Table 1 Clonogenic progenitor assay

Growth factors	Total colony number (% NBT-positive)	
	Transduced BM	Untransduced BM
None	0 (ND)	0 (ND)
IL-3+SCF+G-CSF+Epo	524 (29%)	547 (0%)
E_2	228 (96%)	0 (ND)

Transduced and untransduced X-CGD mouse bone marrow (BM) cells were inoculated onto methylcellulose in duplicate (1×10^5 cells/dish). Colonies were counted at day 10, and an *in situ* NBT test was carried out to detect superoxide production by individual colonies. NBT, nitroblue tetrazolium; ND, not done; IL-3, mouse interleukin-3; SCF, rat stem cell factor; G-CSF, human granulocyte colony-stimulating factor; Epo, human erythropoietin; E_2, estradiol.

combination (SCF, IL-3, erythropoietin (Epo) and G-CSF), 10^{-7} M 17β-estradiol (E_2) alone, or no stimulation. The E_2 concentration that supported optimal growth of the GcRER-transduced murine progenitors was chosen.[21,23] Table 1 summarizes the result of the colony assay at 10 days of growth. No colony was observed in the culture without stimulation, regardless of whether the cells were transduced or untransduced. With the cytokine cocktail, both transduced and untransduced X-CGD BM cells yielded comparable number of colonies (about 500 colonies out of 2×10^5 cells). Most of them were myeloid, and there were a few erythroid and mixed colonies. Thus, transduction with MGK/h91GE did not show positive or negative effect on cytokine-induced colony formation. Finally, the untransduced BM formed no colony in the presence of E_2 alone, as we observed previously.[21,23] In contrast, 10^{-7} M E_2 induced about 200 colonies from 2×10^5 transduced X-CGD BM cells, most of which were granulocyte/monocyte colonies. Considering the very low background colony formation in this assay, these E_2-induced colonies must be derived from vector-transduced progenitors that actually expressed SAG. From the ratio of E_2-induced colonies to cytokine-induced colonies, the *ex vivo* transduction efficiency was estimated to be 44%.

On day 10 of the methylcellulose culture, the colonies were subjected to an *in situ* nitroblue tetrazolium (NBT) test to detect respiratory burst activity. In this assay, most phorbol myristate acetate (PMA)-stimulated wild-type (WT) granulocyte colonies reduced NBT and turned blue (not shown), while the cytokine-induced colonies derived from untransduced X-CGD BM showed no respiratory burst activity (Table 1). As for MGK/h91GE-transduced X-CGD BM, 29% of the cytokine-induced colonies were NBT-positive, while nearly all of the E_2-induced colonies showed a respiratory burst (Table 1 and Figure 2). These results indicated that the SAG/estrogen system selectively expanded genetically modified progenitors *in vitro*, and the estrogen-induced colonies actually coexpressed ΔY703FGcRER and gp91^{phox}. NBT positivity in the cytokine-induced colonies (29%) would represent functional transduction efficiency based on gp91^{phox} expression (see Discussion).

In vivo expansion of functionally corrected neutrophils

In parallel with the *in vitro* progenitor assay, the same batch of MGK/h91GE-transduced Ly5.1-X-CGD BM cells
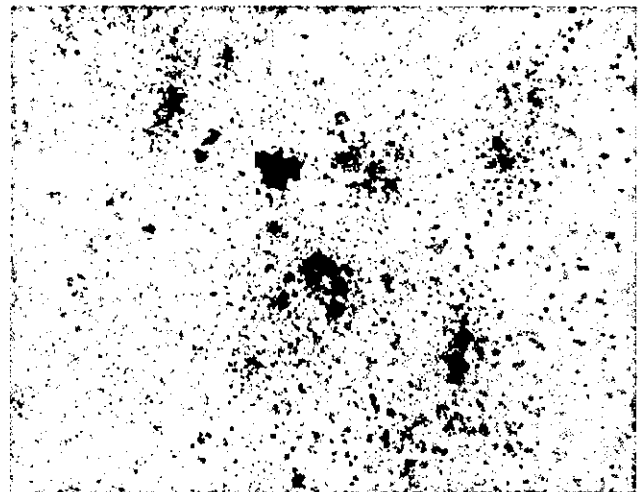

Figure 2 *In situ* colony NBT test. X-CGD bone marrow cells were transduced with MGK/h91GE vector and 2×10^5 cells were subjected to methylcellulose culture with 10^{-7} M estradiol. On day 10, the colonies were overlaid with RPMI medium containing NBT and PMA. Nearly all the estrogen-induced colonies were NBT-positive with blue formazan precipitates.

was transplanted to lethally irradiated male Ly5.2-X-CGD recipients ($n = 8$). Donor-derived Ly5.1 cells rapidly repopulated in the recipients; a series of FACS analysis revealed that the overall white blood cell (WBC) chimerism was 81–91% at 4 weeks post-BMT, and remained above 90% thereafter (FACS data not shown). Following hematopoietic reconstitution, the frequency of oxidase-positive granulocytes in the peripheral blood was monitored by flow cytometry. Leukocytes were loaded with dihydrorhodamine 123 (DHR) and stimulated with PMA.[34] Figure 3 shows representative FACS data of this assay; most PMA-stimulated granulocytes (Gr1^{high}) from a WT C57BL/6 mouse produced superoxide to reduce DHR (Figure 3a and e), while granulocytes from an untreated X-CGD mouse did not (Figure 3b and f).

At 6 weeks post-BMT, when the percentage of DHR-positive neutrophils in the transplants was $9.6 \pm 3.2\%$ (range 7.0–17.0%), four out of eight animals were given E_2 intraperitoneally to address whether the drug would induce an expansion of functionally corrected neutrophils. Our preliminary study showed that about 1 mg of E_2 per mouse (ca. 25 g body weight) was required to achieve a serum estrogen level above 10^{-7} M 24 h after injection (unpublished). Based on this observation, the animals were given 1 mg of E_2 in two doses for 3 days, to ensure trough E_2 levels above 10^{-7} M. This treatment was repeated six times with 4-week intervals until 26 weeks post-BMT.

At 2 weeks after the first course of E_2, three out of four challenged mice had increased levels of DHR-positive neutrophils (from 8.3–17.0 to 12.9–67.3%), while one animal had a lowered DHR positivity (from 9.7 to 5.0%). Figure 3c and g shows an E_2-treated mouse that exhibited the most prominent expansion of oxidase-positive neutrophils. In this animal, oxidase-positive granulocytes were increased from 17.0 to 67.3% (Figure 3g). On the other hand, frequencies of DHR-positive granulocytes in the unstimulated mice were unchanged or
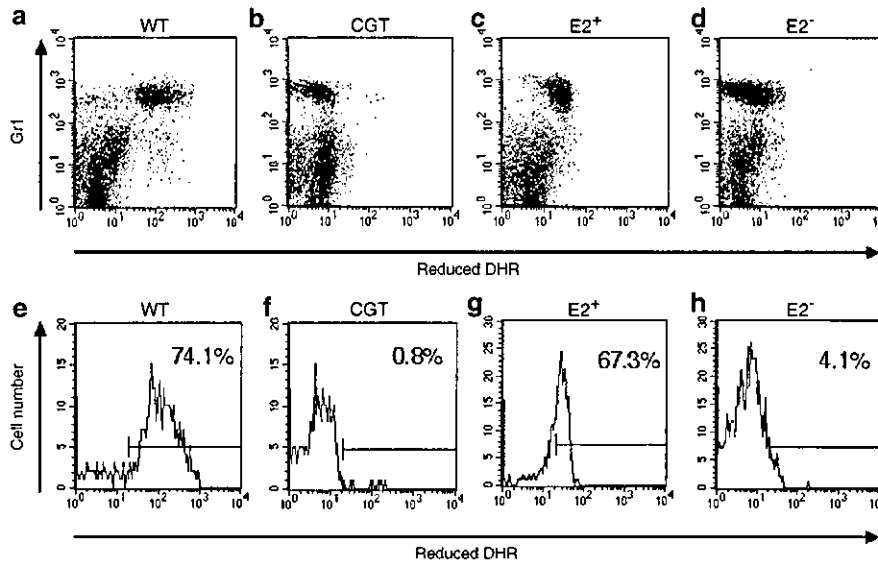
Figure 3 Flow cytometry of DHR assay. Mouse peripheral blood was stimulated with PMA, incubated with DHR and stained with Gr1-PE/Cy5. In the dot plots (a–d), the X-axis represents DHR reduced by superoxide, and the Y-axis represents expression of a granulocyte differentiation marker Gr1. In the histograms (e–h), $Gr1^{high}$-gated cells were shown to highlight superoxide formation by neutrophils. (a and e) A wild-type C57BL/6 mouse (WT). (b and f) An untreated X-CGD mouse (CGD). (c and g) An X-CGD transplant 2 weeks after the first estrogen administration (E_2). (d and h) An X-CGD transplant not administered estrogen (E_2^-).

lowered. Only 1.7–10.1% (4.3±3.9%) of neutrophils produced superoxide, and Figure 3d and h shows a FACS analysis of an unstimulated animal. A parallel NBT slide test showed comparable frequencies of oxidase-positive cells in these mice (NBT slides not shown).

Although the initial response to estrogen varied among transplants, repeated E_2 administration led to an increase in respiratory burst-positive neutrophils in these animals. As shown in Figure 4a, the frequency of DHR-positive neutrophils at 16 weeks post-BMT (2 weeks after the third E_2 administration) was elevated in all the treated animals compared to that seen before the drug challenge (from 11.0±4.0 to 35.7±9.1%), and the increase was significant ($P = 0.014$ by paired t-test). The absolute number of oxidase-positive neutrophils was also significantly increased, as shown in Figure 4b (from 244±211 to 486±302/µl; $P = 0.019$ by paired t-test).

Prolonged increase in oxidase-positive neutrophils

With repeated E_2 administration, the drug-treated X-CGD transplants maintained a higher level of genetically corrected neutrophils than the untreated animals. A difference between groups was observed 2 weeks after the initial treatment; the drug-treated animals (Group 1) showed higher percentages of DHR-positive neutrophils (27.5±27.8%) than the untreated mice (Group 2; 4.3±3.9%) as shown in Figure 5a ($P = 0.043$ by Mann–Whitney U-test). This figure also shows that the levels of oxidase-positive granulocytes were significantly higher in Group 1 than Group 2 at most time points during the repeated course of E_2 administration (asterisks in Figure 5a, $P < 0.05$ by Mann–Whitney U-test). The absolute number of oxidase-positive cells was higher in Group 1 than Group 2 on E_2 treatment as well (asterisks in Figure 5b, $P < 0.05$ by Mann–Whitney U-test).

At a later time point (38 weeks post-BMT), the treatment was switched. That is, the mice in Group 2
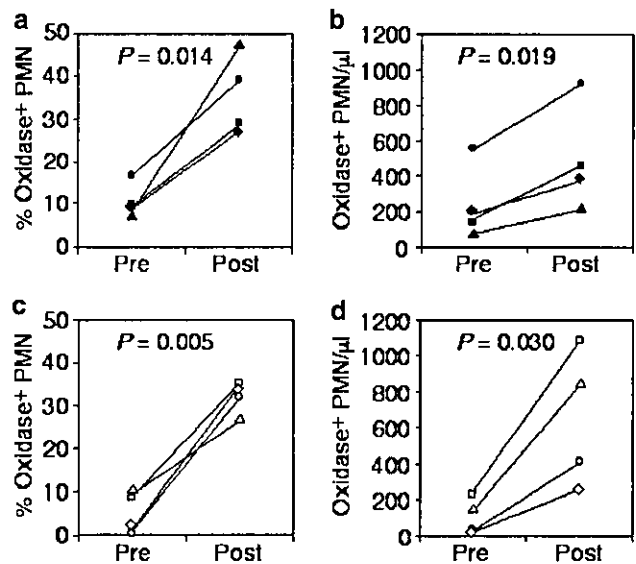

Figure 4 Comparison of oxidase-positive granulocytes before and after estrogen administration. Frequencies (a and c) and absolute numbers (b and d) of oxidase-positive polymorphonuclear leukocytes (PMN) from individual X-CGD transplants are shown. (a and b) Oxidase-positive PMN in Group 1 mice before estrogen administration (Pre; 6 weeks post-BMT) and after the third estrogen injection (Post; 16 weeks post-BMT). The increase was significant by paired t-test (a, $P = 0.014$; b, $P = 0.019$). (c and d) Oxidase-positive PMN in Group 2 mice before estrogen administration (Pre; 38 weeks post-BMT) and after estrogen injection (Post; 40 weeks post-BMT) at a later time point. The increase was significant by paired t-test (c, $P = 0.005$; d, $P = 0.030$). Each animal is represented by a different symbol to track the frequency and number of oxidase-positive PMN.

were given E_2 for 3 days, while the animals in Group 1 were left unchallenged. The E_2-stimulated animals showed a remarkable increase in DHR-positive cells. The percentage of DHR-positive granulocytes rose from
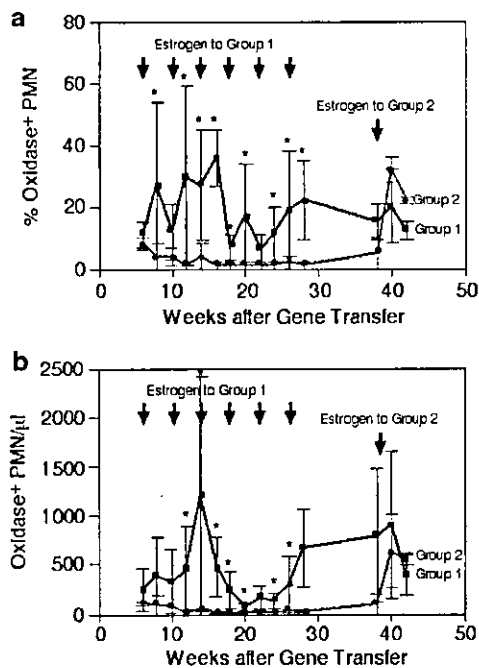
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Figure 5 Estrogen-induced expansion of oxidase-positive granulocytes. Graphs indicate the time course of the change in frequency (a) and absolute number (b) of oxidase-positive granulocytes following exposure to estrogen. To mice in Group 1 (n = 4), estrogen was given in six courses with 4-week intervals from 6 to 26 weeks post-BMT (black line). Mice in Group 2 (n = 4) were given estrogen only once at 38 weeks post-BMT (gray line). Asterisks indicate time points when significantly more oxidase-positive cells existed in Group 1 than Group 2 (P < 0.05 by Mann–Whitney U-test).

4.8 ± 4.7 to $31.7 \pm 3.8\%$ ($P = 0.005$ by paired t-test, Figures 4c and 5a), and the absolute number increased from 96 ± 104 to $638 \pm 378/\mu l$ ($P = 0.030$ by paired t-test, Figures 4d and 5b) in 2 weeks. This result indicated that transduced long-term repopulating cells were maintained in the animals and readily responsive to estrogen, thereby giving rise to an elevated level of corrected neutrophils on drug administration.

During the observation period, the administration of E_2 did not lead to any apparent hematological aberration in the treated mice; none of the recipients of transduced marrow have developed a proliferative disorder, regardless of whether E_2 was administered or not. Apparent feminization was not observed after the periodic estrogen administration in the transplanted male mice.

Discussion

In contrast to successful preclinical gene-transfer studies using mouse models,[13,14] the levels of corrected neutrophils have been too low to impact the CGD phenotype in phase I clinical trials like most gene-transfer attempts targeting human HSCs.[15] In contrast, Fischer and colleagues showed a significant T-lymphocyte reconstitution in a series of patients with X-linked severe combined immunodeficiency (X-SCID) following onco-retrovirus-mediated gene transfer.[35,36] This success largely owes to an extremely strong growth advantage of

lymphocyte precursors transduced with a functional common γ chain (γc) gene.[37] However, an excessive growth stimulation may be harmful. Recently, a lymphoproliferative disorder occurred in patients treated in the X-SCID gene therapy following aberrant activation of LMO2 oncogene by insertional mutagenesis.[38,39] In these patients, a strong and continuous mitogenic stimulation via functional γc may bring about additional events besides LMO2 activation, finally leading to uncontrolled clonal proliferation. Therefore, for most HSC gene therapy candidate diseases in which a therapeutic gene per se does not confer a growth advantage, controlled expansion of transduced stem/progenitor cells is desirable.

For this purpose, we have developed selective amplifier genes,[21] and showed controllable in vivo expansion of marker gene-transduced hematopoietic cells in murine and primate models.[24,25] In the present study, we showed that functionally corrected cells were expandable using the SAG system in an actual disease model of CGD. An in vitro NBT assay showed that estrogen specifically induced functionally corrected colonies. It is currently unclear why gene transfer efficiency based on the total number of colonies (44%) differed from that based on NBT positivity (29%). At present, we consider the latter estimation (29% based on NBT positivity) as more accurate and reliable, because the former is based on an indirect calculation with colony number, which inherently includes fluctuation.

We also showed an in vivo expansion of corrected neutrophils. Following estrogen stimulation, the ratio and number of oxidase-positive granulocytes were elevated, and repeated drug administration maintained an increased level of corrected cells. Furthermore, superoxide-producing cells increased remarkably in the transplants given estrogen at a later time point (Group 2 in Figure 5), suggesting that transduced long-term repopulating cells remain responsive to estrogen and that on-demand expansion of functional neutrophils is feasible in CGD. As mentioned, we observed that the initial response to estrogen varied among transplants in Group 1, and the reason for this variation is yet to be clarified. Considering that the mice in Group 2 responded to E_2 with little deviation at a later time point, the early E_2 administration to Group 1 may account, in part, for this variation. The mice in Group 1 were given E_2 at 6 weeks post-BMT, when the donor-derived hematopoiesis might not have reached a steady state and varied among animals considerably.

Including the present study, we have not encountered a neoplastic outgrowth of SAG-transduced cells in the animals examined thus far, including a primate system.[24,25] Blau and colleagues have presented another conditional expansion system in which an FK506-binding protein 12-based fusion receptor is activated by a dimerizing crosslinker, and no cancerous event has been reported.[19,40,41] Still more extensive studies are required to clear safety issues concerning uncontrolled proliferation. We are carrying out serial transplantation of SAG-transduced BM in an attempt to predict whether such complications would arise in a longer-term follow-up. In addition, large animal studies with clinically relevant protocols are mandatory to address the safety and feasibility of regulated cell expansion in HSC gene therapy.

Materials and methods

Plasmid construction

To transduce X-CGD hematopoietic cells with the hgp91 gene and a modified SAG, a bicistronic retrovirus vector was constructed. The vector, MGK/h91GE, had a hybrid backbone (MGK) comprising the long-terminal repeats (LTRs) and the primer-binding site from MSCV and the *gag* through to the *env* initiation codon from MFG.[26,27,42] The 5'-half of hgp91 cDNA (from the initiation codon to the internal *Ase*I site) was derived from pBS/hgp91,[31] by amplification with the polymerase chain reaction (PCR) (upstream primer, 5'-TCTGCCACCATGGGGAACT-3', and downstream primer, 5'-GCAAGGCCAATGAA GAAGAT-3') to create an *Nco*I site at the initiation codon. The 3'-half of hgp91 (from the internal *Ase*I site to the stop codon) was PCR-amplified on pBS/hgp91 using an upstream primer, 5'-GGCATCACTGGAGTTGTCA-3', and a downstream primer, 5'-GAGGATCCTTA GAAGTTTTCCTTGTTGAA-3', to add a *Bam*HI site at the 3' end. The fragments were cloned into the *Nco*I– *Bam*HI site of MGK by trimolecular ligation to yield MGK/hgp91.[42] The 5' half of the SAG (from the initiation codon to the internal *Dra*III site) was PCR-amplified on pBS/ΔY703FGcRER,[22] with 5'-AAATGGGACCTCTGG GAGCCTGCACCCTG-3' as an upstream primer and a *Dra*III site-linked downstream primer (5'-AGAA CAGCTGCACACTCACT-3') to create a *Ppu*MI site at the initiation codon. The 3' half of the SAG (from the *Dra*III site to the stop codon) was PCR-amplified on pBS/ ΔY703FGcRER with 5'-AAGGCCCCCACCATCAGA CT-3' as an upstream primer and an *Xho*I site-linked downstream primer (5'-CTGGCTCGAGTCAGATGG TGTTGGGGAAG-3') to add an *Xho*I site to the 3' end. The fragments were cloned into the *Ppu*MI–*Xho*I site of pCGI,[43] which contains the encephalomyocarditis virus-derived IRES,[28] by trimolecular ligation. Subsequently, the IRES-ΔY703FGcRER cassette was obtained as a *Bam*HI–*Xho*I fragment, and inserted between the hgp91 and the 3'-LTR of MGK/hgp91, resulting in the final construct MGK/h91GE (Figure 1b).

Animals

Targeted disruption of the X-linked gp91[phox] gene in the mouse was described.[11] The X-CGD mice backcrossed to Ly5.2-C57BL/6 were a gift from Dr MC Dinauer (Indiana University, Indianapolis, IN, USA). The X-CGD mice were crossed with Ly5.1-congenic C57BL/6 mice, and both Ly5.1- and Ly5.2-X-CGD mice were maintained under specific pathogen-free conditions. The animals were given free access to autoclaved food and ultraviolet-irradiated water and treated according to the institutional codes governing animal rights. WT Ly5.2-C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan).

Retroviral transduction

Ecotropic retroviral supernatant was prepared by transient transfection of BOSC23 packaging cells (American Type Culture Collection CRL-11554, Manassas, VA, USA) with MGK/h91GE using Lipofectamine (Invitrogen, Grand Island, NY, USA), following the manufacturer's protocol.[29] X-CGD mouse BM cells were retrovirally transduced using a fibronectin-assisted protocol.[25,33] Male Ly 5.1-X-CGD mice were injected intraperitoneally with 150 mg/kg 5-FU (Kyowa Hakko, Tokyo, Japan), and

BM cells were collected 2 days postinjection. Low-density mononuclear cells were separated using Lympholyte-M (Cedarlane Laboratories, Hornby, Canada) and stimulated for 2 days with α-Minimum Essential Medium (Invitrogen) containing 100 ng/ml recombinant rat SCF (provided by Amgen, Thousand Oaks, CA, USA) and 100 U/ml recombinant human IL-6 (provided by Ajinomoto, Kawasaki, Japan).[44] The cells were then incubated in the fresh viral supernatant on plates precoated with recombinant human fibronectin fragment CH-296 (RetroNectin; provided by Takara Bio, Otsu, Japan) for 2 days under the same conditions. Supernatant infection was repeated five times during transduction, and the manipulated cells were recovered using Cell Dissociation Buffer (Invitrogen). As a negative control, an aliquot of the prestimulated cells was incubated in α-Minimum Essential Medium containing SCF and IL-6 for another 2 days ('untransduced cells').

Clonogenic progenitor assay

Hematopoietic progenitors were assayed using StemPro Methylcellulose Medium (Invitrogen) supplemented with appropriate growth factors. Transduced and untransduced X-CGD mouse BM cells were seeded onto Petri dishes at a density of 1×10^5 cells/dish in 1 ml of StemPro medium containing either no growth factor, 10^{-7} M E_2 (Sigma, St Louis, MO, USA) alone, or a cytokine cocktail of 2 U/ml recombinant human Epo (provided by Chugai Pharmaceuticals, Tokyo, Japan), 100 ng/ml SCF, 20 ng/ml recombinant human G-CSF (provided by Chugai Pharmaceuticals) and 100 U/ml IL-3.[21-23] After 10 days of incubation, colonies were counted and assayed for respiratory burst activity using an *in situ* NBT test (NBT from Sigma).[45] A one-fifth volume of NBT-saturated RPMI-1640 medium (Invitrogen) containing 100 ng/ml PMA (Sigma) and 5% human serum albumin (Baxter Healthcare, Deerfield, IL, USA) was layered onto the methylcellulose culture and incubated at 37°C. After 1 h of incubation, the dishes were examined on an inverted microscope, and the colonies with blue formazan precipitates were scored as NBT-positive.

BMT and estrogen administration

For hematopoietic reconstitution with retrovirally transduced Ly5.1-X-CGD BM cells, 8–10-week-old male Ly5.2-X-CGD recipients were lethally irradiated (split dose of 11 Gy at an interval of 3 h with ^{137}Cs using Gammacell 40, Nordion International, Kanata, Canada) and transplanted with the transduced BM cells. A total of 2–3 × 10^{6} cells per recipient were given by tail vein injection. WBCs were stained with a fluorescein isothiocyanate-conjugated anti-Ly5.2 antibody (Pharmingen, San Diego, CA) and a phycoerythrin (PE)-conjugated anti-Ly5.1 antibody (Pharmingen) to measure chimerism with a FACScan (Becton Dickinson, San Jose, CA, USA). After hematopoietic reconstitution, one half of the transplanted mice were administered with estrogen (Group 1). Starting from 6 weeks post-BMT, the mice were intraperitoneally given 0.5 mg of E_2 dipropionate (Ovahormon Depot from Teikoku Hormone MFG, Tokyo, Japan) twice for 3 days. The E_2 administration was repeated every 4 weeks until 28 weeks after BMT. At 40 weeks, E_2 administration was switched so that the formerly unstimulated mice were challenged with E_2 (Group 2).

Peripheral blood counts and measurement of respiratory burst activity

A complete blood cell count (CBC) was performed using tail vein blood on a PC-608 particle counter (Erma, Tokyo, Japan) according to the manufacturer's recommendations. Blood smears were stained with Wright–Giemsa using standard methods and examined at × 500 for differential analysis.

Superoxide production by peripheral leukocytes was assayed using the NBT slide test of Buescher with slight modification.[10] Fresh whole blood from the tail vein was placed on a glass slide and incubated at 37°C in a humidified chamber until it had clotted. The clot was gently removed, and the slide was rinsed in phosphate-buffered saline (PBS) to free it of erythrocytes, then covered with NBT-saturated RPMI-1640 medium containing 100 ng/ml PMA and 5% human serum albumin. After incubation at 37°C for 20 min, the slide was rinsed in PBS, fixed in absolute methanol for 60 s, and counter-stained with 1% safranine-O (Sigma) to identify nuclear morphology. Superoxide production by peripheral leukocytes was assayed using flow cytometry by loading the cells with DHR (Sigma) as described.[13,14,34] Mouse whole blood was incubated with 30 μM DHR at 37°C for 5 min and stimulated with 5 μg/ml PMA at 37°C for 30 min. After erythrocytes were lysed with Lysis buffer (150 mM NH_4Cl, 20 mM $NaHCO_3$, 1 mM EDTA), the cells were stained with a biotinylated anti-Gr1 antibody (Pharmingen) plus PE/Cy5-conjugated streptavidin (DAKO, Glostrup, Denmark) and analyzed with a FACScan. Data were statistically analyzed using the Mann–Whitney U-test and the paired t-test with StatView software (SAS Institute, Cary, NC, USA).

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RESEARCH ARTICLE

In vivo expansion of gene-modified hematopoietic cells by a novel selective amplifier gene utilizing the erythropoietin receptor as a molecular switch

Takeyuki Nagashima[1]
Yasuji Ueda[1]*
Yutaka Hanazono[2]
Akihiro Kume[3]
Hiroaki Shibata[4]
Naohide Ageyama[4]
Keiji Terao[4]
Keiya Ozawa[3]
Mamoru Hasegawa[1]

[1]DNAVEC Research Inc., Ibaraki
305-0856, Japan

[2]Division of Regenerative Medicine,
Center for Molecular Medicine, Jichi
Medical School, Tochigi 329-0498,
Japan

[3]Division of Genetic Therapeutics,
Center for Molecular Medicine, Jichi
Medical School, Tochigi 329-0498,
Japan

[4]Tsukuba Primate Center, National
Institute of Infectious Diseases,
Ibaraki 305-0843, Japan

*Correspondence to: Yasuji Ueda,
DNAVEC Research Inc., Ibaraki
305-0856, Japan.
E-mail: yueda@dnavec.co.jp

Abstract

Background *In vivo* expansion of gene-modified cells would be a promising approach in the field of hematopoietic stem cell gene therapy. To this end, we previously developed a selective amplifier gene (SAG), a chimeric gene encoding the granulocyte colony-stimulating factor (G-CSF) receptor (GCR), as a growth-signal generator and the hormone-binding domain of the steroid receptor as a molecular switch. We have already reported that hematopoietic cells retrovirally transduced with the SAG can be expanded in a steroid-dependent manner *in vitro* and *in vivo* in mice and nonhuman primates. In this study, we have developed a new-generation SAG, in which the erythropoietin (EPO) receptor (EPOR) is utilized instead of the steroid receptor as a molecular switch.

Methods Two EPO-driven SAGs were constructed, EPORGCR and EPORMpl, containing the GCR and c-Mpl as a signal generator, respectively. First, to compare the steroid-driven and EPO-driven SAGs, Ba/F3 cells were transduced with these SAGs. Next, to examine whether GCR or c-Mpl is the more suitable signal generator of the EPO-driven SAG, human cord blood CD34$^+$ cells were transduced with the two EPO-driven SAGs (EPORMpl and EPORGCR). Finally, we examined the *in vivo* efficacy of EPORMpl in mice. Irradiated mice were transplanted with EPORMpl-transduced bone marrow cells followed by administration of EPO.

Results The EPO-driven SAGs were shown to induce more rapid and potent proliferation of Ba/F3 cells than the steroid-driven SAGs. The EPORMpl induced more efficient EPO-dependent proliferation of the human cord blood CD34$^+$ cells than the EPORGCR in terms of total CD34$^+$ cell, c-Kit$^+$ cell, and clonogenic progenitor cell (CFU-C) numbers. In the transplanted mice the transduced peripheral blood cells significantly increased in response to EPO.

Conclusions The new-generation SAGs, especially EPORMpl, are able to efficiently confer an EPO-dependent growth advantage on transduced hematopoietic cells *in vitro* and *in vivo* in mice. Copyright © 2003 John Wiley & Sons, Ltd.

Introduction

One of the major obstacles associated with hematopoietic stem cell (HSC) gene therapy is the low efficiency of gene transfer into human HSCs with retroviral vectors [1]. The ability to positively select cells containing potentially therapeutic genes *in vivo* would represent an important tool for the clinical application of HSC gene therapy. A promising strategy of *in vivo* positive selection of transduced cells is to confer a direct proliferation advantage on gene-modified cells relative to their untransduced counterparts. We developed a chimeric gene designated 'selective amplifier gene' (SAG) which encodes a chimeric receptor between the granulocyte colony-stimulating factor (G-CSF) receptor (GCR) and the hormone-binding domain of the estrogen or tamoxifen receptor. The GCR moiety is a growth-signal generator and the estrogen receptor (ER) moiety is a molecular switch to regulate (turn on or off) the growth signal generated from the GCR. We previously showed that hematopoietic cells transduced with the SAG can be selectively expanded in an estrogen- or tamoxifen-dependent manner *in vitro* [2–5] and *in vivo* in mice and nonhuman primates [6,7]. In nonhuman primates, however, some animals that received the SAG did not show an increase in transduced cells in response to estrogen or tamoxifen, suggesting that the SAG was not potent enough to achieve *in vivo* expansion in all animals [7].

The utilization of the steroid receptor as a molecular switch may have attenuated the potency of the SAG. The estrogen-mediated dimerization of the chimeric molecule may be less efficient than the natural ligand (G-CSF)-mediated dimerization. In fact, the fusion protein between the GCR and estrogen receptor responds to G-CSF more efficiently than to estrogen [2]. To rectify this problem, we utilized the erythropoietin receptor (EPOR) instead of the steroid receptor as a molecular switch. Since the EPOR is a member of the cytokine receptor superfamily [8], the fusion proteins between the EPOR and other cytokine receptors such as the GCR should be more stable and compatible than the prototype fusion protein. In addition, the EPOR is not expressed on immature hematopoietic cells and thus can be used as a selective switch for these cells [9]. Of note, recombinant human erythropoietin (EPO) has already been used widely in clinical application and can be administered repeatedly to human subjects without serious adverse effects [10,11].

On the other hand, as a growth-signal generator, we tried to use the thrombopoietin (TPO) receptor, c-Mpl, in addition to the GCR. It has been reported that c-Mpl is expressed on very immature hematopoietic cells and that TPO actually stimulates the growth of these cells [12–15]. In fact, the cytoplasmic fragment of c-Mpl has already been used for the purpose of cell expansion [5,16]. The intracellular signal from c-Mpl may thus be more appropriate than that from the GCR for expansion of hematopoietic stem/progenitor cells. In the present study,

we examined the efficacy of these new generation SAGs *in vitro* and *in vivo* in mice.

Materials and methods

Cell lines

Ba/F3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), and 1 ng/ml recombinant mouse IL-3 (rmIL-3; Gibco-BRL). The ecotropic packaging cell line BOSC23 [17] and human embryonic kidney 293T cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin.

Plasmid construction

The wild-type human erythropoietin receptor (EPORwt) cDNA was obtained from pCEP4-EPOR (kindly provided by Dr. R. Kralovics, University of Alabama, USA) [18]. The fragment containing the murine phosphoglycerate kinase (pgk) promoter and neomycin phosphotransferase gene (neo) (EcoRI-BamHI) in the retroviral plasmid pMSCV2.2 (kindly provided by Dr. R. G. Hawley, University of Toronto, Canada) [19] was replaced by the EPORwt cDNA (EcoRI-BamHI) to construct pMSCV-EPORwt.

pMSCV-EPORGCR and pMSCV-EPORMpl were constructed as follows. The cytoplasmic region of murine G-CSF receptor (GCR) cDNA was obtained by PCR using pMSCV-ΔY703FGCRER as a template [3] with the primer pair 5′-AAG GAT CCA AAC GCA GAG GAA AGA AGA CT-3′ and 5′-AAG TCG ACC TAG AAA CCC CCT TGT TC-3′. The cDNA coding to the cytoplasmic region of human TPO receptor (c-Mpl) was obtained by PCR using pcDNA3.1-c-Mpl (provided by Dr. M. Takatoku, Jichi Medical School, Tochigi, Japan) [20] as a template with the primer pair 5′-AAG GAT CCA GGT GGC AGT TTC CTG CA-3′ and 5′-CGG TCG ACT CAA GGC TGC TGC CAA TA-3′. The fragment containing the extracellular plus transmembrane region of the human EPOR cDNA was obtained by PCR using pCEP4-EPOR as a template with the primer pair 5′-CTC GGC CGG CAA CGG CGC AGG GA-3′ and 5′-AAG GAT CCC AGC AGC GCG AGC ACG GT-3′. The fragment containing the extracellular plus transmembrane region of human EPOR cDNA and the fragment containing the cytoplasmic region of murine GCR or human c-Mpl were cloned into the EcoRI-SalI site of pBluescript SK (pSK; Stratagene, La Jolla, CA, USA) to construct pSK-EPOGCR or pSK-EPOMpl, respectively. The pgk promoter/neo cassette (EcoR-SalI) in pMSCV was replaced by the EcoRI-SalI fragment containing the EPORGCR or EPORMpl cDNA each from pSK-EPORGCR or pSK-EPORMpl, respectively. The resultant construct was designated as pMSCV-EPORGCR or pMSCV-EPORMpl, respectively.

pMSCV-EPORwt-ires-mitoEYFP, pMSCV-EPORGCR-ires-mitoEYFP, and pMSCV-EPORMpl-ires-mitoEYFP were

constructed as follows. The internal ribosome entry site (ires) sequence derived from pIRES-EGFP (Clontech, Palo Alto, CA, USA) and the mitoEYFP cDNA derived from pEYFP-Mito (Clontech) were inserted into the PstI-BamHI site and the SpeI-NotI site of pSK, respectively. The resultant plasmid was pSK-ires-mitoEYFP. The mitEYFP cDNA encodes the enhanced yellow fluorescent protein (enhanced YFP, EYFP) linked to a mitochondria localization signal sequence so that EYFP is sequestered inside the mitochondria, thus circumventing the presumed toxicity of YFP [21]. The blunted fragment encoding the ires-mitoEYFP cDNA was ligated into the ClaI blunted site of pMSCV-EPORwt, pMSCV-EPORGCR, and pMSCV-EPORMpl to obtain pMSCV-EPORwt-ires-mitoEYFP, pMSCV-EPORGCR-ires-mitoEYFP, and pMSCV-EPORMpl-ires-mitoEYFP, respectively. The final plasmids were certified as correct by sequence analysis.

Retroviral vectors

To obtain ecotropic retroviral vectors, BOSC23 cells were transfected with mouse stem cell virus (MSCV)-based retroviral plasmids (derivatives from pMSCV, see above) using Lipofectamine Plus (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol and the supernatants containing the ecotropic retroviral vectors were harvested 48–72 h post-transfection. The titers were 1×10^6/ml as assessed by RNA dot-blot. To obtain amphotropic retroviral vectors, 293T cells were transfected with MSCV-based retroviral plasmids along with pCL-Ampho (Imgemex, San Diego, CA, USA) using Lipofectamine Plus and the supernatants containing the amphotropic retroviral vectors were harvested 48–72 h post-transfection. The titers were 1×10^6/ml as assessed by RNA dot-blot.

Retroviral transduction and culture

Ba/F3 cells were suspended in 1 ml retroviral supernatant containing 10 ng/ml rmIL-3 at a density of 1×10^5 cells/ml, and transferred to 12-well plates coated with 20 µg/cm^2 of RetroNectin (Takara Bio, Shiga, Japan) [22]. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h. During this period, culture medium was replaced by fresh viral supernatant twice (every 12 h). After retroviral infection, YFP-positive cells were isolated using an EPICS ELITE cell sorter (Coulter, Miami, FL, USA). The purity of sorted YFP-positive cells was greater than 98%. The sorted Ba/F3 cells were subjected to further liquid culture (described above) or cell proliferation assays (see below).

Human cord blood CD34$^+$ cells (BioWhittaker, Walkersville, MD, USA) were thawed and placed in 12-well plates coated with 20 µg/cm^2 of RetroNectin and cultured for 24 h at 37 °C with 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL) supplemented with

10% FBS (Hyclone, Logan, UT, USA), 50 ng/ml recombinant human interleukin 6 (rhIL-6; Ajinomoto, Osaka, Japan), 100 ng/ml recombinant human stem cell factor (rhSCF; Biosource, Camarillo, CA, USA), 100 ng/ml recombinant human Flt-3 ligand (Research Diagnostic, Flanders, NJ, USA), and 100 ng/ml recombinant human thrombopoietin (rhTPO; Kirin, Tokyo, Japan). The cells were then resuspended in 1 ml viral supernatant containing the same cytokines as described above at a starting density of 1×10^5 cells/ml. During the transduction period (48 h), culture medium was replaced by fresh viral supernatant four times (every 12 h). After retroviral transduction, human cord blood CD34$^+$ cells were washed twice and cultured in IMDM medium containing 10% FBS (Hyclone) and 1% penicillin/streptomycin in the presence of 10 ng/ml recombinant human EPO (rhEPO; Roche Diagnostics, Mannheim, Germany) in a 37 °C 5% CO_2 incubator. The cells were subjected to flow cytometry or colony assay (see below) on indicated days.

Cell proliferation assay

Ba/F3 proliferation assay was performed using the CellTier 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. In brief, 20 µl MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium)-labeling mixture was added to each well of 96-well dishes containing cells to be assayed. Following incubation at 37 °C for 2 h, the spectrophotometric absorbance was measured at wavelengths of 490 and 650 nm. A_{490}-A_{650} values were used to determine Ba/F3 cell proliferation. Experiments were conducted in triplicate.

Flow cytometry

Human cord blood CD34$^+$ cells were washed and resuspended in CellWASH (Becton Dickinson, San Jose, CA, USA). The cells were then incubated with phycoerythrin (PE)-labeled anti-c-Kit (Nichirei, Tokyo, Japan), PE-labeled anti-glycophorin A (Nichirei), PE-labeled anti-CD41 (Nichirei), or PE-labeled anti-CD15 (Immunotech, Marseille, France) at 4 °C for 30 min. The cells were washed once and subjected to a FACSCalibur (Becton Dickinson) using excitation at 488 nm. Untransduced cells served as negative controls.

For mouse blood samples, blood cells were suspended in ACK lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan) to dissolve red blood cells. The cells were washed once and subjected to a FACSCalibur (Becton Dickinson) using excitation at 488 nm.

Colony assay and PCR

Human cord blood CD34$^+$ cells were plated in 35-mm dishes with α-minimum essential medium (Gibco-BRL)

containing 1.2% methylcellulose (Shinetsu Kagaku, Tokyo, Japan) supplemented with 20% FBS (Intergen, Purchase, NY, USA) and 1% bovine serum albumin (Sigma, St. Louis, MO, USA) in the presence of 100 ng/ml rhSCF, 100 ng/ml rhIL-6, and 100 ng/ml recombinant human interleukin 3 (rhIL-3; PeproTech, London, UK), or in the presence of 20 ng/ml of rhEPO alone. After incubation for 14 days at 37 °C in a humidified atmosphere of 5% CO_2 in air, colonies were scored under an inverted microscope. The experiments were performed in triplicate.

Colonies in methylcellulose culture were plucked up under an inverted microscope, suspended in 50 µl of distilled water, and digested with 20 µg/ml proteinase K (Takara) at 55 °C for 1 h followed by incubation at 99 °C for 10 min. PCR was performed to amplify the 351-bp sequence using the EYFP sense primer (5'-CGT CCA GGA GCG CAC CAT CTT C-3') and antisense primer (5'-AGT CCG CCC TGA GCA AAG ACC-3'). To certify the initial DNA amounts, the β-actin genomic DNA fragment was simultaneously amplified using the sense primer (5'-CAT TGT CAT GGA CTC TGG CGA CGG-3') and antisense primer (5'-CAT CTC CTG CTC GAA GTC TAG GGC-3'). Amplification conditions were 95 °C for 1min, 55 °C for 30 s, and 72 °C for 30 s with 35 cycles.

Mouse transplantation

Eight-week-old C57Bl/6 mice (Charles River Japan, Yokohama, Japan) intraperitoneally received 150 µg/kg 5-fluorouracil (Sigma). Forty-eight hours after injection, bone marrow cells were harvested from the femora of each mouse. Cells were cultured in IMDM (Gibco-BRL) containing 20% FBS (Hyclone) and 20 ng/ml rhIL-6 and 100 ng/ml recombinant rat SCF (provided by Amgen) for 48 h. The cells were then placed in 6-well plates coated with 20 µg/cm² of RetroNectin and resuspended in IMDM supplemented with 10% FBS (Hyclone) and the aforementioned cytokines at a starting density of 5×10^5 cells/ml. During the transduction period (48 h), culture medium was replaced by fresh viral supernatant four times (every 12 h). The cells were harvested after a total of 96 h (4 days) in culture, washed with PBS three times, and injected into 8-week-old female C57/Bl6 mice that had been irradiated with 800 cGy. After transplantation, some mice received recombinant mouse EPO (rmEPO; 200 IU/kg, Roche Diagnostics) in a total volume of 100 µl via the tail vein three times a week. To avoid development of anemia after drawing blood from the transplanted mice, blood was transfused into the mice via the tail vein at the time of blood drawing. The blood for transfusion was drawn from donor C57/Bl6 mice and pooled. It was irradiated with 20 Gy and diluted with physiological salt solution prior to transfusion. Peripheral blood mononuclear cells of the recipient mice were analyzed for EYFP expression by flow cytometry.

Results

A new generation SAG

The structure of the SAGs is shown in Figure 1. The prototype SAG (steroid-driven SAG) is a chimeric gene encoding the G-CSF receptor (GCR) and the estrogen receptor hormone-binding domain. In the GCR, the ligand (G-CSF)-binding domain was deleted to remove the responsiveness to endogenous G-CSF [2]. The tyrosine residue at the 703rd amino acid in the GCR was replaced by phenylalanine to hamper the differentiation signal [3]. In addition, another mutation (G525R) was introduced into the estrogen receptor hormone-binding domain to evade the responsiveness to endogenous estrogen without impairing the responsiveness to synthetic hormones such as tamoxifen [4]. In this study, we constructed a new generation SAG, in which the erythropoietin (EPO) receptor (EPOR) is utilized instead of the estrogen or tamoxifen receptor as a molecular switch. Two types of EPO-driven SAG were constructed, EPORGCR and EPORMpl, containing the GCR gene and the thrombopoietin (TPO)
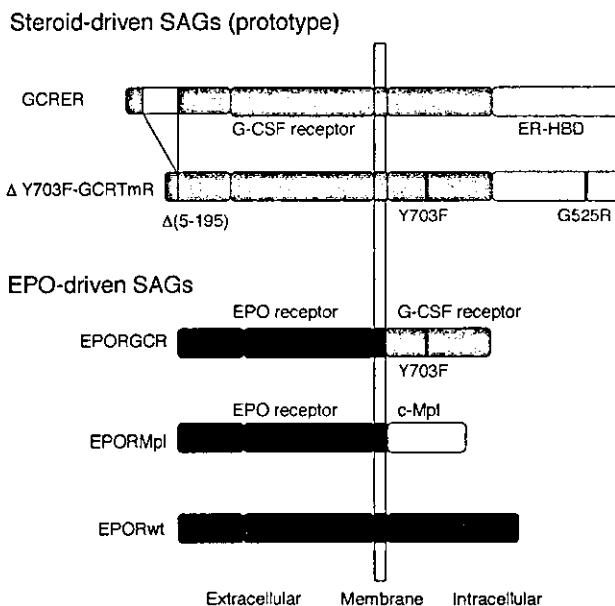
Figure 1. The structure of SAGs. The GCRER is a prototype of the selective amplifier gene (SAG), a chimeric gene encoding the G-CSF receptor (GCR) as a growth-signal generator and the estrogen receptor hormone-binding domain (ER-HBD) as a molecular switch. In ΔY703F-GCRTmR, the G-CSF-binding domain was deleted from the GCR gene to abolish responsiveness to endogenous G-CSF, a point mutation (Y703F) was introduced into the GCR moiety to disrupt the differentiation signal generated by the GCR, and another point mutation (G525R) was introduced into the ER-HBD moiety to evade responsiveness to endogenous estrogen without impairing responsiveness to a synthetic hormone tamoxifen. In the new SAG, the erythropoietin (EPO) receptor (EPOR) was utilized instead of the estrogen or tamoxifen receptor as a molecular switch. To construct it, the intracellular domain of the wild-type EPOR (EPORwt) gene was replaced by that of the GCR or thrombopoietin receptor (c-Mpl) gene as a growth-signal generator

receptor (c-Mpl) gene, respectively, as a growth-signal generator.

In vitro effects of the EPO-driven SAG on Ba/F3

Bicistronic retroviral vectors were generated which express the EPO-driven SAG or wild-type EPOR (EPORwt) gene as the first cistron and the EYFP gene as the second cistron. The vectors were infected into Ba/F3 cells. Ba/F3 is a mouse pro-B cell line and the cells require IL-3 for growth. YFP-positive cells were isolated (>98% purity) and stimulated by rhEPO at various concentrations (Figure 2A). All the cells acquired the ability of EPO-dependent growth and were able to proliferate even in the absence of IL-3. Ba/F3 cells expressing either EPORwt, EPORGCR, or EPORMpl reached the maximum growth levels by adding 1–100 ng/ml EPO (Figure 2A). Endogenous EPO will not induce a significant proliferative response of the cells, since the physiological range of serum EPO concentrations is below 0.1 ng/ml.

We compared the EPO- and steroid-driven SAGs in terms of their ability to expand Ba/F3 cells. The Ba/F3 cells expressing either of the two EPO-driven SAGs proliferated in the presence of EPO to the same extent as the parental Ba/F3 cells in the presence of IL-3. Of note, the EPO-driven SAG (EPORGCR) expanded Ba/F3 cells by around 10^4-fold more than the steroid-driven counterpart (ΔGCRTmR) after 2 weeks of culture (Figure 2B), indicating that the molecular switch using the EPOR is more efficient than that using the tamoxifen

receptor despite the inclusion of the same signal generator (GCR) in the SAGs. Thus, we used EPO-driven SAGs for subsequent experiments.

In vitro effects of the EPO-driven SAGs on human CD34$^+$ cells

To examine whether GCR or c-Mpl is the more suitable signal generator of the EPO-driven SAG, human cord blood CD34$^+$ cells were used as targets. CD34$^+$ cells were transduced with bicistronic retroviral vectors which express the EPO-driven SAG as the first cistron and the EYFP gene as the second cistron. After transduction, $27.3 \pm 4.7\%$ of the cells fluoresced (YFP-positive). The transduced CD34$^+$ cells were then cultured in liquid medium in the presence of EPO. The fraction of YFP-positive cells increased over time, and virtually all (>95%) of the cells became YFP-positive during a 2-week culture with EPO. This suggests that the EPO-driven SAGs are able to confer a growth advantage on human CD34$^+$ cells. As shown in Figure 3, although the cells transduced with EPORwt proliferated most quickly, the cell number already began to decrease within 2 weeks after the culture initiation. The cells transduced with EPORGCR grew slowly compared with the others, but began to decrease in number by week 3. On the other hand, the cells transduced with EPORMpl proliferated the longest (1 month) in the presence of EPO and the cell number increased by 10^4-fold over this period.
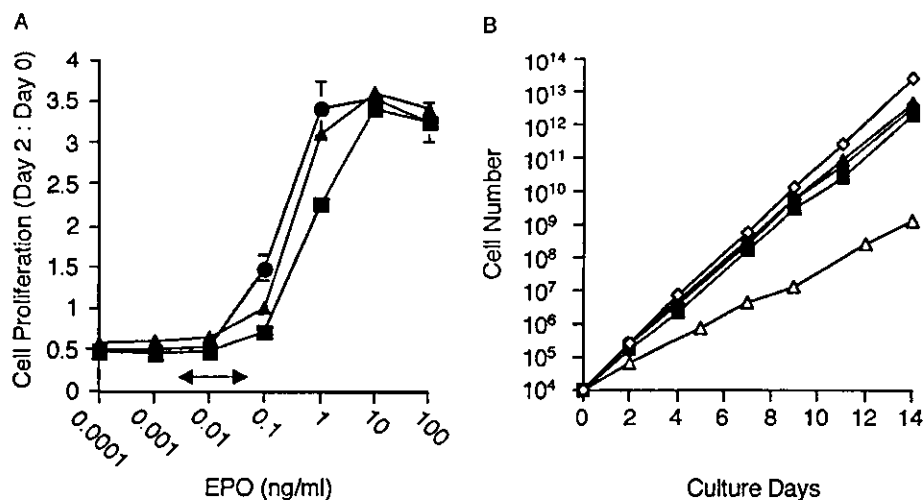
Figure 2. The EPO-driven SAG efficiently stimulates Ba/F3 cell growth. (A) EPO-dependent growth of Ba/F3 cells by introduction of the EPO-driven SAG. BaF3 cells were transduced with the EPORwt (solid triangles), EPORGCR (solid squares) or EPORMpl gene (solid circles) each along with the EYFP gene by bicistronic retroviral vectors. YFP-positive cells were sorted (>98%) and treated with EPO at various concentrations. The proliferation assay (see Materials and Methods) was performed on days 0 and 2, and the ratio of day 2 A_{490}-A_{650} to day 0 A_{490}-A_{650} (means $\pm$ SD of triplicate) is shown. The arrow indicates the physiological range of EPO concentrations in human plasma. (B) The EPO-driven SAG triggers higher levels of cell proliferation than the steroid-driven SAG. The parental Ba/F3 cells (open diamonds) were cultured in the presence of IL-3 (10 ng/ml). Ba/F3 cells transduced with the EPORwt (solid triangles), EPORGCR (solid squares), or EPORMpl gene (solid circles) were cultured in the presence of rhEPO (10 ng/ml). Ba/F3 cells transduced with the ΔGCRTmR gene (open triangles) were cultured in the presence of tamoxifen (10^{-7} M). Accumulative data were calculated by means of a triplicate experiment. Experiments were repeated three times and a representative one is shown
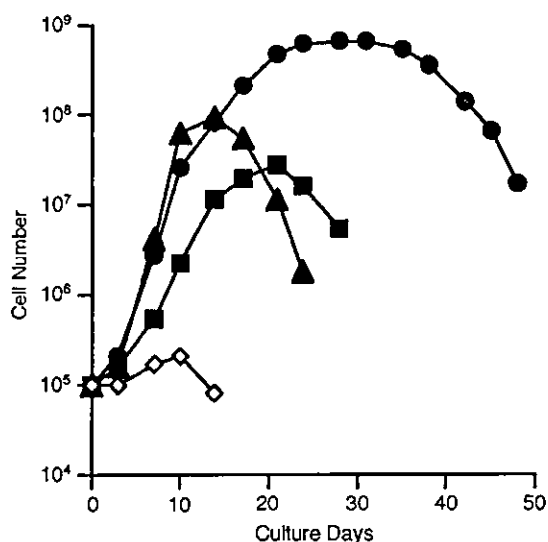
Figure 3. The EPORMpl is the most potent amplifier for human cord blood CD34$^+$ cells. Human cord blood CD34$^+$ cells were transduced with the EPOwt (solid triangles), EPORGCR (solid squares), or EPORMpl gene (solid circles) each along with the EYFP gene by bicistronic retroviral vectors. Untransduced cells are also shown (open diamonds). The cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO. Virtually all the cells (>95%) became YFP-positive by week 2. Accumulative data were calculated by means of a triplicate experiment. Experiments were repeated three times and a representative one is shown

Characterization of the c-Mpl signal of SAG

The transduced CD34$^+$ cells were then examined for the expression of c-Kit, a primitive hematopoietic cell marker, by flow cytometry (Figure 4). The c-Kit$^+$ fraction decreased over time, implying that the cells were differentiated during culture. The c-Kit$^+$ fraction in the cells transduced with EPORMpl, however, was relatively high (33%) at week 3 in liquid culture, whereas the c-Kit$^+$ fraction decreased to 10% or lower in the cells transduced with EPORwt or EPORGCR at the same time point. These results demonstrate that the c-Mpl signal preserved more c-Kit$^+$ immature hematopoietic cells than the other signals.

To examine the EPO-driven SAGs for their ability to expand hematopoietic progenitor cells, CD34$^+$ cells transduced with the EPO-driven SAGs were cultured in semisolid (methylcellulose) media in the presence of multiple cytokines (IL-3, IL-6 and SCF) or EPO alone. Table 1 summarizes the results. The cells transduced with the EPO-driven SAGs formed many colonies in the presence of EPO and almost all of them (94–100%) contained the provirus as assessed by individual colony PCR. In contrast, 25–38% of the colonies formed by cells in the presence of multiple cytokines contained the provirus. This result shows that the EPO-driven SAGs are able to confer an EPO-dependent growth advantage at the level of clonogenic progenitor cells. The cells transduced with the EPO-driven SAGs before (day 0) and after (day 7) liquid culture with EPO were placed in semisolid media in the presence of EPO without other cytokines, and the resultant myeloid and erythroid colonies were counted. As shown in Figure 5, during the liquid culture with EPO, the transduction by EPORMpl resulted in the highest levels of clonogenic progenitor cell expansion by more than 10-fold.

We then examined whether cells transduced with the EPO-driven SAGs would show any specific lineage
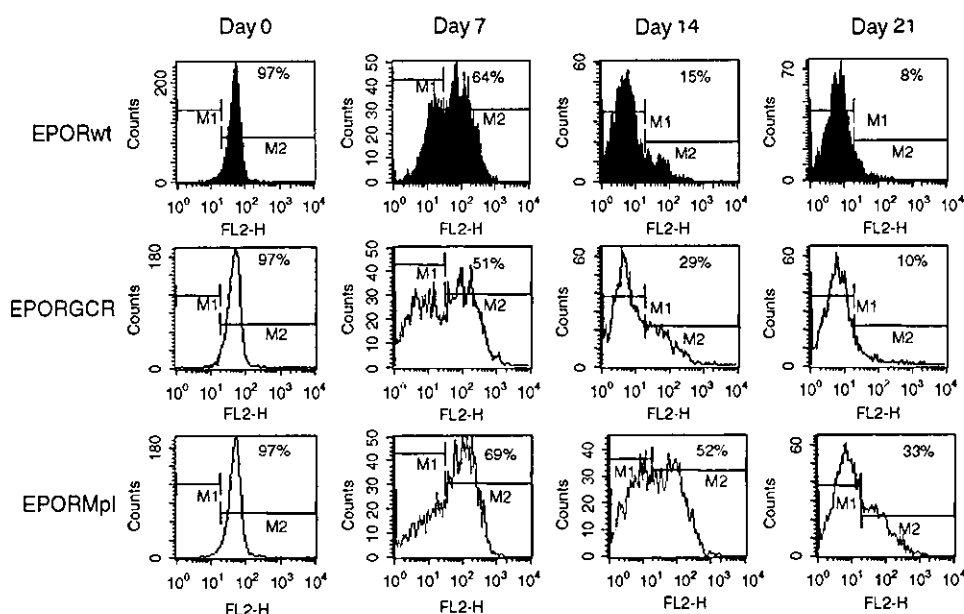
Figure 4. The EPOR-Mpl preserves c-Kit$^+$ cells most efficiently. Human cord blood CD34$^+$ cells were transduced with the EPOwt (black), EPORGCR (gray), or EPORMpl gene (white) by the same retroviral vectors as in Figure 3. The cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO. On the indicated days, aliquots of the cells were examined for c-Kit expression by flow cytometry. The percentage of c-Kit$^+$ cells is shown. Experiments were repeated four times and a representative profile is shown

Table 1. Colony formation by human cord blood CD34+ cells transduced with the EPO-driven SAGs

Transgene	IL-3 (100 ng/ml) IL-6 (100 ng/ml) SCF (100 ng/ml)		EPO (20 ng/ml)	
	Number of colonies*	Provirus-positive colonies†	Number of colonies*	Provirus-positive colonies†
EPORwt-YFP	62 ± 11	5/16 (31%)	15 ± 3	15/16 (94%)
EPORGCR-YFP	54 ± 8	6/16 (38%)	24 ± 1	16/16 (100%)
EPORMpl-YFP	54 ± 9	4/16 (25%)	31 ± 6	15/16 (94%)
YFP	49 ± 4	8/16 (50%)	12 ± 1	9/16 (56%)
Untransduced	53 ± 4	ND	17 ± 1	ND

*Colony number out of 200 cells is shown. Each value represents mean ± SD of triplicate culture.
†Individual colony DNA was subjected to PCR for the proviral YFP and genomic β-actin sequences and the ratio of the provirus-positive colony number to the β-actin-positive colony number is shown.

preference after liquid culture with EPO. The transduced CD34+ cells were cultured in liquid medium containing EPO. During the culture, the expression of various differentiation markers was examined by flow cytometry (Figure 6). As expected, the erythroid marker (glycophorin A) was expressed in almost all (93%) cells transduced with EPORwt at day 14. The myeloid marker (CD15) was expressed in 24% of cells transduced with EPORGCR at day 7 (data not shown), but fell to 1% by

day 14. Thus, EPORGCR induced very few cells to differentiate toward the myeloid lineage despite the inclusion of the GCR moiety as a signal generator. One reason may be that a point mutation (Y703F) was introduced into the GCR cDNA to attenuate the granulocytic differentiation signal (Figure 1) [3]. On the other hand, cells transduced with EPORMpl expressed all of these markers at relatively high levels at day 14; the megakaryocytic marker (CD41) (46%), glycophorin A (58%) and CD15 (11%). Thus, the cells expanded by the c-Mpl signal showed the most balanced expression of myeloid, erythroid, and megakaryocyte markers. We therefore decided to utilize EPORMpl as an SAG for subsequent *in vivo* experiments in mice.

In vivo expansion of gene-modified cells

Finally, we examined the efficacy of the EPOMpl-type SAG *in vivo* in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the MSCV-based vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced cells were transplanted into irradiated mice and, after hematopoietic reconstitution, YFP expression was examined in the peripheral blood by flow cytometry to see whether the EPOMpl-transduced cells would increase in response to EPO administration. In mice, however, even drawing a small volume of blood will result in the elevation of endogenous EPO concentrations [23,24]. We also confirmed that sequential blood drawing caused an elevation of endogenous serum EPO concentrations in mice (data not shown). Therefore, drawing blood from the transplanted mice may result in the expansion of transduced hematopoietic cells. To avoid development of anemia due to blood drawing, we
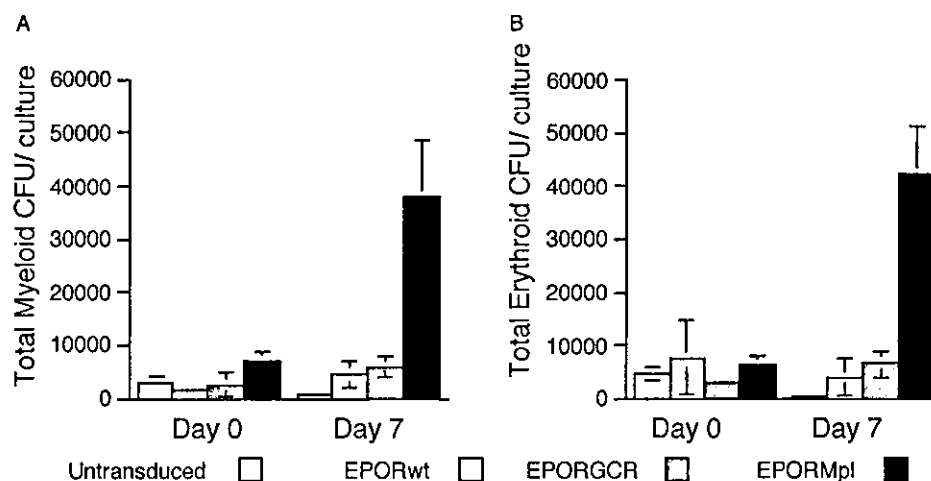
Figure 5. The EPOR-Mpl expands clonogenic progenitor cells most efficiently. Human cord blood CD34+ cells were transduced with the EPOwt, EPORGCR or EPORMpl gene by the same retroviral vectors as in Figure 3. The untransduced and transduced cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml EPO for 7 days. The cells before (day 0) and after (day 7) the liquid culture were plated in methylcellulose medium in the presence of EPO alone and the resultant colonies were counted. (A) Total myeloid clonogenic progenitor cell (colony-forming units, CFU) numbers per culture. (B) Total erythroid CFU numbers per culture. Means ± SD of a triplicate experiment are shown. Experiments were repeated three times and a representative one is shown
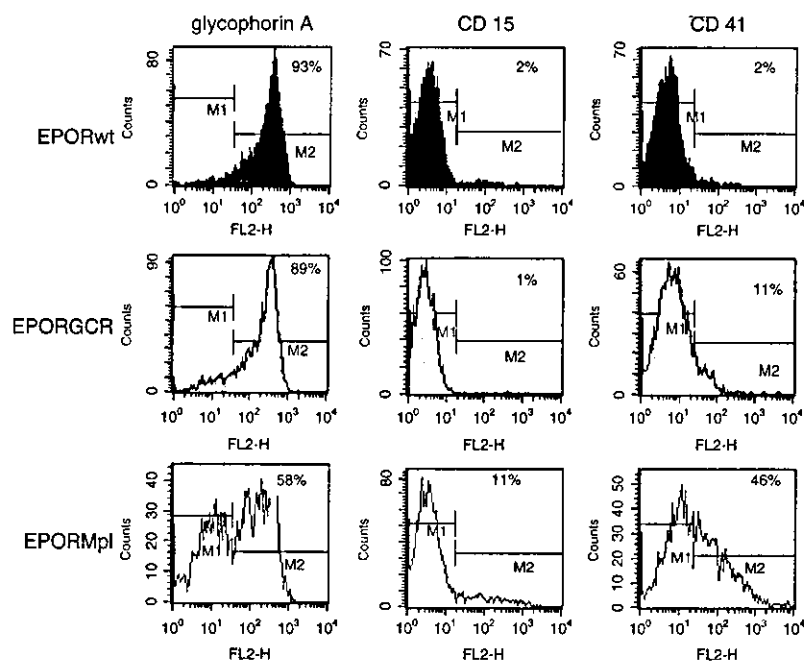
Figure 6. The CD34$^+$ cells expanded by the EPOR-Mpl show the most balanced expression of multilineage surface markers. Human cord blood CD34$^+$ cells were transduced with the EPOwt, EPORGCR, or EPORMpl gene by the same retroviral vectors as in Figure 3. After 14-day liquid culture with 10% FBS and 10 ng/ml EPO, the transduced cells were examined for the expression of glycophorin A (erythroid marker), CD15 (myeloid marker), and CD41 (megakaryocyte marker) by flow cytometry. The percentages of marker-positive cells are shown. Experiments were repeated four times and a representative profile is shown

transfused mice at the time of blood drawing. As a result, the mice did not develop anemia, and thus the elevation of endogenous EPO concentration was prevented.

In the group receiving EPORMpl, YFP-positive cells increased in response to the EPO administration (n = 6), although YFP-positive cells remained unchanged without EPO administration (n = 4) (Figure 7A). A significant increase (paired t-test, $p < 0.05$) in YFP-positive cells was observed 4 weeks after the initiation of EPO administration. The increase was attributable to that in granulocytes and monocytes (data not shown). We could not detect any significant change in other lineages. The increase seemed transient, as a significant increase was no longer observed at further time points. On the other hand, in the control group receiving YFP alone without EPORMpl, YFP-positive cells remained unchanged at around 10% in the peripheral blood regardless of EPO administration (n = 6 for a subgroup with EPO, n = 6 for a subgroup without EPO; Figure 7B).

Discussion

Although a few HSC gene therapy trials have proven successful [25,26], most attempts have been hampered by the low efficiency of gene transfer into HSCs. To overcome the problem, we have previously developed a method of selective *in vivo* amplification of transduced hematopoietic cells using a 'selective amplifier gene' (SAG) which encodes a fusion protein consisting of a growth-signal generator and its molecular switch. The prototype SAG encodes a fusion protein between the

GCR and the estrogen or tamoxifen receptor, and confers a growth advantage on gene-modified hematopoietic cells in an estrogen- or tamoxifen-inducible fashion *in vivo* [6,7]. In the present study, we developed a new generation SAG which utilizes the EPOR as a molecular switch instead of the steroid receptor. The EPO-driven SAG encodes a fusion protein between the extracellular plus transmembrane domain of the EPOR and the cytoplasmic domain of the GCR or c-Mpl. The results reported here indicated that the SAG utilizing the EPOR as a molecular switch is more efficient for hematopoietic cell proliferation than that utilizing the steroid (or tamoxifen) receptor despite the inclusion of the same signal generator in the SAGs.

Cytokine receptors generate the growth signal through ligand-induced dimerization. Dimerization is necessary but not sufficient for optimal signal generation [27,28]. The EPO-driven SAG might have allowed more effective ligand-induced conformation change than the steroid-driven SAG. Similar to our chimeric receptors, Blau *et al.* developed a cell growth switch that is a cytokine receptor-FK506 binding protein (FKBP) fusion gene to confer inducible proliferation to transduced cells [29,30]. In their system, cytokine receptor signal is turned on by treatment with a synthetic dimerizer FK1012 or its derivatives. However, it remains unclear whether their chimeric protein would allow effective ligand-induced conformation change to the same extent as the EPO-driven SAG.

We also showed that the c-Mpl signal expanded clonogenic progenitor cells (CFU) far more efficiently than the EPOR or GCR signal. In addition, the cells expanded
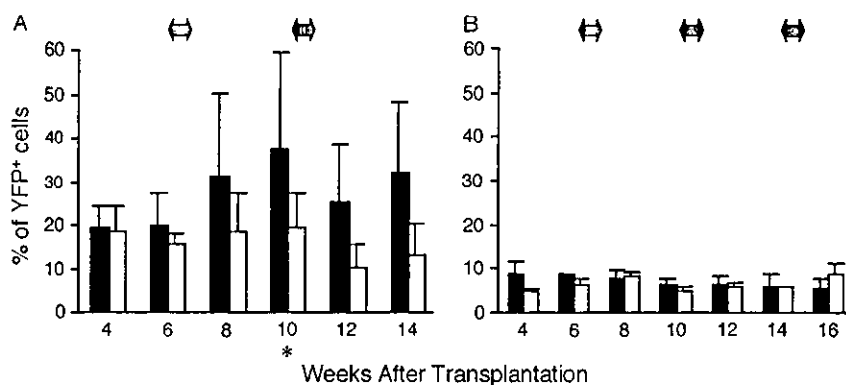
Figure 7. The gene-modified hematopoietic cells can be expanded by treatment with EPO *in vivo* in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the retroviral vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced cells were transplanted into irradiated mice. The percentages of YFP-positive cells in the peripheral blood are shown for the EPOR-Mpl group (A) or the YFP control group (B). In each group, mice were divided into two subgroups: EPO-treated subgroup (solid bars, 200 IU/kg, three times a week, n = 6 each for A and B) and EPO-untreated subgroup (open bars, n = 4 for A and n = 6 for B). The gray arrows in A and B indicate the week of EPO administration. Means ± SD of each subgroup are shown. The increase in YFP-positive cells in the EPO-treated mice was statistically significant at week 10 (4 weeks after the initiation of EPO administration) (*$p < 0.05$)

by the c-Mpl signal showed the most balanced expression of myeloid, erythroid, and megakaryocyte markers. Other investigators have also shown that the c-Mpl signal is able to efficiently support the growth of transduced murine bone marrow cells [31]. Taken together, the intracellular signal from c-Mpl may be suitable for reliable expansion of immature hematopoietic cells.

We have demonstrated that EPORMpl can confer an EPO-dependent growth advantage on the transduced hematopoietic cells *in vivo* in a mouse transplantation model. It should be noted that EPORMpl contains the human c-Mpl and may not have worked well in mouse cells. It would be more predictive to examine the efficacy of the EPORMpl in nonhuman primates. We are evaluating the efficacy of EPOMpl-type SAGs in the setting of a nonhuman primate transplantation protocol. In mice, the increase of transduced cells with EPORMpl seemed transient, as was the case with chimeric genes reported by other investigators [32,33]. The method may not result in the selection of transduced cells at the HSC level. The long terminal repeat (LTR) promoter may not express the transgene in HSCs. Alternatively, the c-Mpl signal may not induce proliferation of HSCs. Thus, the selection of transduced cells may occur only within the differentiated progeny of transduced HSCs, not at the level of transduced HSCs themselves. In order to obtain clinically relevant effects, repeated EPO administration would be required. Polycythemia may take place, but it can be treated by occasional phlebotomy safely. Given that our earlier version of SAG utilized estrogen receptor as a molecular switch, we believe that EPO is much safer than estrogen to turn on a molecular switch, since side effects induced by estrogen may not be well treated or controlled.

With the EPO-driven SAG, therapeutic effects may result from continuously elevated levels of endogenous EPO in patients with chronic anemia such as thalassemia. When anemia is ameliorated and endogenous EPO levels

return to physiological levels, the positive selection system is then 'automatically' turned off. This 'leave it to patients' system would be convenient. However, a safety concern may be raised regarding leukemogenesis, as the SAG proliferation signal is persistently turned on *in vivo* by endogenous EPO, although physiological levels of EPO will not induce a significant proliferative response. Since a set of EPO-mimetic peptides or a modified EPO such as the erythropoiesis stimulating protein (NESP) has been developed [34,35], it may be possible to develop an EPO-driven SAG containing a mutant EPOR which does not bind to endogenous EPO but binds to the EPO-mimetic peptides or modified EPO.

Acknowledgements

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Detection of CD3ε polymorphism in cynomolgus monkeys by a method based on RFLP

Uda A, Tanabayashi K, Mukai R, Terao K, Yamada A. Detection of CD3ε polymorphism in cynomolgus monkeys by a method based on RFLP. J Med Primatol 2004; 33:34–37. © Blackwell Munksgaard, 2004

Akihiko Uda[1],
Kiyoshi Tanabayashi[1],
Ryozaburo Mukai[1], Keiji Terao[1],
Akio Yamada[2]

[1]Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Ibaraki, [2]Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan

Abstract: We previously reported that peripheral lymphocytes from about 12% of cynomolgus monkeys lacked reactivity with anti-rhesus monkey CD3 monoclonal antibody (FN18). The nucleotide sequence analysis of the genes encoding CD3 component proteins revealed that a single amino acid substitutions found in the CD3ε chain determined the phenotype. In this study, we attempted to develop a method based on the restriction fragment length polymorphism (RFLP) and apply it for determination of the genotypes of individual monkeys. Comparison of the phenotype determined by fluorescence-activated cell sorter analysis with the genotype determined by RFLP analysis revealed that the FN18 -positive trait was dominant over the FN18-negative trait. It was also revealed that allele frequency was significantly different among macaques depending on the geographical region where their ancestors were derived from.

Introduction

Cynomolgus monkeys (*Macaca fascicularis*) are important experimental animals for biomedical research and understanding immunobiology of these animals is essential for interpretation of experimental data. The FN18 monoclonal antibody (mAb), which was raised against CD3 molecules of rhesus monkey (*Macaca mulatta*), is also able to be used for identification of T cells of cynomolgus monkey; however, it was shown that T cells from some cynomolgus monkeys and rhesus monkeys did not react with FN18 mAb [1–3, 7, 8].

The nucleotide sequence analysis of cDNAs coding for CD3 components showed that CD3ε chain from FN18 non-reactive cynomolgus monkeys had two common amino acid substitutions at positions 67 and 72 [8]. We have further shown that the amino acid at position 67 played a key role in determining the FN18 responsiveness by *in vitro* experiments using several mutated CD3ε genes [7].

In this study, we attempted to establish a method for genotyping individual monkeys based on the

polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method and used the method to determine the allele frequencies among cynomolgus monkeys derived from different geographical regions.

Materials and Methods

Animals

All the cynomolgus monkeys studied here were raised and reared in the Tsukuba Primate Center for Medical Science, NIID. Both genders were involved and the ages of the monkeys were between 2 and 16 years. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Disease.

RFLP analysis

PolyA mRNA extracted from peripheral blood mononuclear cells (PBMCs) of FN18-reactive

cynomolgus monkeys using the Quickprep micro mRNA purification kit (Amersham, Uppsala, Sweden) was converted into cDNA using high fidelity RNA PCR kit (Takara, Shiga, Japan). Genomic DNA was extracted form PBMCs using the Gene-TLE solution (Takara). PCR of CD3ε chain was performed in 20 μl reaction mixture containing ε-sense primer (5'-CTC CAT CTC TGG AAC CAC AGT A-3') and ε-anti-sense primer (5'-CAG GTA GAG ATG ATG GCT CG-3'), 0.6–0.8 μg of genomic DNA or cDNA and 0.6 U of ExTaq polymerase (Takara). The size of PCR products was expected to be 207 bp. The reaction mixtures were heated at 95°C for 5 minutes, and then 40 cycles of amplification consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s was carried out followed by an additional extension at 72°C for 7 minutes. About 0.3–0.5 μg of amplified DNA were digested with 2.5 U of MboII at 37°C for 2 hour followed by agarose gel electrophoresis using 4% gel. As similar results were obtained using both cDNA and genomic DNA as templates for PCR, most part of this study was conducted using the genomic DNA as template.

Fluorescence-activated cell sorter (FACS) analysis

The PBMCs were isolated from fresh blood by standard Ficoll-Hypaque gradient centrifugation method. PBMCs were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 containing 100 U/ml, 0.1 mg/ml streptomycin, and 10% foetal calf serum at a concentration of 10^6 cells/ml. PBMCs (10^5) were incubated at 4°C for 1 hour with FN18 mAb (Biosource, Camlio, CA, USA), and washed twice with PBS containing 1% bovine serum albumin. After fixation with 1% paraformaldehyde at 4°C for 30 minutes, FACS analysis (FACS Caliber; Becton Dickinson, Cockeysville, MD, USA) was performed.

Results

Differentiation of genotype by the PCR-RFLP

As the nucleotide at position 200 of the FN18+ and FN18– cDNA clone was A and G,

respectively, and the flanking sequence, GAAGA, gave rise to a recognition site by restriction enzyme MboII (Fig. 1), it was likely that the nucleotide difference could readily be differentiated by MboII digestion. As expected, the PCR amplicons derived from cloned FN18+ DNA was digested by MboII yielding two smaller bands, but that from FN18– clone was resistant to the digestion with MboII (Fig. 2A). We therefore attempted to apply this technique to determine the genotype of individual monkeys. There should be three distinct electrophoreic patterns of MboII-digested fragments. The PCR amplicons from homozygotes bearing A/A at position 200 should be resistant to MboII digestion, whereas those bearing G/G must be cut into two fragments. The PCR fragments from heterozygotes, however, would give rise to three fragments of 207, 113 and 94 bp, if properly digested. As incomplete digestion may be misleading, the PCR amplicons were subjected to complete digestion. In order to accomplish complete digestion, we used sufficient amount (2.5 U) of the enzyme to digest 0.3–0.5 μg of a short DNA fragment containing only one cutting site. Furthermore, the PCR amplicons from presumed heterozygotes were subjected to the nucleotide sequencing to assure that appearance of three fragments were due indeed to the heterozygocity. The results showed that both A and G were present at position 200 (data not shown), indicating that the presence of a 207-bp fragment after MboII digestion was because of the absence of MboII recognition site but not of incomplete digestion. These results indicate that RFLP analysis with MboII could be used as a powerful tool to determine the genotype of macaques.

Inheritance of the polymorphism

To analyse how this polymorphism is inherited, a family consisted of three parents (one sire and two dams) and four offspring were selected and subjected to the PCR–RFLP analysis. They were bred and raised in the Tsukuba Primate Center for Medical Science, NIID. By FACS analysis it was shown that the sire (3028) was FN18 negative while the two dams (3032 and 1159) were positive


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                                     67              72
            61   H N G K N K E  D S G  D R L  F L P E  F S E  80
FN18 +/+   181   CACAATGGTA AAAACAAAGA AGATTCTGGG GATCGGCTGT TTCTGCCGGA ATTTTCAGAA 240
FN18 -/-   181   ---------- --------G ---------- ----AA---- ---------- ---------- 240
            61   - - - - - - G  - - - - Q  - - - - - - - - -   80
```

Fig. 1. Nucleotide sequence of CD3ε. The nucleotide and the deduced amino acid sequences around polymorphic region were shown. Dots indicated identical nucleotide or amino acids. Recognition and cleavage site of MboII are indicated by underline and arrowhead, respectively.
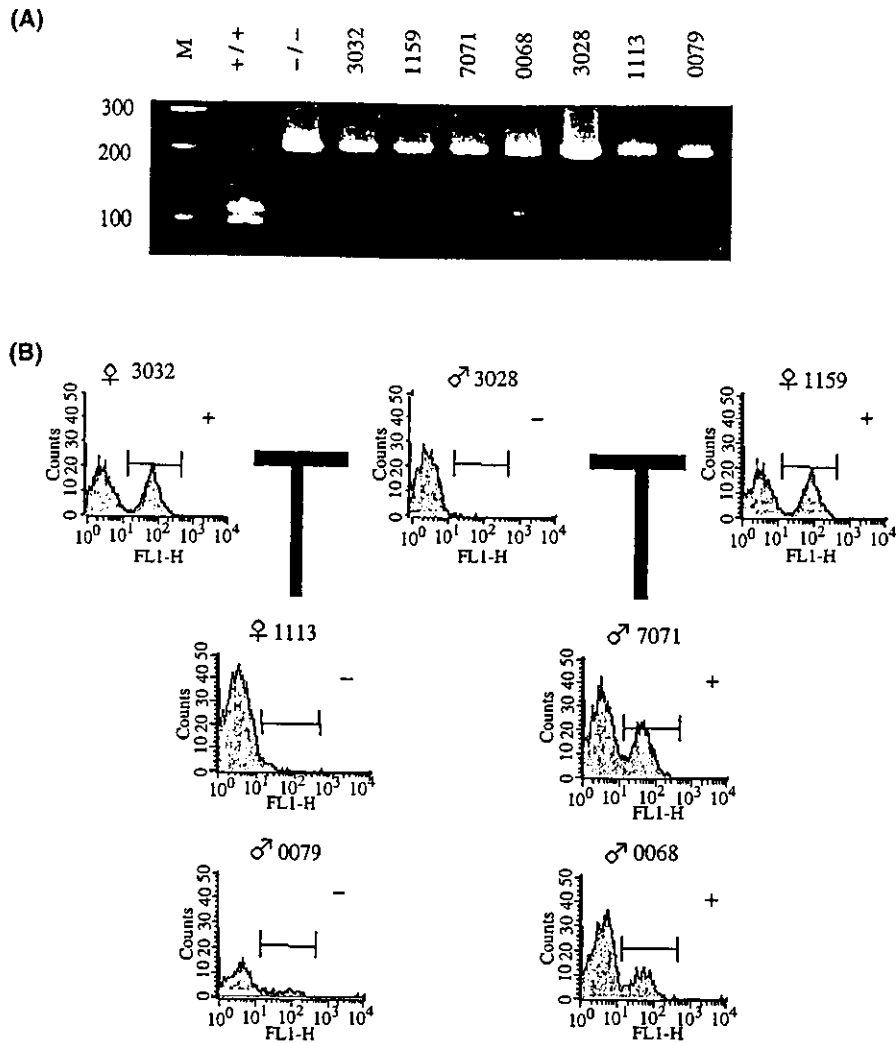
(A)

(B)

Fig. 2. The family pedigree demonstrating the inheritance of CD3ε genotypes and phenotypes. (A). The PCR products (207 bp) amplified from the genomic DNA of PBMCs were digested with *Mbo*II. The PCR products from cloned FN18 +/+ or –/– were also included as a positive or negative control. (B) The phenotypes regarding the reactivity with FN18 mAb were determined by FACS analysis. 1-H (*x*-axis): the fluorescence intensity of FN18 mAb.

(Fig. 2B). Similarly two offspring (1113 and 0079) were negative whereas the others (7071 and 0068) were positive. As the FACS profile of 0079 was rather ambiguous, we stained PBMC of this monkey with an mAb directed to monomorphic epitope of CD3ε (SP34). It was shown that SP34 positive cells did not react with FN18 confirming that 0079 was FN18 negative. The PCR–RFLP analysis using cDNA as templates showed that three monkeys (3028, 1113 and 0079) were homozygous (–/–) while the other monkeys (3032, 1159, 7071 and 0068) were heterozygous (+/–) (Fig. 2A). This finding indicates that these genes were co-dominantly expressed on RNA level. As there is no antibody available that would react with the protein expressed from the FN18 –/– genotype, the FN18-positive phenotype appeared to be

inherited according to the Mendelian rules, and dominant over FN18.

Allele frequency of monkeys from different geographical areas

We then applied the RFLP technique to determine the allele frequency of this particular single nucleotide polymorphism among cynomolgus monkeys whose ancestors were introduced from three different countries – Malaysia, Indonesia and Philippines. As shown in Table 1, the frequency of three genotypes, FN18 +/+, FN18 +/– and FN18 –/– in total, was 0.576, 0.339 and 0.085, respectively. It was noted, however, that the frequency of FN18 –/– was significantly higher (0.208) in the monkeys derived from Philippines

Table 1. The frequency of CD3ε genotype in cynomolgus monkeys

| Genotype | Country | | | | | | Total | |
| | Malaysia | | Indonesia | | Philippine | | | |
	Frequency	n	Frequency	n	Frequency	n	Frequency	n
FN18 +/+	0.808	38	0.675	52	0.226	12	0.576	102
FN18 +/–	0.149	7	0.299	23	0.566	30	0.339	60
FN18 –/–	0.043	2	0.026	2	0.208	11	0.085	15
Total	1.000	47	1.000	77	1.000	53	1.000	177

than in those from Malaysia (0.043) and Indonesia (0.026).

Discussion

In this study, we established a simple method for the detection of CD3 polymorphism, and applied the method to analyse the mode of inheritance of the CD3ε polymorphism. We also determined allele frequency among monkeys originated from different countries. We found that the frequency of FN18 –/– genotype was higher in the Philippine population. This might be caused by bottleneck effect as mating was carried out among monkeys of the same origin. Another possibility was that FN18-negative gene had diffused widely into Philippine population as FN18-negative phenotype might be advantageous in reproduction or adaptation, in particular environment of Philippines.

There are several reports suggesting that the polymorphism found in human CD3 might be related to type I diabetes [4–6], but controversial results are also reported. It would be interesting to see whether there are any differences in biological properties between macaques of Philippine and other places. It also seems important to look at whether there are linkages between this polymorphism and certain diseases in cynomolgus monkeys.

In conclusion, we established a simple method to identify the polymorphism of CD3ε by genotyping using RFLP. The RFLP analysis of a large number of monkeys demonstrated that the frequency of the genotype of the CD3ε differed among cynomolgus monkeys of different origin of country.

Acknowledgments

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