plantation model [20]. The expansion was transient, as is the case with other chimeric genes containing c-Mpl as a signal generator [20], although basal marking levels seemed to increase gradually after repeated EPO administration as shown in Fig. 3A. The method largely results in the selection of transduced cells, not at the level of HSCs, but within the differentiated progeny of transduced HSCs.

In the clinical setting, even if the expansion of gene-modified cells is transient, patients can expect therapeutic effects from EPO administration when used as necessary, such as for infection events in patients with chronic granulomatous disease. EPO is a safe drug and can be administered repeatedly with minimal adverse effects. Polycythemia was the only side effect observed in the present study but was manageable by periodic phlebotomy. Therapeutic effects might also be expected from continuously elevated levels of endogenous EPO, such as in patients with thalassemia. When anemia is ameliorated by the gene therapy and endogenous EPO levels return to physiological levels, then the positive selection system is "automatically" turned off, making this a convenient system in such disorders.

Although this "leave it to patients" system would be convenient, a safety concern may be raised regarding leukemogenesis [21]. The SAG proliferation signal that is persistently turned on *in vivo* by endogenous EPO could trigger a secondary event in addition to possible retroviral insertional mutagenesis, although physiological levels of EPO will not induce a significant proliferative response of SAG [12]. Since a set of EPO-mimetic peptides or a modified EPO such as the erythropoiesis stimulating protein has been developed [22,23], it may be possible to develop an SAG containing a mutant EPO receptor that does not bind to endogenous EPO but binds to such EPO-mimetic peptides or modified EPO.

Materials and Methods

Animals. Cynomolgus monkeys (*Macaca fascicularis*) were housed and handled in accordance with the rules for animal care and management of the Tsukuba Primate Center and the guiding principles for animal experiments using nonhuman primates formulated by the Primate Society of Japan. The animals (2.5–5.6 kg, 3–5 years) were certified free of intestinal parasites and seronegative for simian type-D retrovirus, herpesvirus B, varicella-zoster-like virus, and measles virus. The protocol of experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Collection of cynomolgus CD34⁺ cells. Cynomolgus monkeys received recombinant human (rh) SCF (50 μg/kg; Amgen, Thousand Oaks, CA, USA) and rhG-CSF (50 μg/kg; Chugai, Tokyo, Japan) as daily subcutaneous injections for 5 days prior to blood cell collection. Peripheral blood or bone marrow cells were then collected by leukapheresis or by aspiration from iliac bones, respectively. From the harvested cells, the leukocyte cell fraction was obtained after red blood cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan). Enrichment of CD34⁺ cells was performed using magnet beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY, USA), which cross-reacts with cynomolgus CD34 [24]. The purity of CD34⁺ cells ranged from 90 to 95% as assessed with another anti-human CD34 (clone 563; PharMingen, San Diego, CA, USA) which cross-reacts with cynomolgus CD34 [24]. Mean CFU enrichment was 48-fold as assessed by colony-forming progenitor assays performed before and after enrichment.

Retroviral transduction. We used a retroviral vector expressing SAG (a chimeric gene of the human EPO receptor extra- plus transmembrane region and c-Mpl cytoplasmic region) [12] and PLI nonexpression retroviral vector containing untranslated *neo*^{R} and *β-gal* sequences [13]. The titers of the viral supernatants used in the present study were both 1×10^6 particles/ml, as assessed by RNA dot blot. CD34⁺ cells were cultured at a starting concentration of $1–5 \times 10^5$ cells/ml in fresh vector supernatant of PLI or SAG with rhSCF (Amgen), rh thrombopoietin (Kirin, Tokyo, Japan), and rh Flt-3 ligand (Research Diagnostics, Flanders, NJ, USA), each at 100 ng/ml in dishes coated with 20 μg/cm^2 of RetroNectin (Takara, Shiga, Japan). Every 24 h, culture medium was replaced with fresh vector supernatant and cytokines. After 96-h transduction, cells were washed and continued in culture (Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) containing 10% fetal calf serum (Gibco) and 100 ng/ml rhSCF alone) for 2 additional days in the same RetroNectin-coated dishes [25].

Intrabone marrow transplantation. Cynomolgus monkeys were anesthetized. Two needles were inserted into both ends of the femur or humerus [26]. A syringe containing 50 ml of heparin-added saline was connected to one needle and an empty syringe was connected to the other. Normal saline was irrigated gently from one syringe to another through the marrow cavity twice (Fig. 1). Gene-modified cells were suspended in 1 ml of phosphate-buffered saline containing 10% autologous serum and then injected into the marrow cavity and the needle holes were sealed with bone wax (Lukens, Reading, PA, USA). We measured the internal pressure in the marrow cavity during the procedure in some animals and carefully performed saline irrigation and iBMT without inflicting extra pressure on the marrow cavity. No animals suffered from neutropenia, thrombocytopenia, infection, or pulmonary embolism and there was no morbidity. After transplantation, rhEPO (Chugai) was administered to some animals at a dose of 200 IU/kg once or twice daily subcutaneously. Administration of cyclosporin A (Novartis, Basel, Switzerland) to animals was started a week prior to the EPO administration to prevent the development of anti-human EPO antibody [27].

Clonogenic hematopoietic progenitor assays. Cells were plated in a 35-mm petri dish in 1 ml of α-minimum essential medium containing 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan) supplemented with 100 ng/ml rh interleukin-3 (PeproTech, Rocky Hill, NJ, USA), 100 ng/ml rh interleukin-11 (PeproTech), 100 ng/ml rhSCF (Biosource, Camarillo, CA, USA), 2 U/ml rhEPO (Roche, Basel, Switzerland), 20% fetal calf serum, 1% bovine serum albumin, 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). RhEPO was not added to the culture for colony formation from SAG-transduced cells, to avoid excess proliferative response of the transduced cells to EPO. After incubation for 14 days at 37°C with 5% CO_2, colonies containing more than 50 cells were counted using an inverted light microscope. Experiments were conducted in triplicate.

Quantitative PCR. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA). DNA (250 ng) was amplified in triplicate with *neo*-specific primers for PLI (5′-TCCATCATGGATGCAATGCGGC-3′ and 5′-GATAGAAGGCGATGCGCTGCGAATCG-3′) or with SAG-specific primers (5′-GACGCTCTCCCTCATCCTCGT-3′ and 5′-GAGGACTTGGGGAGGATTTCA-3′). Standards consisted of DNA extracted from an SAG- or PLI-producer cell line (which has a known copy number of the proviral sequence) serially diluted with control cynomolgus genomic DNA. Negative controls consisted of DNA extracted from peripheral blood cells of naive monkeys. A β-actin-specific primer set (5′-

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CCTATCAGAAAGTGGTGGCTGG-3′, 5′-TTGGACAGCAAGAAAGTGAGCTT-3′) was used to certify equal loading of DNA per reaction. Reactions were run using the Qiagen SYBR Green PCR Master Mix (Qiagen) on the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using the following conditions: 50°C for 2 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 62°C for 30 s, 72°C for 30 s, and 83°C for 15 s. The quantitative PCR was certified each time to yield linear amplifications in the range of the intensity of a positive control series (0.01–100%, correlation coefficient >0.98). For calculating the transduction efficiencies, the C_t value of the vector sequence was normalized based on the C_t value of the internal control β-actin sequence on the same sample as directed in the manufacturer's protocol. Gene marking percentages were calculated given that each provirus-positive cell contains one copy of the vector sequence.

Colony PCR. Well-separated, individual colonies at day 14 were plucked into 50 μl of distilled water, digested with 20 μg/ml proteinase K (Takara) at 55°C for 1 h followed by 99°C for 10 min, and assessed for the SAG or nonexpression PLI vector sequence by nested PCR. The outer primer sets were the same as were used in the quantitative PCR described above. Amplification conditions for the outer PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 20 cycles. The outer PCR products were purified using MicroSpin S-400 HR Columns (Amersham, Piscataway, NJ, USA). The inner primer set for the SAG vector was 5′-CCACCCCTAGCCCTAAATCTTATG-3′ and 5′-GGTGGTTCAGCATCCAATAAGG-3′, and that for the PLI vector was 5′-ATACGCTTGATCCGGCTACCTG-3′ and 5′-GATACCGTAAAGCACGAGGAAG-3′. Amplification conditions for the inner PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 20 cycles. Simultaneous PCR for the β-actin sequence was also performed to certify DNA amplification of the sample in each colony. The primer set for β-actin was the same as was used in the quantitative PCR described above. Amplification conditions for β-actin PCR were 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min with 30 cycles. The final PCR products were separated on 2% agarose gels. The sizes of the products were 206, 483, and 232 bp for SAG, nonexpressing PLI vector, and β-actin sequences, respectively. The transduction efficiency of CFU was calculated by dividing the number of colonies positive for the vector sequence by the number positive for the β-actin sequence. Plucked methylcellulose not containing colonies served as negative controls.

In situ PCR. *In situ* detection of transplanted cell progeny was performed by amplifying the SAG sequence as previously reported [28]. Peripheral blood nucleated cells were spun down to glass slides. The SAG-specific primer sequences were the same as were used for the quantitative PCR described above. The reaction mixture consisted of 420 μM dATP, 420 μM dCTP, 420 μM dGTP, 378 μM dTTP, 42 μM digoxigenin-labeled dUTP (Roche), 0.8 μM each SAG primer, 4.5 mM $MgCl_2$, PCR buffer (Mg^{2+} free), and 4 U Takara *Taq* DNA polymerase (Takara). Slides were covered with the Takara Slide Seal for *in situ* PCR (Takara). PCR was performed using the PTC100 Peltier thermal cycler (MJ Research, Watertown, MA, USA) under the following conditions: 94°C for 1 min and 55°C for 1 min with 15 cycles. The digoxigenin-incorporated DNA fragments were detected using the horseradish peroxidase (HRP)-conjugated rabbit F(ab′) anti-digoxigenin antibody (Dako). Slides were then stained for HRP using the Vector SG Substrate Kit. Finally, slides were counterstained with Kernechtrot dye that stains nucleotides, mounted in glycerol, and examined under a light microscope.

LAM-PCR. The LAM-PCR was performed as previously described [15]. The genomic-proviral junction sequence was preamplified by repeated primer extension using 0.25 pmol of vector-specific, 5′-biotinylated primer LTR1 (5′-AGCTGTTCCATCTGTTCTTGGCCCT-3′) with *Taq* polymerase (2.5 U; Qiagen) from 100 ng of each sample DNA. One hundred cycles of amplification were performed with the addition of fresh *Taq* polymerase (2.5 U) after 50 cycles. Biotinylated extension products were selected with 200 μg of magnetic beads (Dynabeads Kilobase BINDER Kit; Dynal). The samples were incubated with Klenow polymerase (2 U; Roche), dNTPs (300 μM; Pharmacia, Uppsala, Sweden), and a random hexanucleotide mixture (Roche) in a volume of 20 μl for 1 h at 37°C. Samples were washed on the magnetic particle concentrator (Dynal) and incubated with *TaqI* (Fermentas, Hanover, MD, USA) to cut the 5′ long terminal repeat-flanking genomic DNA for 1 h at 65°C. After an additional wash step, 100 pmol of a double-stranded asymmetric linker cassette and T4 DNA ligase (6 U; New England Biolabs, Beverly, MA, USA) was incubated with the beads in a volume of 10 μl at 16°C overnight. Denaturing was performed with 5 μl of 0.1 N NaOH for 10 min at room temperature. Each ligation product was amplified with *Taq* polymerase (5 U; Qiagen), 25 pmol of vector-specific primer LTR2a (5′-AACCTTGATCTGAACTTCTC-3′), and linker cassette primer LC1 (5′-GACCCGGGAGATCTGAATTC-3′) by 35 cycles of PCR (denaturation at 95°C for 60 s, annealing at 60°C for 45 s, and extension at 72°C for 60 s). Of each PCR product, 0.2% served as a template for a second, nested PCR with internal primers LTR3 (5′-TCCATGCCTTGCAAAATGGC-3′) and LC2 (5′-GATCTGAATTCAGTGGCACAG-3′) under identical conditions. Final products were separated on a 2% agarose gel.

Flow-cytometric sorting. We used the FSC/SSC profile (forward and side scatter) to sort granulocytes (purity 95%). Anti-CD3 and anti-CD20 were used to sort T lymphocytes (purity 99%) and B lymphocytes (purity 95%), respectively. Cells were sorted using an EPICS Elite cell sorter equipped with an argon-ion laser (Beckman Coulter, Fullerton, CA, USA). Data acquisition and analysis were performed using the EXPO2 software (Beckman Coulter).

Cellular immune response assay. Peripheral blood mononuclear cells and bone marrow stromal cells were isolated from monkey D8058. The stromal cells were transduced with a retroviral vector carrying the PLI, SAG, or human EPO receptor cDNA. The transduced stromal cells were irradiated with 4000 cGy and used as stimulator cells. Untransduced stromal cells irradiated with 4000 cGy served as a control. The peripheral blood mononuclear cells (responder cells, 2×10^5/well) were cocultured with the stimulator or control cells (5×10^4/well) in 96-well, flat-bottom plates with RPMI 1640 medium (Sigma) containing 10% fetal calf serum and 20 IU/ml rh interleukin-2 (Shionogi, Osaka, Japan). After 5 days in culture, the blastogenesis of responder cells was assessed. Briefly, the cells were labeled with 1 μCi/well of [*methyl*-^{3}H]thymidine (Amersham) for 16 h and harvested with an automated cell harvester (Laboratory Science, Tokyo, Japan) onto glass-fiber filters (Molecular Devices, Sunnyvale, CA, USA). The incorporation of [*methyl*-^{3}H]thymidine into responder cells was quantified in a liquid scintillation counter (Aloka, Tokyo, Japan). All experiments were performed in triplicate.

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RESEARCH ARTICLE

Expansion of genetically corrected neutrophils in chronic granulomatous disease mice by cotransferring a therapeutic gene and a selective amplifier gene

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Hematopoietic stem cell gene therapy has not provided clinical success in disorders such as chronic granulomatous disease (CGD), where genetically corrected cells do not show a selective advantage in vivo. To facilitate selective expansion of transduced cells, we have developed a fusion receptor system that confers drug-induced proliferation. Here, a 'selective amplifier gene (SAG)' encodes a chimeric receptor (GcRER) that generates a mitotic signal in response to estrogen. We evaluated the in vivo efficacy of SAG-mediated cell expansion in a mouse disease model of X-linked CGD (X-CGD) that is deficient in the NADPH oxidase gp91^{phox} subunit. Bone marrow cells from X-CGD mice were transduced with a bicistronic retrovirus encoding GcRER and gp91^{phox}, and transplanted to lethally irradiated X-CGD recipients. Estrogen was administered to a cohort of the transplants, and neutrophil superoxide production was monitored. A significant increase in oxidase-positive cells was observed in the estrogen-treated mice, and repeated estrogen administration maintained the elevation of transduced cells for 20 weeks. In addition, oxidase-positive neutrophils were increased in the X-CGD transplants given the first estrogen even at 9 months post-transplantation. These results showed that the SAG system would enhance the therapeutic effects by boosting genetically modified, functionally corrected cells in vivo.
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Keywords: *chronic granulomatous disease; selective amplifier gene; respiratory burst; estrogen-binding domain*

Introduction

Gene transfer to hematopoietic stem cells (HSCs) holds promise to provide a long-standing cure of many lymphohematological diseases. One of the candidate disorders is chronic granulomatous disease (CGD), a rare inherited phagocyte dysfunction that renders patients particularly susceptible to catalase-positive microorganisms.[1] The disease is caused by a defect in microbicidal oxidant production, resulting from mutations in the genes encoding four essential subunits of the phagocyte NADPH oxidase (*phox*). The X-linked form of CGD (X-CGD), accounting for about 70% of all cases, is due to genetic mutations in the large subunit of the oxidase cytochrome b_{558}, which is a 91 kDa glycoprotein referred to as gp91^{phox}.[2] A rare autosomal recessive form of CGD results from a defect in the gene encoding p22^{phox}, the small subunit of the cytochrome (about 5%). Other patients have an autosomal recessive trait with a deficiency of either p47^{phox} (20–25%) or p67^{phox} (<5%), which are two soluble proteins in the oxidase complex.

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Although prophylactic antibiotics and interferon γ constitute a cornerstone of CGD management and have brought about a better outlook,[3,4] morbidity caused by infection or granulomatous complications remains significant. Allogeneic bone marrow transplantation (BMT) has not been well adopted because of procedure-associated risks and difficulty in finding a suitable donor, but this therapeutic option is increasingly considered for young patients with histocompatible siblings.[5] Recently, a study of patients who underwent nonmyeloablative stem cell transplantation was published, with a better outcome with young patients as well.[6]

Somatic gene therapy targeted at autologous HSCs can bypass problems involved in allotransplantation such as acute graft rejection and graft-versus-host disease.[7] For CGD, correction of only a minority of phagocytes is likely to provide clinical benefit, because a partial chimerism after BMT has freed patients from severe infections and female carriers of X-CGD with as few as 5–10% oxidase-positive neutrophils are often asymptomatic.[8–10] Likewise, preclinical studies with mouse models have provided a rationale for this approach.[11–14] So far, a few phase I clinical gene therapy trials have been conducted, but the percentages of corrected neutrophils have been too low to impact the disease phenotype.[15]

A potential transgene-induced immune reaction remains to be discussed extensively, but maintenance of low-level chimerism in some transplants suggests that rejection by this mechanism is less likely to occur.

Even with the recent refinement of transduction protocols, transducing enough human HSCs is a major challenge to gene therapy for inherited and acquired blood cell disorders.[16] Thus, it is desirable to expand genetically corrected cells in the body, to improve the therapeutic efficacy of stem cell gene therapy. One strategy to achieve this goal is to help their preferential outgrowth through drug selection. On transduction of the target cells with a therapeutic gene and a drug-resistance gene, administering the corresponding cytotoxic drug leads to an increase of genetically modified cells.[17,18] An alternative approach is to confer a direct proliferative advantage on the genetically modified cells, provided that the mitogenic stimulation is restricted to the genetically modified cells in a controllable manner.[19,20]

We have developed a novel system for the selective expansion of transduced cells to compensate for the low frequency of genetically corrected cells.[21–23] The expansion system comprises a fusion protein and a stimulator drug. As a growth signal generator, a chimeric receptor (GcRER) was constructed with the granulocyte colony-stimulating factor (G-CSF) receptor (GcR) and the hormone-binding domain of the estrogen receptor (ER-HBD). The artificial gene encoding the fusion protein was referred to as a 'selective amplifier gene (SAG)'. We showed that transduced hematopoietic stem/progenitor cells were expandable with this system in murine and primate models.[24,25] In the present study, a bicistronic retroviral vector carrying the human gp91^{phox} (hgp91) gene and a modified SAG was evaluated in a mouse model of X-CGD.

Results

Retroviral vector carrying the gp91^{phox} gene and a selective amplifier gene

Figure 1a shows the structure of fusion proteins comprising GcR and ER-HBD. The prototype SAG encodes a fusion protein made up of the full-length mouse GcR and the rat ER-HBD.[21] In ΔGcRER, the G-CSF binding domain (amino acids 5–195 in the full-length GcR) was deleted to free it from the endogenous G-CSF.[21] In addition, the most proximal cytoplasmic tyrosine (position 703) of the mouse GcR was replaced with phenylalanine in ΔY703FGcRER to attenuate the differentiation signal, based on the result that the tyrosine residue was strongly involved in granulocyte maturation.[22]

Figure 1b shows the structure of the retroviral vector used in this study. The vector, MGK/h91GE, was constructed with MFG and MSCV backbones,[26,27] the hgp91 gene and the picornavirus-derived internal ribosome entry site (IRES)-linked ΔY703FGcRER gene.[28] Ecotropic BOSC23 packaging cells were transfected with the MGK/h91GE vector plasmid and the viral supernatant was harvested.[29] Viral titer of the supernatant was estimated to be 5×10^5 particles/ml, by a simplified RNA dot blot protocol along with the plasmid as a reference.[30] Ba/F3 cells and gp91^{phox}-deficient PLB-985 myeloid cells

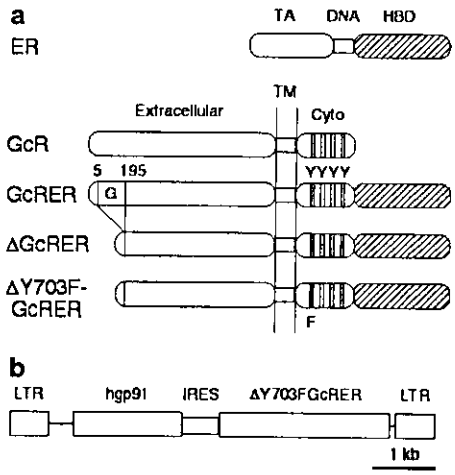

Figure 1 Structure of selective amplifier gene-encoded proteins and gene transfer vector. (a) GcRER is a fusion protein comprising the full-length mouse G-CSF receptor (GcR) and the hormone-binding domain of the rat estrogen receptor (ER). ΔGcRER is deleted of the G-CSF binding domain of GcR (amino acids 5–195). ΔY703FGcRER has a substitution of phenylalanine for tyrosine 703 in GcR. TA, transactivating domain; DNA, DNA-binding domain; HBD, hormone-binding domain; Extracellular, extracellular domain; TM, transmembrane domain; Cyto, cytoplasmic domain; G, G-CSF binding domain; Y, tyrosine residue; F, phenylalanine substitution for Y703. (b) Schematic representation of bicistronic vector (MGK/h91GE) carrying the human gp91^{phox} gene and a selective amplifier gene. LTR, long-terminal repeat; hgp91, human gp91^{phox} gene; IRES, internal ribosome entry site.

were transduced with the viral supernatant, and the expression of the vector-encoded hgp91 was confirmed by fluorescence-activated cell sorting (FACS) with 7D5 monoclonal antibody (a gift from Dr M Nakamura, Nagasaki University, Nagasaki, Japan; FACS data not shown).[31,32]

Transduction of X-CGD progenitors

The efficiency of the MGK/h91GE vector was evaluated by transducing X-CGD mouse bone marrow (BM) cells. The X-CGD mouse was created by targeted disruption of the X-linked gp91^{phox} gene, and its phagocytes are devoid of respiratory burst activity.[11] As a result, these mice share many characteristics of the human CGD phenotype, including an elevated susceptibility to *Aspergillus* species. The mice were backcrossed to C57BL/6; subsequently, the X-CGD allele was introduced into the Ly5.1-C57BL/6 congenic background to allow Ly5.1/5.2 chimerism to be analyzed in the BM transplants.

We assessed the *in vitro* responsiveness of vector-transduced cells to estrogen using a clonogenic progenitor assay. BM cells were harvested from male Ly5.1-X-CGD mice treated with intraperitoneal 5-fluorouracil (5-FU) 2 days before. Following prestimulation with stem cell factor (SCF) and interleukin-6 (IL-6) for 2 days, a major part of BM cells was transduced with the MGK/h91GE viral supernatant according to a standard fibronectin-assisted protocol.[33] The remainder part was incubated in the same culture condition as the prestimulation for another 2 days, instead of being transduced with the viral supernatant ('untransduced cells'). Then, untransduced cells and an aliquot of transduced cells were subjected to methylcellulose culture with a cytokine

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

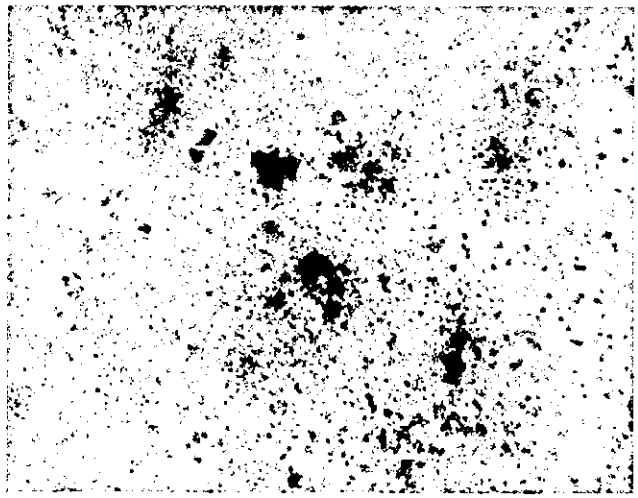

Figure 2 In situ colony NBT test. X-CGD bone marrow cells were transduced with MGK/h91GE vector and 2×10^5 cells were subjected to methylcellulose culture with 10^{-7} M estradiol. On day 10, the colonies were overlaid with RPMI medium containing NBT and PMA. Nearly all the estrogen-induced colonies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

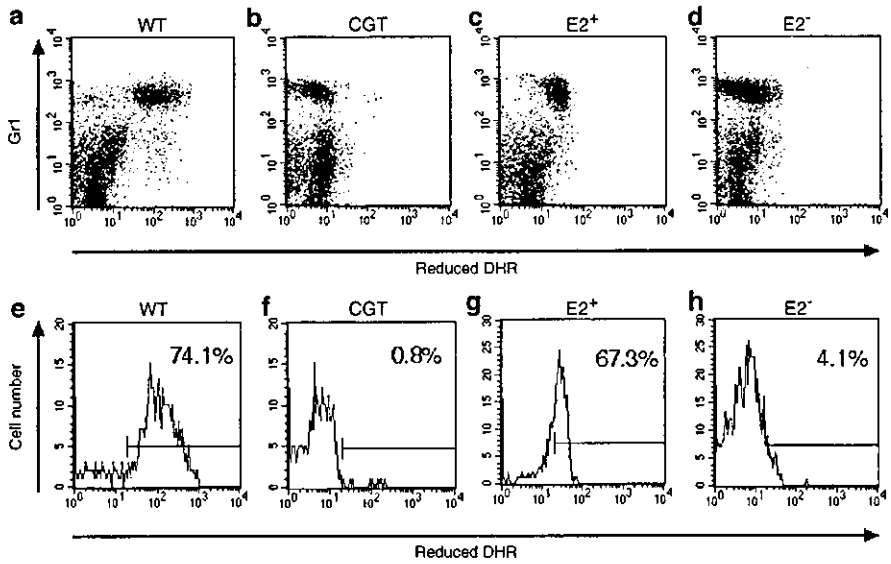

Figure 3 Flow cytometry of DHR assay. Mouse peripheral blood was stimulated with PMA, incubated with DHR and stained with Gr1-PE/Cy5. In the dot plots (a–d), the X-axis represents DHR reduced by superoxide, and the Y-axis represents expression of a granulocyte differentiation marker Gr1. In the histograms (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

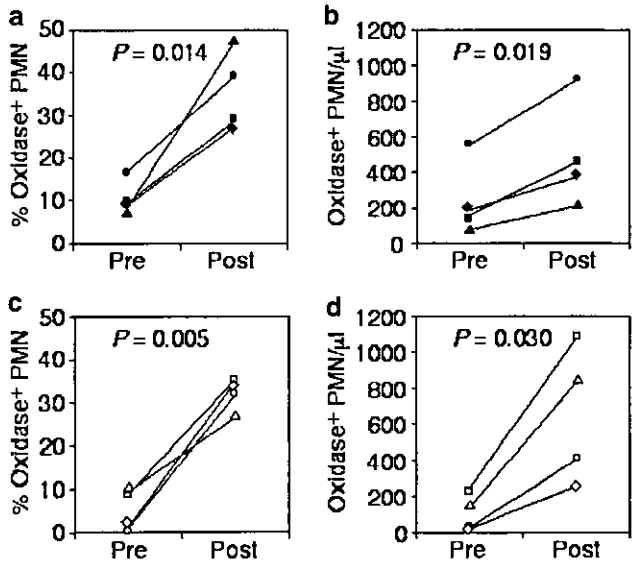

Figure 4 Comparison of oxidase-positive granulocytes before and after estrogen administration. Frequencies (a and c) and absolute numbers (b and d) of oxidase-positive polymorphonuclear leukocytes (PMN) from individual X-CGD transplants are shown. (a and b) Oxidase-positive PMN in Group 1 mice before 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

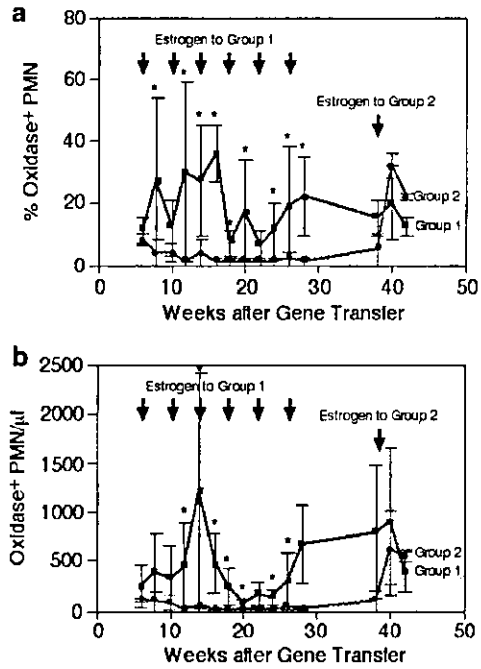

Figure 5 Estrogen-induced expansion of oxidase-positive granulocytes. Graphs indicate the time course of the change in frequency (a) and absolute number (b) of oxidase-positive granulocytes following exposure to estrogen. To mice in Group 1 ($n=4$), estrogen was given in six courses with 4-week intervals 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 $31.7\pm3.8\%$ ($P=0.005$ by paired *t*-test, Figures 4c and 5a), and the absolute number increased from 96 ± 104 to $638\pm378/\mu l$ ($P=0.030$ 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 oncoretrovirus-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 $2–3 \times 10^6$ 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 counterstained 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

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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.

Keywords hematopoietic stem cells; gene therapy; $CD34^+$ cells; selective amplifier gene; *in vivo* expansion; retroviral vector

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 $\mu g/cm^2$ 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 $\mu g/cm^2$ 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]-2*H*-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/cm^2 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)

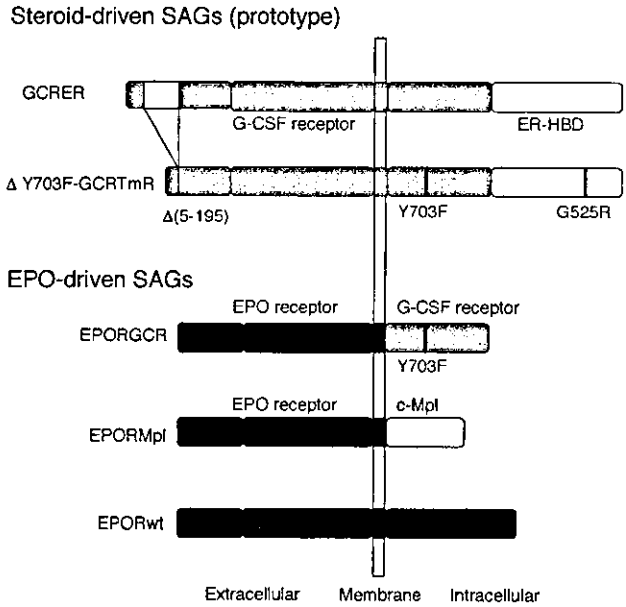

Figure 1. The structure of SAGs. The GCRER is a prototype of the selective amplifier gene (SAG), a chimeric gene encoding the G-CSF receptor (GCR) as a growth-signal generator and the estrogen receptor hormone-binding domain (ER-HBD) as a molecular switch. In ΔY703F-GCRTmR, the G-CSF-binding domain was 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 ± 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.

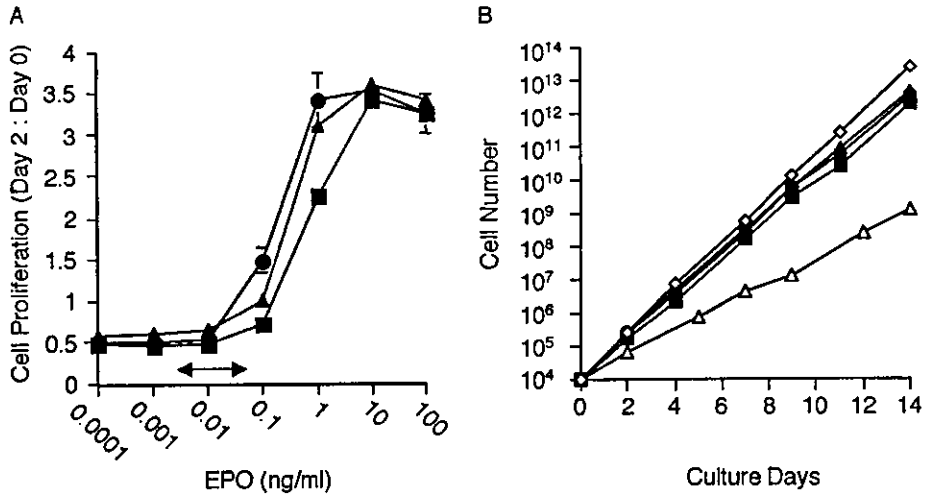

Figure 2. The EPO-driven SAG efficiently stimulates Ba/F3 cell growth. (A) EPO-dependent growth of Ba/F3 cells by introduction of the EPO-driven SAG. BaF3 cells were transduced with the EPORwt (solid triangles), EPORGCR (solid squares) or EPORMpl gene (solid circles) each along with the EYFP gene by 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 ± 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

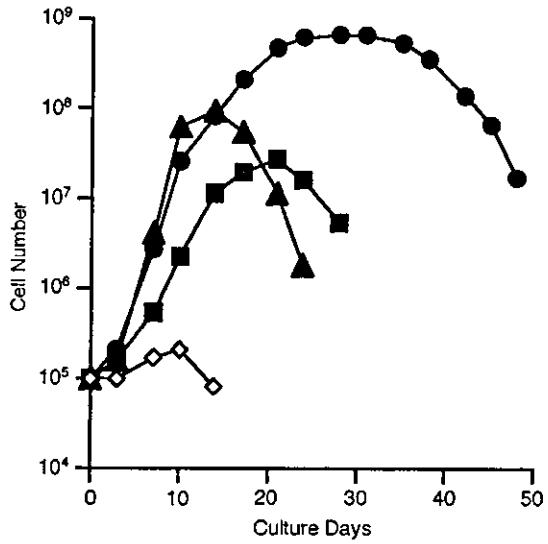

Figure 3. The EPORMpl is the most potent amplifier for human cord blood CD34$^+$ cells. Human cord blood CD34$^+$ cells were transduced with the EPOwt (solid triangles), EPORGCR (solid squares), or EPORMpl gene (solid circles) each along with the EYFP gene by bicistronic retroviral vectors. Untransduced 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

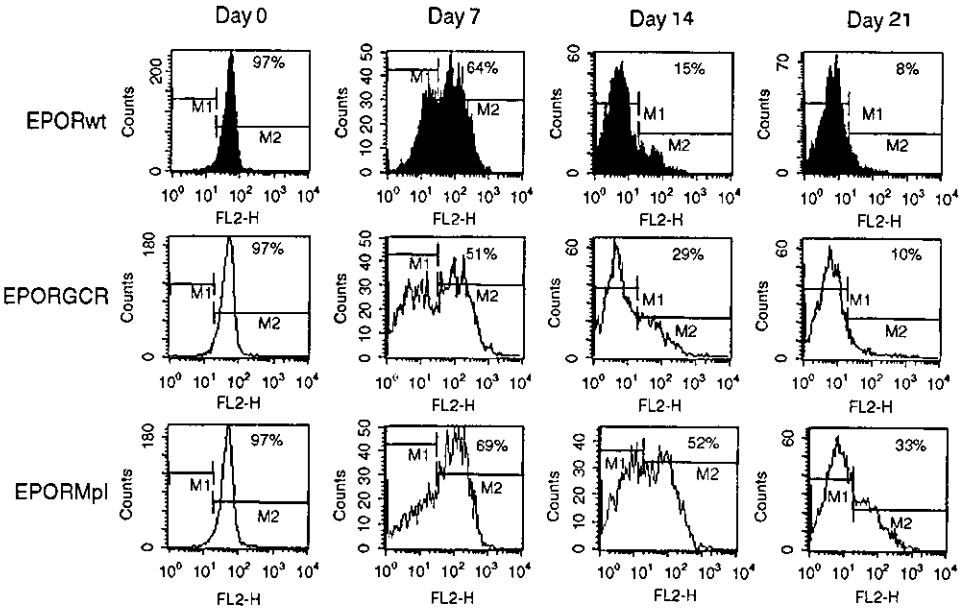

Figure 4. The EPOR-Mpl preserves c-Kit$^+$ cells most efficiently. Human cord blood CD34$^+$ cells were transduced with the EPOwt (black), EPORGCR (gray), or EPORMpl gene (white) by the same retroviral vectors as in Figure 3. The cells were then cultured in IMDM supplemented with 10% FBS and 10 ng/ml 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

	IL-3 (100 ng/ml) IL-6 (100 ng/ml) SCF (100 ng/ml)		EPO (20 ng/ml)	
Transgene	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

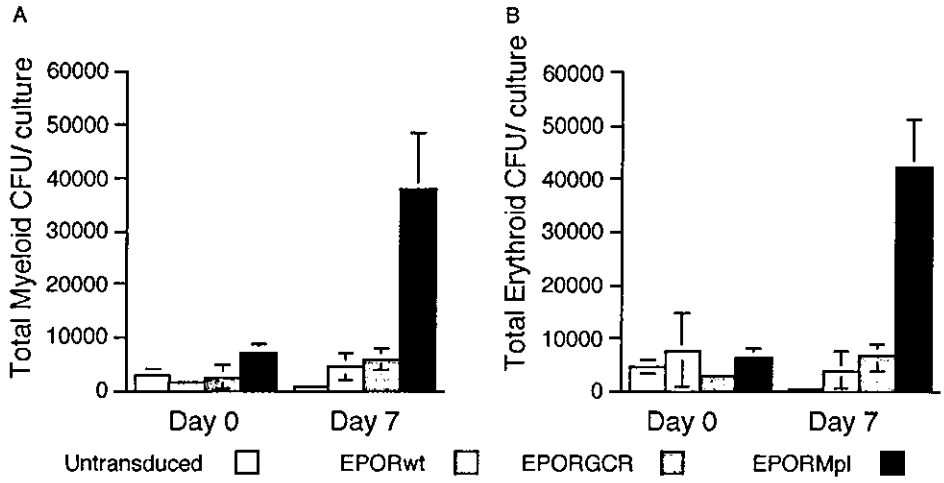

Figure 5. The EPOR-Mpl expands clonogenic progenitor cells most efficiently. Human cord blood CD34$^+$ cells were transduced with the EPOwt, EPORGCR or EPORMpl gene by the same retroviral vectors as in Figure 3. The untransduced and transduced cells were then cultured in IMDM supplemented with 10% FBS 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

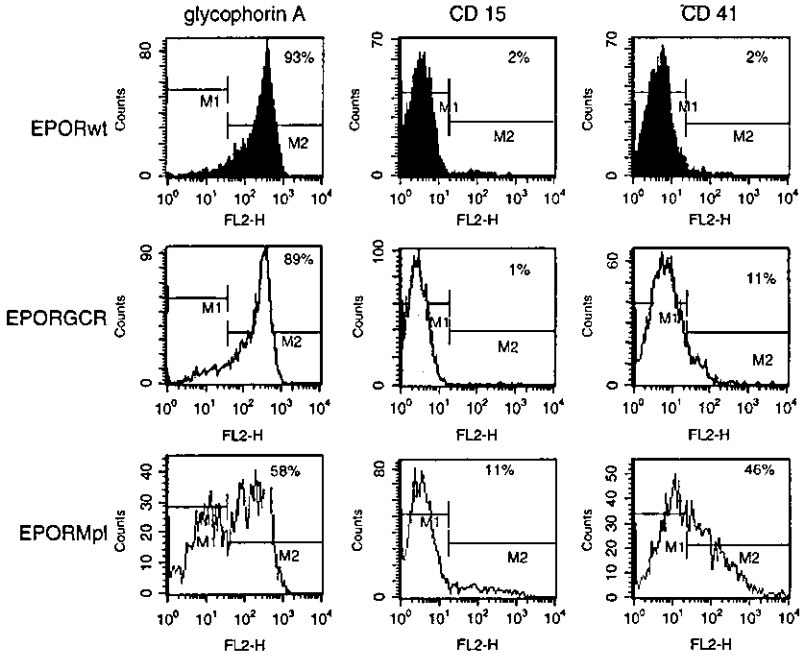

Figure 6. The CD34$^+$ cells expanded by the EPOR-Mpl show the most balanced expression of multilineage surface markers. Human cord blood CD34$^+$ cells were transduced with the EPOwt, EPORGCR, or EPORMpl gene by the same retroviral vectors as in Figure 3. After 14-day liquid culture with 10% FBS and 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

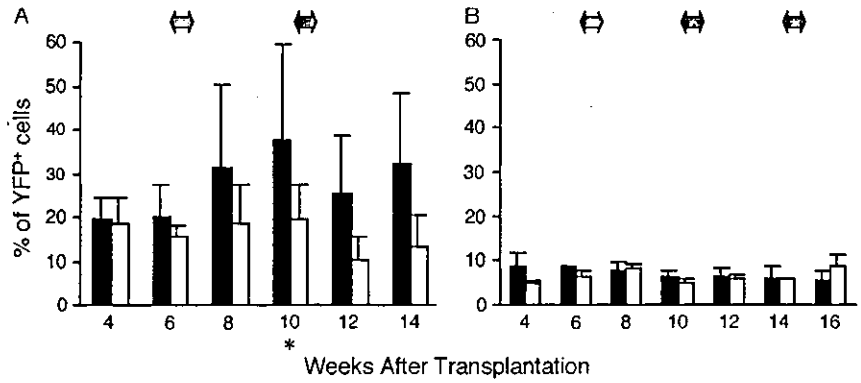

Figure 7. The gene-modified hematopoietic cells can be expanded by treatment with EPO *in vivo* in mice. Murine bone marrow cells were harvested from 5-fluorouracil-treated mice and transduced with the retroviral vector expressing both EPORMpl and YFP, or expressing YFP alone as a control. The transduced 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.

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