid reduction in hematocrit (Ht) level during leukapheresis, which poses a risk for the patient, it is necessary to evaluate the safety and efficacy of leukapheresis using nonhuman primates as a model for human infants.

In this study, we modified a standard procedure for the collection of PBSCs from rhesus monkeys (*Macaca mulata*) using a blood cell separator (Fenwall CS3000+; Baxter, Deerfield, IL) adapted for a

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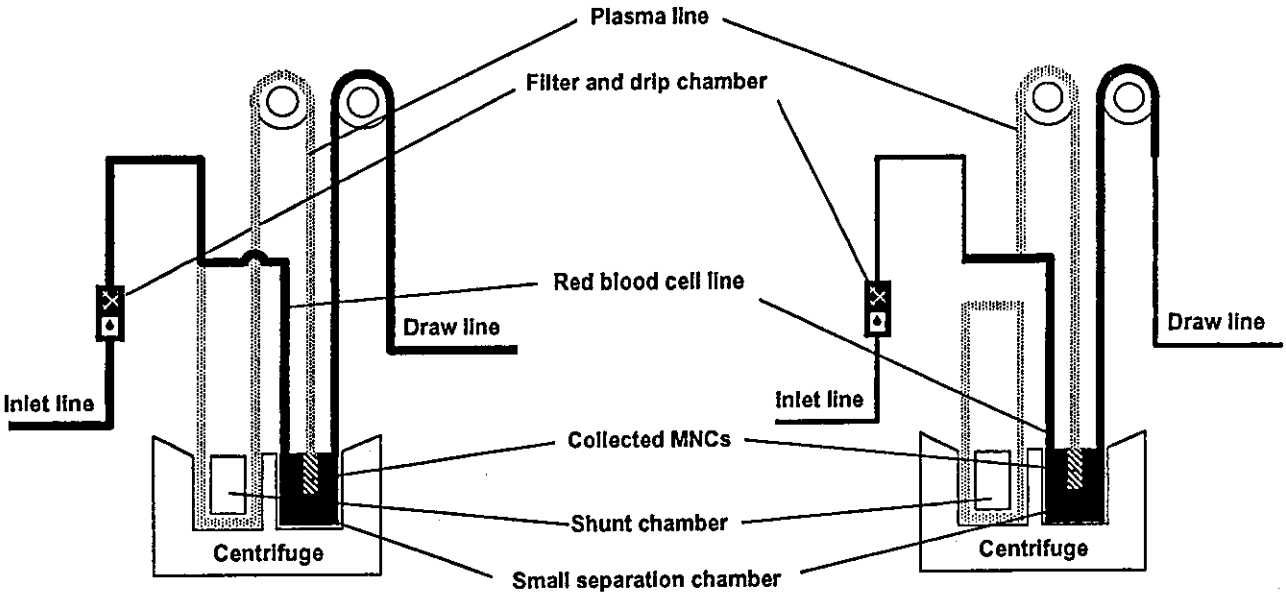

Fig. 1. Diagram of apheresis kit before and after modification. Modifications included the use of a small S25A separation chamber and the shortening of the standard apheresis kit. The plasma line (shadow line) was cut and connected under sterile conditions to the red blood cell line (solid line) using 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 extracorporeal blood volume as much as possible. This modification made it possible to reduce the volume of extracorporeal circulation from 130 to 70 ml.

small chamber and short apheresis kit as shown in Figure 1. This modified procedure made it possible to reduce ECV and perform safe and effective leukapheresis with monkeys whose body weight is similar to that of human infants. This study provides information on the modified leukapheresis process for those investigators who want to perform leukapheresis in neonates. This nonhuman primate model will be useful to test new approaches for PBSCs transplantation, HSC gene therapy, and organ transplantation.

MATERIALS AND METHODS

A total of 12 leukapheresis procedures were performed in nine male rhesus monkeys, aged 3 to 6 years, with a mass of 4.2 to 8.5 kg, which is a range that includes the mass of many human newborns. Three of the monkeys (nos. 099036, 000033, and 099030) underwent leukapheresis twice over two consecutive days.

Animals and Husbandry

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 (TPC) of the National Institute of Infectious Diseases. They were housed individually in stainless steel cages at a temperature of 23–27°C and humidity level between 50 and 70%. There were 12 air changes per hour in the room and the light/dark cycle was 12 h/12 h. Each day, the animals were fed 70 g commercial monkey chow (Type AS; Oriental Yeast, Chiba, Japan), 200 g of fruit, and tap water ad libitum. They were apparently healthy and showed no abnormal sign in a periodic health check.

This study was strictly subject to the Rules for Animal Care and Management of TPC [16] and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [17]. The protocol of the experimental procedure was approved by the National Institute of Infectious Diseases (Tokyo, Japan).

Preparative Regimen

Fifty micrograms/kilogram 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 2 weeks preceding leukapheresis. Autologous blood (20–25 ml) was collected once a week 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 [18]. To provide vascular access for leukapheresis, the right or left femoral artery was cannulated with a 19-gauge catheter. The saphenous vein was catheterized with a 19-gauge intracath. This cannulation was performed under general anesthesia by administration of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Seraktar; Bayer, Leverkusen, Germany). Animals received 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. [3]. All procedures were performed under general anesthesia (A.D.S.1000; Shin-ei, Tokyo, Japan) with isoflurane gas. Collection was accomplished using a small S25A separation chamber and a shunt chamber in place of a standard collection chamber. For the purpose of reducing extracorporeal blood volume, the plasma line of a standard apheresis kit was cut and connected under sterile conditions to the red blood cell line using a polypropylene tubing connector (Iuchi, Osaka, Japan) (Fig. 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, 1.4 ml, 2.5 mm diameter; TOP, Tokyo, Japan) to reduce extracorporeal blood volume as much as possible. A blood component inlet set with a 170-μm filter and drip chamber was sterilely connected to the packed red blood cell 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-citrate-dextrose (ACD) line. The apheresis kit was primed with autologous blood that had been collected 3 weeks before leukapheresis. The animal received a dose of 100 U/kg heparin 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 the total blood volume of the 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 [19]. 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 reinfuse into the treated animal. Immediately after leukapheresis, the animals were given an appropriate dose of protamine sulfate.

Analyses of Leukapheresis Products

The leukapheresis products were collected in a small S25A separation chamber. The product (40–45 ml) was collected and mixed with 7 ml of ACD. The numbers of recovered white blood cells (WBCs) and mononuclear cells (MNCs) were counted with a Sysmex K-4500 instrument (Toa-iyoudenshi, Kobe, Japan). The blood cells were collected after centrifugation at 1,200 rpm for 10 min and treated with ammonium chloride for lysis of erythrocytes. The $CD34^+$ cells were then isolated as a fraction containing HSCs with immunomagnetic beads (Dynal, Lake Success, NY) conjugated to a monoclonal antibody (clone 561) that reacts to both human and monkey CD34 [20,21]. CD34 is a cell-surface marker of undifferentiated HSCs, and $CD34^+$ cell transplantation is widely performed in patients with cancer or other disorders [22]. The purity of the $CD34^+$ cells obtained using this technique ranged from 90 to 95%, a value similar to that reported by other investigators [23]. Progenitor cell enrichment was assessed from colony-forming progenitor assays performed before enrichment. Cells were suspended in α-minimum essential medium (MEM; Gibco) containing 1.2% methylcellulose (Shinetsu Kagaku, Tokyo, Japan) supplemented with 2 U/ml recombinant human erythropoietin (EPO; Roche Diagnostics, Mannheim, Germany), 100 ng/ml recombinant human interleukin-3 (PeproTech, London, U.K.), 100 ng/ml interleukin-11 (IL-11; PeproTech), 100 ng/ml recombinant human stem cell factor (SCF; Biosource, Camarillo, CA), 20% fetal calf serum (FCS; Intergen, Purchase, NY), 1% bovine serum albumin (Sigma, St. Louis, MO), 5 × 10-5M 2-mercaptethanol (Sigma), and antibiotics (100 U/ml penicillin, Banyu, and 0.1 mg/ml streptomycin, Meiji Seika). On day 14 of the culture, colonies containing more than 50 cells were counted as colony-forming units (CFUs). The total numbers of WBCs, MNCs, $CD34^+$ cells, and

TABLE I. Characteristics of Rhesus Monkeys Subjected in Leukapheresis

			Processed blood		Harvested cells			
Animal ID	Body weight (kg)	Estimated total blood volume (ml)[a]	Total volume (ml)	ml/kg	Total nucleated cells ($\times 10^9$/kg)	MNCs ($\times 10^8$/kg)	$CD34^+$ cells ($\times 10^6$/kg)	CFU ($\times 10^6$/kg)
099033	4.9	310	1,000	200	2.99	10.45	27.17	—
099038	4.9	300	1,000	210	1.41	10.17	26.44	98.97
000026	7.5	420	1,600	210	1.12	1.90	—	—
000030	7.3	410	1,600	220	0.94	1.69	—	—
000031	8.5	470	1,300	150	0.47	1.66	—	—
000029	6.5	380	1,000	150	2.21	1.46	—	—
099036[b]	4.5	290	1,000	220	1.68	0.55	1.44	14.44
099036[b]	4.2	270	900	210	1.29	6.22	16.16	247.96
000033[b]	7.4	420	1,150	150	0.83	2.10	—	9.57
000033[b]	7.4	420	1,150	150	0.75	1.13	—	4.15
099030[b]	4.3	400	1,050	150	0.89	3.78	—	13.75
099030[b]	4.3	400	1,100	160	1.61	4.06	—	7.19
Average	6.5	380	1,200	180	1.38	3.67	17.80	75.02

[a]The total blood volume was estimated by the following formula: Y (total blood volume) = 44.07 × (body weight) + 90.25 [Ref. 26].
[b]Leukapheresis was performed in these three animals on two consecutive days.

CFUs in the apheresis products were calculated by multiplying the percentage of lymphocytes, $CD34^+$ cells, and CFUs by the total blood cell count in the leukapheresis products.

RESULTS

We administered G-CSF to monkeys for 5 days prior to leukapheresis. Administration of G-CSF increased peripheral WBC counts to 42,000 (24,000–66,000) cells/ml 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 [24,25]. 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, we 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 products 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 adverse effects such as blood pressure reduction, and sufficient numbers of $CD34^+$ cells, MNCs, and CFUs 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 I, mean processed blood volume was 1,200 ml (180 ml/kg), which was approximately three times the estimated total blood volume of an individual animal [26].

No complications, such as severe anemia or trombocytopenia, developed in the three monkeys that underwent leukapheresis on two consecutive days. Serial changes in circulating WBC counts, 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. In contrast, WBC counts decreased to below the target value in all monkeys before leukapheresis was completed.

No viral or fungal organisms were isolated from culture of the leukapheresis products after cutting and sterile docking of the tube, and no microbial contamination occurred in colony-forming progenitor assays. In addition, we performed successful transplantations using these leukapheresis products without any complications such as the development of infectious disease (data not shown).

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. We succeeded in reducing ECV, but still needed autologous blood for priming. However, reduction of the ECV allowed priming with autologous blood in these nonhuman primates. Therefore, the modified procedure enables safe and effective leukapheresis in nonhuman primates and other small animals. Sufficient numbers of $CD34^+$ cells, MNCs, and CFUs could be collected for stem cell transplantation as shown in Table I. In addition, an adequate number of cells were also collected from monkeys (Monkey nos. 099036, 000033, and 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 nonhuman primates. The results clearly indicate that this modification enables the safe and effective application of the blood cell separator for leukapheresis in rhesus monkeys. Although the minimum blood volume (150 ml/kg) was processed in leukapheresis, sufficient numbers of cells were harvested without complications in either the animals or 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, indicating that this procedure allows the collection of PBSCs with negligible contamination of platelets and RBCs in the apheresis products. The decrease in Ht value observed after leukapheresis was a complication of the cannulation operation, but did not necessitate treatment with a transfusion. Also, Ht and platelet counts fell transiently during leukapheresis but a blood transfusion was not necessary.

Although clinical trials in humans using this modified procedure would be difficult, it is clear that our modified procedure makes it possible to perform safe and effective leukapheresis in nonhuman primates or other small animals. This animal model will be useful for testing new approaches to PBSC transplantation, HSC gene therapy, and organ transplantation. Despite the need to modify the tube each time in a clinical setting, we believe this modification will be helpful to researchers who may need a noninvasive method of collecting PBSCs from human newborn infants [27,28]. We are now applying this modified leukapheresis procedure to juvenile cynomolgus monkeys (*Macaca fascicularis*) that weighed approximately 2 kg, a mass equivalent to that of a human newborn infant. Our nonhuman primate model will provide an important framework for such future clinical studies.

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Molecular cloning, functional characterization, and enzyme-linked immunosorbent assay of cynomolgus monkey Fas ligand

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Abstract

Fas ligand (FasL) cDNAs were cloned and sequenced from cynomolgus, rhesus, and pig-tailed monkeys. The 840-bp cDNAs were identical among these three species of monkeys except for one nucleotide. The deduced 280 amino acids were completely identical and displayed 97% homology with human FasL (hFasL). Recombinant soluble FasL obtained from COS cells transfected with cynomolgus monkey FasL (cm-FasL) cDNA induced apoptosis in cells displaying human or cynomolgus monkey Fas-expressing cells. Several anti-human FasL monoclonal antibodies (mAbs) were able to neutralize the cytotoxic activity of monkey FasL, and a combination of mAbs was selected to obtain the most sensitive detection of monkey soluble FasL (sFasL) under sandwich enzyme-linked immunosorbent assay (ELISA). Plasma from normal monkey did not contain detectable levels of sFasL, whereas plasma from monkeys acutely infected with simian immunodeficiency virus (SIV) displayed increased levels of sFasL.

Keywords: Nonhuman primates; Fas; Fas ligand; Molecular cloning; Apoptosis; ELISA

1. Introduction

Fas ligand (FasL) belongs to the tumor necrosis factor (TNF) family that includes TNF-α, lymphotoxin, TNF-related apoptosis-inducing ligand (TRAIL), CD40 ligand, CD27 ligand, CD30 ligand, and OX40 ligand (Nagata, 1997). Most members of the TNF family are type II membrane proteins, with the exception of lymphotoxin-α. FasL induces apoptotic cell death by binding to its receptor, Fas (also called APO-1 or CD95), which is a member of the TNF receptor family (Nagata and Golstein, 1995).

Abbreviations: mAb, monoclonal antibody; FasL, Fas ligand; sFasL, soluble FasL; ELISA, enzyme-linked immunosorbent assay; cm-FasL, cynomolgus monkey FasL; hFasL, human FasL; MMP, matrix metalloproteinase; SIV, simian immunodeficiency virus; RACE, rapid amplification of cDNA ends; HSCF, CD4-positive T cell line immortalized by *Herpesvirus saimiri*; PHA, phytohemagglutinin.

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FasL is predominantly expressed in activated T and NK cells (Tanaka et al., 1996; Arase et al., 1995), while Fas is ubiquitously expressed in various cells (French et al., 1995; Watanabe-Fukunaga et al., 1992). FasL-mediated cell death is involved in T or NK cell-mediated cytotoxicity and regulation of lymphocyte homeostasis (Nagata and Golstein, 1995). FasL-induced apoptosis has also been implicated in diseases such as hepatitis (Kondo et al., 1997), insulin-dependent diabetes (Chervonsky et al., 1997), and human immunodeficiency virus (HIV)-induced acquired immunodeficiency syndrome (AIDS) (Hosaka et al., 1998). FasL is also expressed in the testis (Bellgrau et al., 1995), eye (Griffith et al., 1995), and some malignant tumor cells (Hahne et al., 1996; Strand et al., 1996), and has been proposed as a mediator of immune privilege in such tissues to kill invading Fas-expressing effector cells.

A soluble form of FasL (sFasL) can be released from human FasL (hFasL) transfectants and induces apoptosis in Fas-expressing cells, indicating that hFasL can be processed from the membrane, like TNF-α (Tanaka et al., 1995; Kayagaki et al., 1995). Since matrix metalloproteinase (MMP) inhibitors block the processing of both FasL and TNF-α, MMP-like enzymes are considered responsible for processing membrane-bound FasL (Tanaka et al., 1996; Kayagaki et al., 1995) and TNF-α (Gearing et al., 1994; McGeehan et al., 1994). Recently, ADAM17 (TNF-α converting enzyme) and ADAM10, belonging to the ADAM (a disintegrin and metalloproteinase) family, have been shown to specifically process TNF-α (Black et al., 1997; Moss et al., 1997; Rosendahl et al., 1997). The processing enzyme for FasL has remained unidentified. Enzyme-linked immunosorbent assay (ELISA) systems for measuring human sFasL have already been established using anti-human FasL monoclonal antibodies (mAbs) (Tanaka et al., 1996; Kayagaki et al., 1995), and have been applied to detect sFasL levels in clinical samples. Elevated levels of sFasL have been reported in sera from patients with large granular lymphocytic leukemias and NK cell lymphomas (Tanaka et al., 1996), asymptomatic stage HIV infection (Bahr et al., 1997), and aggressive nasal lymphoma (Sato et al., 1996). These results have provided useful information in elucidating the role of sFasL and FasL/Fas-mediated apoptosis in human immune diseases.

No sensitive ELISA system has been available for detecting sFasL in nonhuman primates, although nonhuman primates serve as valuable models for studying (1) the efficacy of various vaccines and drugs against infectious diseases, including AIDS; (2) the pathogenic mechanisms of numerous infectious diseases; and (3) the biology of allogeneic and xenogeneic organ and tissue transplantation (King et al., 1988; Wolf et al., 1985; Letvin et al., 1985; Meisenberg et al., 1992; van Beusechem et al., 1992; Nakajima et al., 1995).

In this context, we cloned monkey FasL cDNA, generated monkey FasL transfectants, purified recombinant monkey sFasL, and finally established a sensitive ELISA system for monkey sFasL. Recombinant monkey sFasL was functional in inducing apoptosis in human and monkey Fas-expressing cells and the newly established ELISA system was sensitive to picogram concentrations of sFasL. This system allows increasing levels of sFasL in plasma to be monitored in cynomolgus monkeys infected with simian immunodeficiency virus (SIV).

2. Materials and methods

2.1. Chemical

Synthesis of the MMP inhibitor KB-R8301 ([4-(*N*-hydroxyamino)-2R-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphtyl) alanine-*N*-methylamide) was performed as previously described (Yamamoto et al., 1998).

2.2. Cloning and expression of monkey FasL cDNA

Peripheral blood was obtained from healthy cynomolgus, rhesus, and pig-tailed monkeys. Peripheral blood lymphocytes (PBL) were isolated by centrifugation on Ficoll–Pauque solution (Amersham Pharmacia Biotech, Buckinghamshire, UK) and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) containing 5 μg/ml of concanavalin A (ConA) (Sigma, St. Louis, MO) and 60 U/ml of human IL-2 (Invitrogen, Carlsbad, CA) for 7 days. ConA-activated T cell blasts were finally stimulated with 10 ng/ml phorbol myristic acetate (Sigma) and 500 ng/ml ionomycin (Sigma) for 4 h. Poly(A)$^+$

RNA was prepared using an mRNA isolation kit (Amersham Pharmacia Biotech). Single-strand cDNA was synthesized using either random hexamer oligonucleotides or oligo (dT)12-18 primer as primers in a cDNA synthesis kit (Invitrogen). Monkey FasL cDNA was amplified by PCR using an oligonucleotide corresponding to the first six codons as the sense primer and an oligonucleotide corresponding to the last six codons as the antisense primer, according to the published human FasL (hFasL) sequence (Takahashi et al., 1994). Sense (GGCCTCGAGAGATGCAGCAGCCCTTCAATTAC) and antisense (CGAGCGGCCGCTTAGAGCTTAGAGCTTATATAAGCCGAA) primers were tagged with a *Xho*I or a *Not*I site, respectively. The reaction mixture for PCR contained 10 pmol each of the sense and antisense primers and the reaction was initiated with the addition of 2.5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Conditions for PCR were 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C for 40 cycles. After *Xho*I and *Not*I digestion, PCR product was subcloned into pBluscriptII SK+ (Stratagene). Three independent clones from each species of monkey were sequenced using an ALFII DNA sequencer (Amersham Pharmacia Biotech). To determine the monkey FasL sequence in the region corresponding to the sense and antisense primers for hFasL, cDNA was synthesized from monkey poly(A)$^+$ RNA using a rapid amplification of cDNA ends (RACE) kit (Clontech, Palo Alto, CA) according to the manufacturer's instruction. Amplified 5′ and 3′ RACE cDNAs were subcloned into PCR-Script SK+ (Stratagene) and three independent clones were sequenced. Sequenced cynomolgus monkey FasL (cm-FasL) cDNA was then transferred into *Xho*I and *Not*I sites of the CDM8 (Invitrogen) expression vector. Transient expression of monkey FasL cDNA (cm-FasL/CDM8) in COS cells was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Culture supernatant containing soluble FasL (sFasL) was collected after 5 days. Transient expression of hFasL cDNA was also performed using hFasL/CDM8 (Kayagaki et al., 1995) in the same manner.

2.3. Cytotoxic assay

Cytotoxic activity of sFasL in culture supernatants was tested against human Fas transfected WR19L (hFas/WR19L) using alamar Blue (Alamar Biosciences, Sacramento, CA) as described previously (Kayagaki et al., 1995). Briefly, 4×10^4 hFas/WR19L or WR19L cells were cultured with serially diluted culture supernatant of cm-FasL/COS or hFasL/COS cells in a total volume of 200 μl. After 16 h, 10 μl of alamar Blue was added and further incubated for 4 h. Fluorescence of the reduced alamar Blue was measured on a fluoroscan (MTP-32; Corona Electric, Tokyo, Japan) at 590 nm by excitation at 544 nm. To compare sensitivity to FasL-induced apoptosis, cynomolgus monkey T cell blasts activated by phytohemagglutinin (PHA), the CD4-positive T cell line immotalized by *Herpesvirus saimiri* (HSCF) CD4-positive T cell line immortalized by *H. saimiri* (Akari et al., 1996), and hFas/WR19L cells were treated with serially diluted cynomolgus monkey soluble FasL (cm-sFasL). Cell viability was determined using the alamar Blue method. For selecting anti-human FasL mAbs that cross-react with monkey sFasL, two mAbs, NOK-2 (mouse IgG2a, κ) and NOK-3 (mouse IgM, κ), generated previously (Kayagaki et al., 1995) and two mAbs (4A5 and 4H9, hamster IgG) purchased from MBL (Nagoya, Japan) were screened. In a neutralization test, serially diluted mAbs were added to the mixture of hFas/WR19L cells (4×10^4 cells) and cm-FasL/COS supernatant and cell viability was determined using the alamar Blue method.

2.4. Western blotting

Supernatant of COS cells transfected with cm-FasL/COS or hFasL/COS was heated in 1 × SDS buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM dithiothreitol). Samples were electrophoresed on 10–15% gradient polyacrylamide gel. Proteins were then transferred to PVDF membranes (Millipore, Bedford, MA). After blocking in PBS containing skimmed milk solution (Block Ace; Snow Brand Milk, Sapporo, Japan), the membrane was incubated for 1 h at room temperature with 200-fold diluted anti-human FasL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS supplemented with 25% Block Ace. After washing three times with PBS containing 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with 1000-fold diluted peroxidase-conjugated anti-rabbit IgG (Jack-

son ImmunoResearch Laboratories). The membrane was washed three times with PBS containing 0.05% Tween 20 and the proteins recognized by the antibody were visualized using the ECL system (Amersham Pharmacia Biotech).

2.5. Establishment of ELISA for monkey sFasL

To establish the sandwich ELISA for monkey sFasL, various combinations of capture antibody and detection antibody were set using mAbs cross-reacting with cm-sFasL. Immulon 600 (Greiner Japan, Tokyo) plates were incubated overnight with 100 μl/well of capture mAb (10 μg/ml). After washing with PBS containing 0.05% Tween-20 (PBS–Tween), wells were blocked by 200 μl/well of PBS with 10% Block Ace for 1 h at 37 °C. Following the removal of 10% Block Ace, 50 μl of the sample was added and incubated for 1 h at room temperature. After washing with PBS–Tween, 100 μl/well of biotinylated detection mAb (5 μg/ml) was added and the plate was incubated for 1 h at room temperature. Plates were washed with PBS–Tween, and 50 μl of 1:1000 diluted streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech) was then added and incubated for 30 min. After washing with PBS–Tween, plates were developed with 100 μl of 1 mg/ml orthophenylene diamine in 50 mM citrate-phosphate buffer (pH 5.0) containing 0.03% H_2O_2, and stopped using 100 μl of 2N H_2SO_4. Optical density (OD) at 490 nm was measured using an automatic ELISA reader (Thermo Max; Molecular Devices, Sunnyvale, CA). The cm-sFasL was subsequently purified from the supernatant of cm-FasL/COS cells using an anti-hFasL (NOK-2) affinity column, and serial dilutions of purified cm-sFasL were used as the standard.

To evaluate the blocking ability of KB-R8301 for cm-FasL processing from COS cells transfectant, cm-FasL/COS or hFasL/COS cells (5×10^5) were cultured with or without KB-R8301 (from 0.625 to 10 μM) for 24 h and sFasL in the supernatant was determined by ELISA as described above.

2.6. Measuring sFasL levels in monkey plasma

To measure concentrations of sFasL in monkey plasma, plasma samples were obtained from normal monkeys and SIVmac239-infected monkeys. These monkeys were bred in the Tsukuba Primate Center of the National Institute of Infectious Diseases, and were serologically negative for B virus, simian T cell leukemia virus-type I, and simian-type D retrovirus. The plasma was obtained from two male monkeys aged 5 years, immediately before and 10 and 17 days after intravenous inoculation of 100 tissue culture infectious doses ($TCID_{50}$) of a pathogenic clone, SIVmac239 (Akari et al., 1998). Plasma was separated by centrifuge and stored at −80 °C until use. The sFasL levels were determined by the ELISA method using 4H9 for capture and biotinylated 4A5 for detection.

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Monkey  MQQPFNYPYP QIYWVDSSAS SPWAPPGTVL PCPTSVPRRP GQRR-PPPPP   49
Human   .......... .......... .......... .......... ....P.....   50

Monkey  PPPPLPPPPP SPLPPLPLPP LKKRGNHSTG LCLLVMFFMV LVALVGLGLG   99
Human   ...LP..... P......... .......... .......... ..........  100

Monkey  MFQLFHLQKE LAELRESTSQ KHTASSLEKQ IGHPSPPPEK KEQRKVAHLT  149
Human   .......... .......... M......... .......... ..L.......  150

Monkey  GKPNSRSMPL EWEDTYGIVL LSGVKYKKGG LVINETGLYF VYSKVYFRGQ  199
Human   ..S....... .......... .......... .......... ..........  200

Monkey  SCTNLPLSHK VYMRNSKYPQ DLVMMEGKMM SYCTTGQMWA HSSYLGAVFN  249
Human   ..N....... .......... .......... .......... R.........  200

Monkey  LTSADHLYVN VSELSLVNFE ESQTFFGLYK L  280
Human   .......... .......... .......... .  281
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Fig. 1. Comparison of amino acid sequences for monkey and human Fas ligands. Identities are indicated by dot plot. The putative transmembrane domain is underlined. The amino acids at the position for hFasL processing are indicated by asterisks.

3. Results and discussion

3.1. Cloning of monkey FasL cDNA

The nucleotide sequences of cynomolgus, rhesus, and pig-tailed monkeys FasL have been deposited to DDBJ under accession numbers AB035138, AB035139, and AB035140, respectively. Monkey FasL cDNA nucleotide sequences among these three species of macaque monkeys were almost identical except for one nucleotide change at nucleotide position 522 (cynomolgus: G; rhesus: A; pig-tailed: A), a silent change which does not change the encoding amino acid. The monkey FasL cDNA sequence contained a long open reading frame (ORF) of 840 bp and displayed 97% homology to human FasL. The ORF codes a polypeptide comprising 280 amino acids, and is completely identical for all three species. The predicted amino acid sequence of monkey FasL was one amino acid shorter than human FasL, with a calculated Mr of 31 kDa. As with human FasL (Takahashi et al., 1994), monkey FasL is a type II membrane protein, with high homology to human FasL. An alignment of the amino acid sequences of monkey and human FasL is shown in Fig. 1. Eight amino acid changes (five in extracellular domains and three in intracellular domains) and one amino acid deletion (position 45) are present in monkey FasL compared to human FasL. Compared to the previously reported rhesus FasL cDNA (Wang et al., 1998), six nucleotide changes (our G^{150}, A^{192}, G^{218}, C^{459}, T^{825}, C^{829} vs. their A^{150}, G^{192}, A^{218}, T^{459}, C^{825}, T^{829}) and one amino acid change (our R^{43} vs. their K^{43}) were identified. This may be attributable to polymorphism. Conservation of these residues among the other two species of monkeys and human suggests a more likely sequence of G^{150}, A^{192}, G^{218}, C^{459}, T^{825}, C^{829} in the rhesus monkey, as determined in the present study.

3.2. Functional characterization of monkey FasL

Since FasL cDNA sequences of three species of macaques were identical, cynomolgus monkey FasL (cm-FasL) cDNA was inserted into a mammalian expression vector CDM8 and transiently transfected into COS cells as a representative. Recent studies have reported the presence of a functional soluble form of human FasL (Tanaka et al., 1995; Kayagaki et al., 1995). We therefore collected supernatant from the cm-FasL/COS cell culture at 5 days after transient transfection, and examined cytotoxicity against the human Fas-expressing cell line, hFas/WR19L. Both cm-FasL/COS cell supernatant and hFasL/COS cell supernatant exhibited specific cytotoxicity against hFas/WR19L cells in a dose-dependant manner (Fig. 2A). This result indicated that cm-FasL/COS cells could produce functional monkey recombinant FasL that might undergo processing

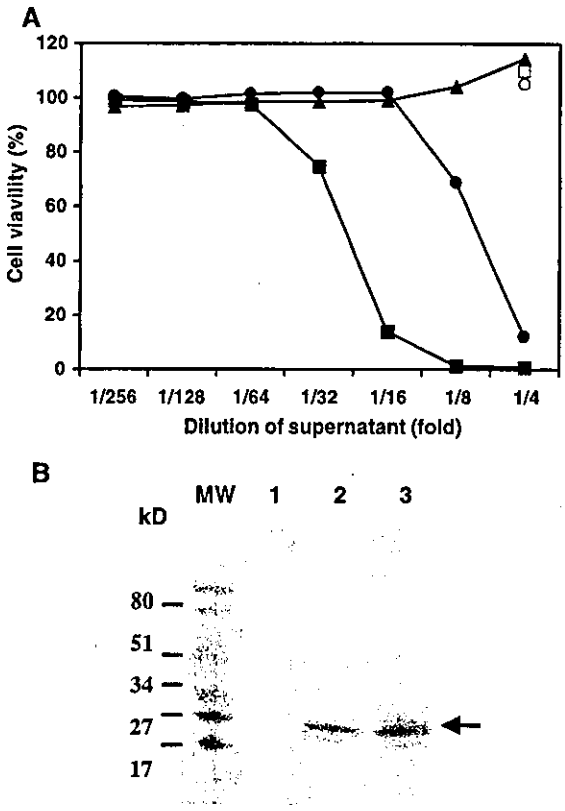

Fig. 2. Release of functional sFasL from cm-FasL-transfected COS cells. (A) Cytotoxic activity of sFasL in supernatant of vector (▲), cm-FasL (■, □), or hFasL (●, ○)-transfected COS cells was tested against hFas/WR19L cells (■, ●) or WR19L cells (□, ○) according to the alamar Blue method. (B) Western blotting. Supernatant (10 μl) from vector-transfected (Lane1), cm-FasL (Lane2), or hFasL (Lane3)-transfected COS cells was analyzed using anti-hFasL antibody as described in the Materials and methods, heated in SDS sample buffer and resolved by electrophoresis on a 10–15% gradient polyacrylamide gel. After blotting to a PVDF membrane, sFasL was detected using ECL system. The arrow indicates the band corresponding to monkey sFasL protein.

from membrane-bound FasL. Recently, Schneider et al. (1998) reported the cleavage site of hFasL, located between Ser 126 and Leu 127 in hFasL protein. As shown in Fig. 1, monkey FasL protein displayed the same amino acids surrounding the cleavage site of hFasL protein. We observed a 27-kDa protein in the supernatant of cm-FasL/COS cells as well as of hFasL/COS cells by Western blotting using anti-hFasL polyclonal Ab (Fig. 2B). These results suggest that monkey FasL can be processed to create a functional soluble form (sFasL) like human FasL. As shown in Fig. 3, monkey sFasL also exhibited potent cytotoxicity against Fas-expressing cynomolgus monkey T cell blasts and the HSCF cell line (Akari et al., 1996).

3.3. Characterization of anti-human FasL mAbs against monkey FasL

Cross-reactivity of four different anti-hFasL mAbs (NOK-2, NOK-3, 4A5, and 4H9) was characterized with cm-sFasL on the basis of neutralization of cm-sFasL cytotoxicity against hFas/WR19L cells. In these mAbs, four mAbs, NOK-3 (mouse IgM, κ), 4A5 (hamster IgG), and 4H9 (hamster IgG), neutralized the cytotoxic activity of cm-sFasL in a dose-dependent manner (Fig. 4). As presented in Fig. 4, among these four mAbs cross-reacting with cm-sFasL, 4A5 mAb neutralized cm-sFasL from 50- to 100-fold more efficiently than 4H9, NOK-3, and NOK-2.

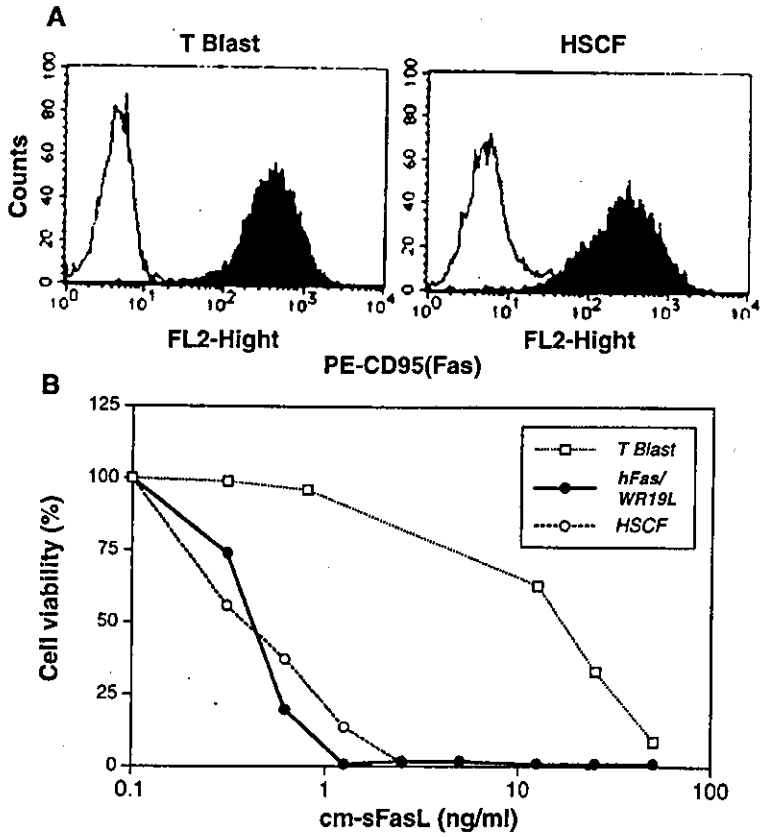

Fig. 3. Recombinant monkey sFasL induced apoptosis in Fas-positive cynomolgus monkey cells. (A) Cynomolgus monkey PHA-activated T cell blasts and T cell line (HSCF) were stained with PE-conjugated anti-human CD95 mAb (filled diagrams) and analyzed by flow cytometry. Open histograms represent staining with isotype-matched control mAb. (B) Cytotoxic activity of purified recombinant cynomolgus monkey soluble FasL (cm-sFasL) was tested against hFas/WR19L (●), HSCF (○), and PHA-activated T cell blasts (□) according to the alamar Blue method.

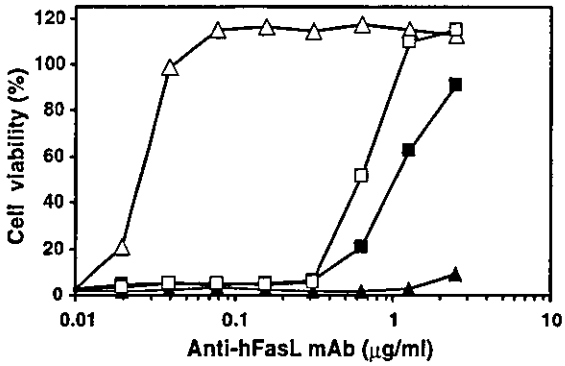

Fig. 4. Comparison of neutralizing activity among anti-human FasL mAbs cross-reacting with monkey sFasL. Cytotoxic activity of sFasL in the supernatant of cm-FasL-transfected COS cells was tested against hFas/WR19L cells in the presence of serially diluted NOK-2 (▲), NOK-3 (■), 4A5 (△), and 4H9 (□). Cytotoxicity was determined according to the alamar Blue method.

3.4. Establishment of an ELISA system for monkey sFasL measurement

Next, establishment of a sandwich ELISA system for monkey sFasL was attempted using all combinations of the four mAbs for capture and detection. As a result, 4A5 was most efficient for detection, in combination with 4H9 for capture. A sandwich ELISA system was therefore established using 4H9 for capture and biotinylated 4A5 for detection. The correlation between optical density and cm-sFasL contents ranging from 5 to 400 pg/ml was significant ($r>0.9$, $P<0.001$), indicating that this assay system is very sensitive for measuring both monkey sFasL (Fig. 5A) and human sFasL (Fig. 5B). Using this ELISA system, we examined the effect of MMP inhibitor (KB-R8301) on monkey sFasL release from cm-FasL/COS cells. When cm-FasL/COS cells were cultured for 24 h in the presence of KB-R8301, KB-R8301 inhibited sFasL release in a dose-dependent manner (60% inhibition at 10 μM and 36% inhibition at 2.5 μM) (data not shown), in addition to inhibiting human sFasL release from hFasL/COS (Kayagaki et al., 1995). This result indicated that release of monkey FasL is likewise processed by an MMP-like enzyme. Finally, this assay system was utilized to evaluate levels of sFasL in plasma from SIV-infected cynomolgus monkeys. The sFasL was undetectable in normal monkey plasma (data not shown). Conversely, levels of sFasL in SIV-infected cynomolgus monkeys increased and became detectable at 10–17 days after infection with pathogenic SIV (Table 1). These results demonstrated that the presently established sandwich ELISA system is applicable to monitor changes to sFasL concentrations in monkey disease

Table 1
Soluble FasL levels in SIV-infected monkey plasma

Monkey #	Dpi	CD4+ T cells (%)	Plasma p27 (ng/ml)	sFasL (pg/ml)
#028	0	47.9	0.00	0.0
	10	47.3	3.15	4.1
	17	30.5	2.91	14.3
#323	0	43.9	0.00	0.0
	10	48.9	1.63	0.0
	17	47.3	0.71	24.8

Dpi: days post infection of SIVmac239 into cynomolgus monkeys.
CD4+ T cells: percentages of CD4+ lymphocyte subsets.
Plasma p27: p27 SIV antigen level in plasma.
sFasL: sFasL level by ELISA using anti-hFasL antibodies (4H9 and biotinylated 4A5).

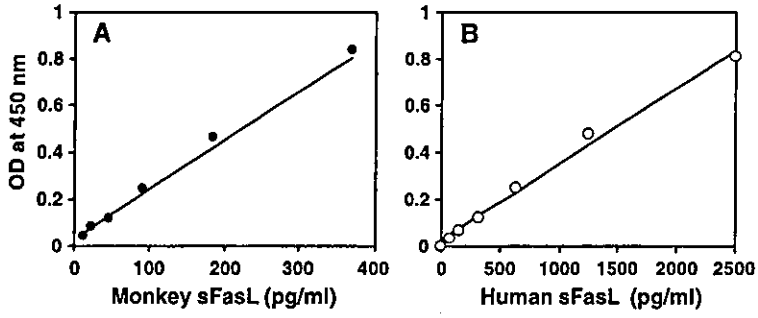

Fig. 5. Standard curves for monkey sFasL and human sFasL determined by ELISA. Purified monkey (A) and human (B) recombinant sFasL were serially diluted and the reactivity was determined by ELISA using 4H9 for capture and biotinylated 4A5 for detection.

models. Since the amino acids sequence is completely identical among three species of macaque monkeys, this ELISA system is also potentially useful for rhesus and pig-tailed monkeys. Although the role of sFasL in the progression of disease remains controversial, shedding of FasL from the membrane plays an important role in controlling Fas/FasL-induced apoptosis. Both the in vitro assay system for apoptosis induced by recombinant monkey sFasL and the highly sensitive monkey sFasL measuring system established in the present study will be useful for exploring the significance of Fas/FasL-induced apoptosis in monkey disease models, including AIDS (Letvin et al., 1985), malaria (Matsumoto et al., 2000), and organ transplantation (van Beusechem et al., 1992).

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PII S1050-1738(02)00255-4 **TCM**

Genetic Manipulation of Primate Embryonic and Hematopoietic Stem Cells with Simian Lentivirus Vectors

Yutaka Hanazono,* Takayuki Asano, Yasuji Ueda, and Keiya Ozawa

During the past several years, many articles have described how human embryonic stem (ES) cells and adult hematopoietic stem cells (HSCs) can differentiate into cardiac muscle, blood vessels, and various other types of cells. The articles raised the expectation that these stem cells may become useful for the treatment of a variety of diseases, including cardiovascular diseases. Genetic manipulation of ES cells and HSCs would be important for such future applications of the cells. Until now, retroviral vectors have been used primarily for stable expression of transgenes in murine ES cells and HSCs. Because murine models may not predict reliably the biology of ES cells and HSCs in humans, we have utilized primate ES cells and HSCs as targets of gene transfer. We have shown that primate ES cells and HSCs can be transduced efficiently with lentiviral vectors derived from the simian immunodeficiency virus, and that the high transgene expression persists without transcriptional silencing. This highly efficient gene transfer method allows for safe and faithful gene delivery to primate ES cells and HSCs to test potential research and therapeutic applications. (Trends Cardiovasc Med 2003;13:106–110) © *2003, Elsevier Science Inc.*

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• Stem Cell Therapy and Animal Models

With the establishment of embryonic stem (ES) and embryonic germ (EG) cell lines (Reubinoff et al. 2000, Shamblott et al. 1998, Thomson et al. 1998), great attention has been given to the therapeutic potential of these cells from both a biologic and medical perspective. This potential is based on their remarkable ability to differentiate into most of the specialized cells of the three EG layers and their ability

to divide for indefinite periods in culture. These extraordinary features have stimulated many researchers to consider using human pluripotent stem cells as cellular therapy for a variety of diseases and injured states. Human ES cells have been reported to differentiate into neurons, cardiac myocytes, and blood cells (Kaufman et al. 2001, Kehat et al. 2001, Reubinoff et al. 2000). Of particular note, ES cell-derived cardiomyocytes have been engrafted following implantation in mice (Klug et al. 1996), suggesting that ES cells can be used as a source of donor cardiomyocytes.

In animal studies, primarily using murine models, researchers also have recently observed that hematopoietic cells have the capacity to form other kinds of cells, such as liver, muscle, and blood vessel cells (Graf 2002); although cell fusions may, in part, account for such a change of phenotype (Terada et al. 2002, Ying et al. 2002). Several articles have suggested the possible use of bone marrow cells for the treatment of cardiac ischemia (Jackson et al. 2001, Kocher et al. 2001, Orlic et al. 2001), and bone marrow cells have been successfully used for the treatment of patients with ischemic limbs (Tateishi-Yuyama et al. 2002).

Clearly, animal models will prove to be important in the study of ES cells and hematopoietic stem cells (HSCs). Although mouse models have been used widely, they may not predict reliably the biology of ES cells and HSCs in humans. Nonhuman primate ES cells and HSCs may reflect human ES cell and HSC characteristics and behavior more closely, due to their close phylogenetic relationship (Wagner and Storb 1996). We have used cynomolgus ES cells and HSCs for the study of genetic manipulation and transplantation, with the future goal of transplantation-based applications. This article focuses on genetic manipulation of primate ES cells and HSCs using simian lentivirus vectors.

• Primate Lentivirus Vectors

Genetic manipulation of human ES cells and HSCs likely will be required for their future clinical application, at least in some disorders. Until now, murine oncoretroviral vectors (henceforth referred to as retroviral vectors) have been used predominantly for the purpose of stable expression of transgenes in murine ES cells and HSCs (Cherry et al. 2000). In an attempt to improve transgene expression in either ES cells or HSCs, retroviral vectors derived from the mouse stem cell virus (MSCV) have been developed (Grez et al. 1990, Hawley et al. 1994). These vectors differ from the standard Moloney mouse leukemia virus vectors in the long terminal repeat (LTR) promoter-enhancer region and the primer-binding site. However, even with MSCV-based vectors, transgene expression has remained low and expression is repressed over time (Cherry et al. 2000).

Lentiviral vectors are another group of integrating vectors. Unlike retroviral vectors, lentiviral vectors can transduce nondividing cells (Naldini et al. 1996). They have been developed by modifying human immunodeficiency virus 1 (HIV-1) (Nadini et al. 1996), feline immunodeficiency virus (Poeschla et al. 1998), equine infectious anemia virus (Olsen 1998), simian immunodeficiency virus (SIV) (Nakajima et al. 2000, Schnell et al. 2000), or bovine immunodeficiency virus (Berkowitz et al. 2001). Although the HIV-1-based vectors are the most widely used among them, our lentiviral vector is based on an SIV derived from an African green monkey (SIVagm) (Nakajima et al. 2000). The advantage of SIVagm vectors over HIV-1 vectors is in safety. Replicative HIV-1 is severely pathogenic in humans, and thus HIV-1 vectors have potential safety concerns. A series of safety modifications have been made in HIV-1 vectors to decrease the chance of recombination events resulting in replication-competent viruses. Increasingly more HIV-1 sequences have been removed from the vectors, but the ability to produce high-titer vectors that can transduce nondividing cells and integrate efficiently is dependent on the inclusion of at least some residual HIV-1 elements (such as *gag*, *pol*, and *rev*) in addition to the LTRs (Dull et al. 1998). Thus, some safety concerns remain. In contrast, SIVagm has no pathogenicity in its immunocompetent natural host and in experimentally inoculated macaque monkeys (Honjo et al. 1990). In addition, the sequence homology between HIV-1 and SIVagm is relatively low (about 50%) (Figure 1). The generation of replication-competent virus by recombination between SIVagm vectors and HIV-1 in human subjects is therefore highly unlikely. This provides an advantage in safety, especially if target cells are already infected with HIV or are permissive to HIV infection.

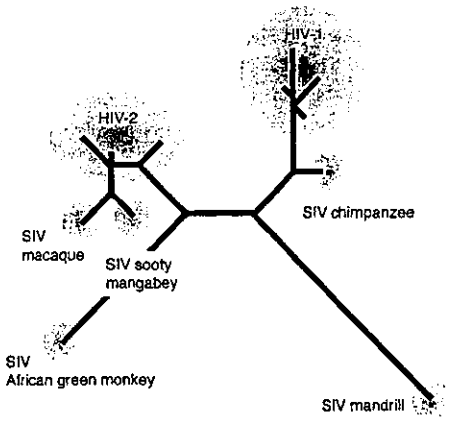

Figure 1. Primate lentiviruses. Primate lentiviruses are divided into several subgroups. The simian immunodeficiency virus (SIV) African green monkey (SIVagm) is phylogenetically most distant to the human immunodeficiency virus 1 (HIV-1), and the homology between the two viruses is rather low (around 50%). The generation of replication-competent virus by recombination between SIVagm vectors and HIV-1 in vitro or in human subjects is therefore unlikely. Thus, SIVagm vectors should offer a safety advantage over HIV-1 vectors. This tree was constructed from the nucleotide sequences of the *rev* gene by the Neighbor-Joining Method in Genetyx-Mac (Software Development Co., Ltd., Tokyo, Japan). Twelve sequences of the virus samples (4 HIV-1, 3 HIV-2, and 5 SIV strains) were analyzed.

• Gene Transfer into Primate ES Cells

Successful therapeutic use of human ES cell-derived donor cells would require the generation of essentially pure differentiated cell cultures, because the engraftment of undifferentiated pluripotent ES cells could result in teratoma formation. In addition, evasion of host immune responses to allogeneic ES cell-derived donor cells would be required to maintain prolonged engraftment. Nonhuman primate ES cell lines (rhesus, common marmoset, and cynomolgus ES cell lines) have been established (Suemori et al. 2001, Thomson et al. 1995 and 1996) and are a useful model system to study these issues. These cells were derived from blastocysts and show prolonged undifferentiated proliferation while maintaining a normal karyotype and developmental potential to differentiate into all three EG layer cells. Nonhuman primate ES cells differ from mouse ES cells, but

are remarkably similar to human ES cells in morphology, surface marker expression, and lack of leukemia inhibitory factor dependency.

We have utilized cynomolgus ES cells and explored the potential of SIV vectors to introduce new genetic material into the cells (Asano et al. 2002). When cynomolgus ES cells were transduced once with an SIV vector carrying the enhanced green fluorescent protein (EGFP or GFP) gene, most cells fluoresced after a single transduction (Figure 2). The GFP expression persisted for months without selection procedures and was not repressed during embryoid body formation. On the other hand, retroviral GFP expression in cynomolgus ES cells was low (Asano et al. 2002).

Taking advantage of the fact that the 3'LTR duplicates and becomes the 5'LTR in vivo after reverse transcription and integration, the SIV vector was constructed with a deletion of the U3 region in the 3'LTR, allowing for production of a self-inactivating (SIN) vector. The SIN SIV vector loses the transcriptional capacity of the LTR once it is integrated into the target genome. One possible explanation for the sustained lentiviral transgene expression is that the transcriptionally inactive state of the lentiviral LTR might allow the provirus to escape epigenetic mechanisms of silencing (Yoder et al. 1997). Stable transgene expression, however, also was observed when a normal SIV vector with an intact LTR was utilized (Asano et al. 2002). Lentiviral vectors might have less intrinsic susceptibility to silencing than do retroviral vectors.

Recently, it has been reported that human ES cells can be transduced efficiently by HIV-1-based lentiviral vectors (Pfeifer et al. 2002). The lentiviral gene transfer method should allow for efficient introduction of a variety of genes of interest into primate ES cells and for their sustained expression.

• Gene Transfer into Primate CD34$^+$ Cells

If plasticity of adult HSCs can be applied to human cells, it eventually may be possible to use HSCs to repair a wide array of cells and tissues. We have utilized cynomolgus monkeys (*Macaca fascicularis*) as predictive preclinical HSC transplantation models (Ageyama et al. 2002). We also have developed a cynomolgus model of acute cardiac infarction. This model will provide an important framework for future clinical studies using hematopoietic cells aimed at regeneration of ischemic cardiac muscle.

CD34 is a cell-surface marker of undifferentiated HSCs. Although all HSCs may not express CD34 (Osawa et al. 1996), clinical CD34$^+$ cell transplantation has been conducted successfully in HSC gene therapy studies with retroviral vectors (Aiuti et al. 2002, Cavazzana-Calvo et al. 2000). CD34$^+$ cells can be isolated from bone marrow, peripheral blood, or cord blood. For genetic manipulation, the cells usually are cultured ex vivo for several days prior to reinfusion to allow for cell cycling, because retroviral transduction requires cell division. We have utilized the standard culture conditions that include several cytokines (stem cell factor, Flt-3 ligand, and thrombopoietin) for retroviral transduction of primate CD34$^+$ cells (Hanazono et al. 2002). Prolonged cytokine treatment of HSCs, however, may result in the loss of multilineage differentiation and engraftment abilities (Dunbar et al. 2001). On the other hand, lentiviral vectors can transduce nondividing cells, and thus cytokine treatment is not necessarily required for lentiviral transduction. This is the great advantage of using lentiviral vectors over retroviral vectors for transduction of HSCs.

In our laboratory, human CD34$^+$ cells were transduced with SIV vectors carrying the GFP gene. Twenty percent to forty percent of cells fluoresced after a single infection with the vector (Figure 3A). The transduced CD34$^+$ cells were transplanted into sublethally irradiated nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. This model takes advantage of the immunologically naive state of the mice. Because the NOD/SCID mice are severely immuno-

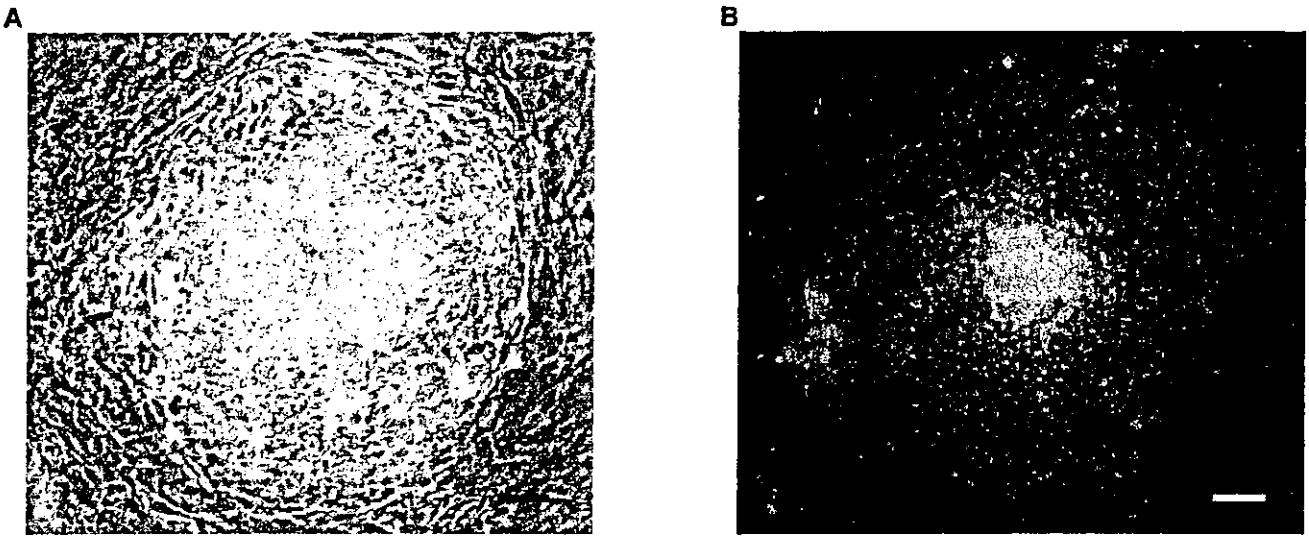

Figure 2. Gene transfer into cynomolgus embryonic stem (ES) cells by simian immunodeficiency virus (SIV) vector. Cynomolgus ES cells (CMK6) were transduced once with the SIV vector expressing the green fluorescent protein (GFP) gene at 60 transducing units per target cell. The transduced cells were observed at day 6 after transduction in a bright field **(A)** and in a dark field **(B)** under a fluorescence microscope. Cells that form colonies are cynomolgus ES cells, and background cells are mouse embryonic fibroblast feeder cells. Fluorescence was observed in many ES cells. Scale bar = 100 μm.