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Quantitative PCR

Genomic DNA was extracted using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany). DNA (250 ng) was amplified in triplicate with PLI primers (5′-TCC ATC ATG GAT GCA ATG CGG C-3′ and 5′-GAT AGA AGG CGA TGC GCT GCG AAT CG-3′). Standards consisted of DNA extracted from a PLI-producer cell line (which has one copy of the proviral sequence per cell) serially diluted with control cynomolgus genomic DNA. Negative controls consisted of DNA extracted from peripheral blood cells of naive monkeys. A β-actin primer set (5′-CCT ATC AGA AAG TGG TGG CTG G-3′ and 5′-TTG GAC AGC AAG AAA GTG AGC TT-3′) was used to certify equal loading of DNA per reaction. Reactions were run using 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 sec, 62°C for 30 sec, 72°C for 30 sec, and 83°C for 15 sec. The quantitative PCR was certified each time to yield linear amplifications in the range of the intensity of positive control series (0.01–100%, correlation coefficient >0.98). For calculating transduction efficiencies, Ct values of the vector sequence were normalized based on Ct values of the internal control β-actin sequence of the same samples as directed by the manufacturer. Gene marking percentages were calculated given that each vector-positive cell contains one copy of the vector sequence.

Colony PCR

Cells were plated in a 35-mm petri-dish containing 1 ml of α-minimum essential medium (Invitrogen), 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan), 100 ng/ml of rh interleukin-3 (PeproTech, Rocky Hill, NJ, USA), 100 ng/ml of rh interleukin-11 (PeproTech), 100 ng/ml of rhSCF (Biosource), 2 U/ml of rh erythropoietin (Roche, Basel, Switzerland), 20% fetal calf serum, 1% bovine serum albumin, 5×10^{-5} M 2-mercaptoetanol (Sigma, St. Louis, MO, USA) and antibiotics (100 U/ml of penicillin and 0.1 mg/ml of streptomycin; Meiji, Tokyo, Japan). Well-separated, individual colonies at day 14 were removed to 50 μl of distilled water, digested with 20 μg/ml of proteinase K (Takara) at 55°C for 1 hour then 99°C for 10 min, and assessed for the vector sequence using PLI primers by nested PCR. The outer primer set was 5′-TCC ATC ATG GAT GCA ATG CGG C-3′ and 5′-GAT AGA AGG CGA TGC GCT GCG AAT CG-3′. The inner primer set was 5′-ATA CGC TTG ATC CGG CTA CCT G-3′ and 5′-GAT ACC GTA AAG CAC GAG GAA G-3′. The outer PCR products were purified using MicroSpin S-400 HR columns (Amersham, Piscataway, NJ, USA) before being subjected to the inner PCR. Amplification conditions for both outer and inner PCR were 20 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min. Simultaneous PCR for the β-actin sequence was also performed to certify amplification of the sample DNA in each colony. The primer set for β-actin PCR was the same as described for the quantitative PCR. Amplification conditions for β-actin PCR were 30 cycles of

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95°C for 1 min, 54°C for 1 min, and 72°C for 2 min. PCR products were run on 2% agarose gel. The product sizes were 483 and 232 bp for the PLI vector and β-actin sequences, respectively. Plucked methylcellulose not containing colonies served as negative controls. Transduction efficiency was calculated by dividing the number of colonies positive for the vector sequence by the number positive for the β-actin sequence.

RESULTS

Gene marking of cynomolgus CD34$^+$ cells

Cynomolgus CD34$^+$ cells were collected either from iliac bones or peripheral blood and purified to 90–95% homogeneity. They were genetically marked with a non-expression retroviral vector, *i.e.* aforedescribed PLI which contains non-translated sequences (Heim *et al.*, 2000). The transduction results are summarized in Table 1.

Autologous iBMT

Instead of the conventional intra-venous transplantation (iVT), we injected the gene-marked, autologous CD34$^+$ cells directly into the bone marrow cavity of four proximal limb bones: the femurs and humeri (Fig. 1). Needles were inserted into both ends of the femur or humerus of two anesthetized cynomolgus monkeys (IB3048 and IB3053). A syringe containing 50 ml of heparin-added normal saline was connected to one needle and an empty syringe was connected to the other. The plunger of the empty syringe was pulled to collect marrow, and consequently normal saline in the other syringe was drawn into the marrow cavity (Fig. 1A). Normal saline was thus irrigated gently from one syringe to the other through the marrow cavity without inflicting extra-pressure. This saline irrigation was done twice.

Table 1.
Ex vivo transduction of purified CD34$^+$ cells

Animal no.	CD34$^+$ cell source	Sex, age, body weight	No. of infused CD34$^+$ cells/kg	Transduction efficiency*
IB3048	Bone marrow	Male, 3.1 years, 3.1 kg	4.5×10^7	34/46 (74%)
IB3053	Peripheral blood	Male, 3.2 years, 2.5 kg	8.1×10^6	49/78 (63%)
V0065	Peripheral blood	Male, 5.5 years, 4.4 kg	1.2×10^7	3/45 (6.7%)
V1007	Peripheral blood	Male, 4.1 years, 3.7 kg	1.5×10^6	14/41 (34%)

* As assessed by colony PCR and published in Ueda *et al.*, 2004.

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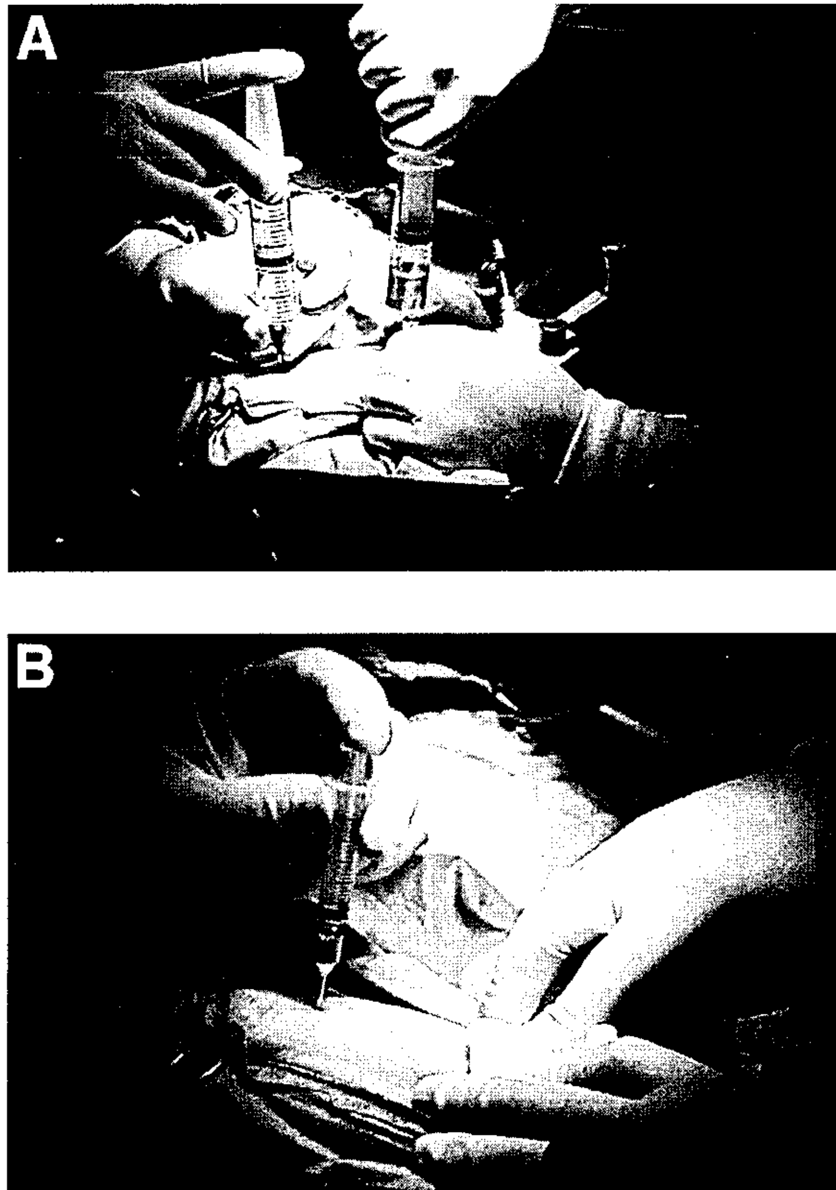

Figure 1. iBMT method. Needles were inserted into either end of the femur and humerus. Normal saline was irrigated gently from one syringe to the other through the marrow cavity (A). Gene-marked cells were then injected into the marrow cavity through one needle (B).

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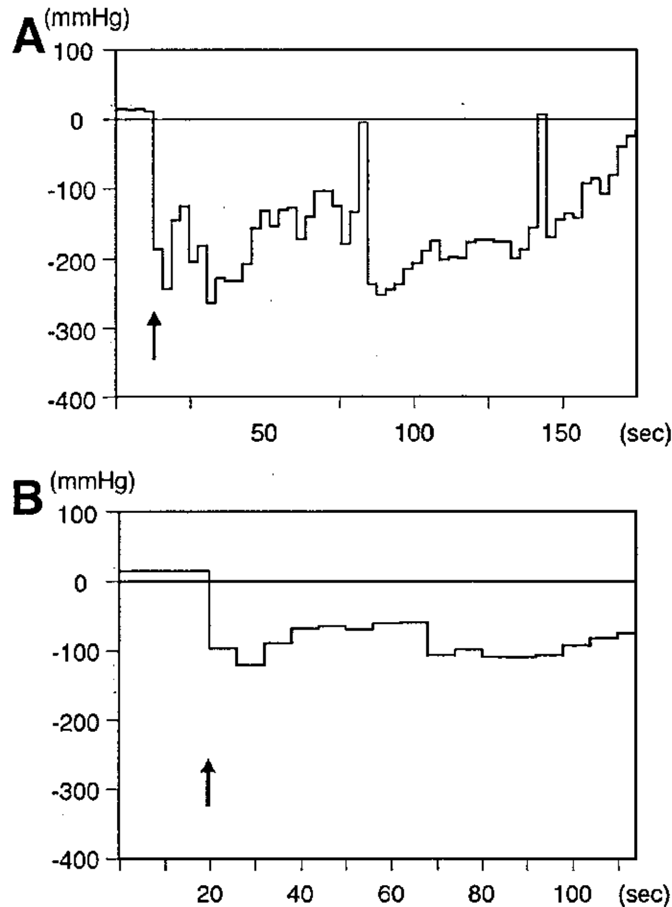

Figure 2. Intraosseous pressure. The internal pressure in the marrow cavity was measured throughout the irrigation of saline in the femur (A) and humerus (B). Representative time-course profiles are shown. Arrows indicate time points of the initiation of saline irrigation. Saline irrigation was conducted without inflicting positive pressure on the marrow cavity.

Gene-marked cells suspended in 1 ml of phosphate-buffered saline containing 10% autologous serum were then injected into the marrow cavity through one needle (Fig. 1B).

In order to rule out the possibility that excessive positive pressure on the marrow cavity during the saline irrigation could cause pulmonary embolism, we tried not to inflict extra-pressure during the procedure. In fact, we have confirmed that saline irrigation was conducted without inflicting positive pressure on the marrow cavity by measuring the intraosseous pressure in one animal (Fig. 2). A third needle was inserted in the middle of the femur or the humerus between the two needles described above, and connected to a pressure transducer. As shown in Fig. 2,

Table 2.
Low-level systemic delivery and long-term engraftment of transplanted cells after iBMT without conditioning

Animal no.	Fractions of gene-marked cells in peripheral blood		Fractions of gene-marked CFU in bone marrow after transplantation	
	One hour after transplantation	One day after transplantation	Implanted femurs	Non-implanted iliac bones*
iBMT				
IB3048	Not detectable (< 0.01%)	Not detectable (< 0.01%)	15/48 (31%) at day 175 1/44 (2.3%) at day 355	3/48 (6.3%) at day 175 1/44 (2.3%) at day 335
IB3053	Not detectable (< 0.01%)	Not detectable (< 0.01%)	1/32 (3.1%) at day 255	4/40 (10%) at day 255
iVT controls				
V0065	0.02%	Not detectable (< 0.01%)	NA#	0/47 at day 125
V1007	0.02%	Not detectable (< 0.01%)	NA	0/4 at day 87

* As published in Ueda *et al.*, 2004.
\# NA, non applicable.

the original intraosseous pressure was 10–20 mmHg; after the initiation of saline irrigation, the intraosseous pressure of both femur and humerus stayed below zero mmHg.

The transplant procedure was thus safely performed without pulmonary embolism or other complications. Importantly enough, myeloablative conditioning treatment such as irradiation was not conducted prior to transplantation. The same held true for the two control animals (V0065 and V1007) that were subjected to standard iVT.

Peripheral blood monitoring after iBMT

One hour and one day after iBMT, gene-marked cells were not detectable in the peripheral blood as assessed by quantitative PCR (detectable range > 0.01%; Table 2), indicating that a systemic delivery of transplanted cells hardly occurred following iBMT. On the other hand, gene-marked cells were detectable, albeit at low levels, one hour after control iVT.

However, after a few days, very low but significant levels (0.01–0.1%) of gene-marked circulating cells were clearly monitored (Ueda *et al.*, 2004). Notably enough, unlike iVT controls, iBMT IB3048 monkey maintained a detectable level of circulating marked cells until the end of the experiment, *i.e.* a whole year (Ueda *et al.*, 2004).

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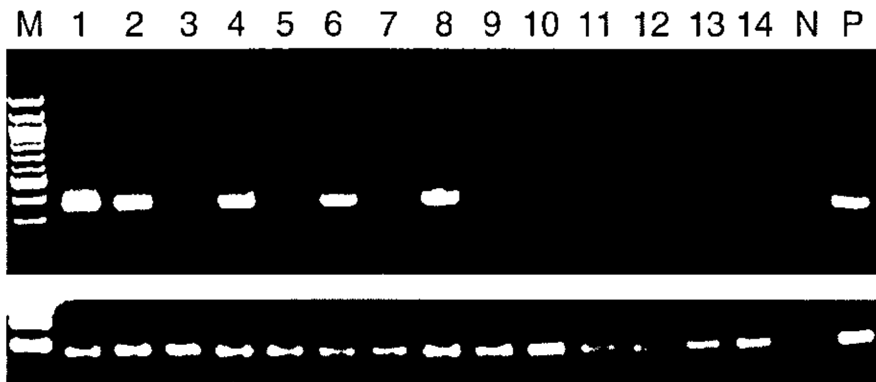

Figure 3. Engraftment of transplanted cells after iBMT without prior conditioning. Cells taken from the implanted femoral bone marrow were subjected to a clonogenic progenitor assay at 6–12 months after iBMT. Individual colonies were examined for the vector sequence (upper panel) and internal control β-actin sequence (lower panel) by PCR. In representative results shown here, five colonies (no. 1, 2, 4, 6, and 8) were vector-positive among 14 of β-actin-positive colonies. M, molecular weight marker: lanes 1–14, individual colony numbers; N, negative control (methylcellulose medium alone); P, positive control.

Long-term engraftment of transplanted cells after iBMT

Cells taken from the implanted femoral bone marrow were subjected to a clonogenic progenitor assay at 6 to 12 months after iBMT. The resulting colonies (colony-forming units, CFU) were examined for the vector sequence by PCR (Fig. 3). As summarized in Table 2, 2 to 30% of CFU were found gene-marked. This result suggests that transplanted cells can engraft in non-conditioned recipient monkeys after iBMT.

Importantly enough, gene-marked CFUs were also found in nonimplanted iliac marrow (Table 2). Indeed, we have shown that such a translocation of marked progenitor cells to nonimplanted bone marrow was detectable within two weeks after iBMT (Ueda *et al.*, 2004). In contrast to iBMT, control iVT is not associated with long-term detection of marked CFUs in iliac marrow as clearly shown in the monkey V0065 (Table 2 and Ueda *et al.*, 2004).

DISCUSSION

In this article, we have detailed our iBMT method for long-term engraftment of autologous CD34$^+$ cells without prior myeloablative conditioning in a primate model. We showed here and in another article (Ueda *et al.*, 2004) that autologous CD34$^+$ cells transplanted directly into both femoral and humeral bone marrow engrafted without prior conditioning in cynomolgus monkeys. Such an engrafment extended until the end of the experiment, *i.e.* lasted 12 months and over 8 months respectively in the two monkeys that have been used in this experiment. Importantly

enough, we did not find any gene-marked CFU (0/47) at 4 months post-transplant in the relevant monkey control in which marked autologous $CD34^+$ cells were intravenously infused under non-myeloablative conditions. Although gained with statistically non-significant numbers of cynomolgus monkeys and low level marking of iVT cells (Table 1), our data suggest that, under non-myeloablative conditions, iBMT is more effective than conventional iVT in our primate model.

There are several possible explanations for the relatively efficient engraftment after iBMT as compared to iVT. The iBMT method may allow many more HSCs and progenitor cells to reach the bone marrow without being trapped at other sites in the body such as the liver, spleen, and lung, than the iVT method. Directly injected cells may also be able to find a proper bone marrow niche for engraftment relatively easily as compared to intravenously injected cells. In fact, it has been reported that some fraction of HSCs are unable to reach the bone marrow niche via conventional venous route (Wang *et al.*, 2003; Mazurier *et al.*, 2003). In the current study, toxic conditioning such as irradiation therapy was not conducted. Thus, there was no conditioning-related injury of stroma cells. This might increase engraftment, given that the injury of stroma cells may result in impaired engraftment (Plett *et al.*, 2002). In addition, it is possible that the physical elimination of endogenous marrow with saline before injection increased engraftment.

Interestingly, although we showed that transplanted cells did not detectably enter the systemic flow (Table 2), gene-marked CFU were also found in the non-implanted iliac marrow at comparable levels after iBMT (Table 2, and Ueda *et al.*, 2004). Thus, transplanted cells relocated from an implanted bone to another, although iBMT in itself did not create a systemic distribution of transplanted cells. A similar translocation post-transplantation has also been reported in mouse syngeneic and allogeneic iBMT and human-mouse xeno-iBMT models (Askenasy *et al.*, 2002; Zhong *et al.*, 2002; Mazurier *et al.*, 2003; Yahata *et al.*, 2003; Wang *et al.*, 2003).

Importantly enough, the very low level of gene-marked cells that has been detected long-term after a few days post-iBMT in peripheral blood of the monkey IB3048 (Results section and Ueda *et al.*, 2004) is consistent with the long-term engraftment of active HSCs. Like for bone marrow CFUs, gene-marking of circulating cells has been maintained until the end of the experiment, *i.e.* a whole year. Such a low level of gene-marked circulating cells is not enough for a therapeutic trial in which a non-conditioning regimen would be required such as for the gene therapy of chronic granulomatous disease and Gaucher disease. However, when the iBMT method is combined with an *in vivo* selection method such as the use of a selective amplifier gene, engraftment of transduced cells at low levels may allow successful expansion to clinically relevant levels even without marrow conditioning (Ueda *et al.*, 2004). Furthermore, this iBMT method may be applicable to allogeneic HSC transplantation (Askenasy *et al.*, 2002; Esumi *et al.*, 2003; Nakamura *et al.*, 2004). It might also be effective for mesenchymal stem cell transplantation, given that these cells express low levels of homing receptors

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(Wynn *et al.*, 2004): iBMT would allow such cells to engraft in bone marrow more efficiently than iVT.

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REFERENCES

Ageyama, N., Hanazono, Y., Shibata, H., Ohto, K., Ono, F., Nagashima, T., Ueda, Y., Donahue, R.E., Hasegawa, M., Ozawa, K., Yoshikawa, Y. and Terao, K. (2002). Safe and efficient methods of autologous hematopoietic stem cell transplantation for biomedical research in cynomolgus monkeys. *Comp. Med.*, **52**: 445–451.

Askenasy, N. (2002). Localized bone marrow transplantation leads to skin allograft acceptance in nonmyeloablated recipients: comparison of intra-bone marrow and isolated limb perfusion. *Stem Cells*, **20**: 86–93.

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

Esumi, T., Inaba, M., Ichioka, N., Kushida, T., Iida, H. and Ikehara, S. (2003). Successful allogeneic transplantation in rats in conjunction with intra-bone marrow injection of donor bone marrow cells. *Transplantation*, **76**: 1543–1548.

Dunbar, C.E., Kohn, D.B., Schiffmann, R., Barton, N.W., Nolta, J.A., Esplin, J.A., Pensiero, M., Long, Z., Lockey, C., Emmons, R.V., Csik, S., Leitman, S., Krebs, C.B., Carter, C., Brady, R.O. and Karlsson, S. (1998). Retroviral transfer of the glucocerebrosidase gene into $CD34^+$ cells from patients with Gaucher disease: *in vivo* detection of transduced cells without myeloablation. *Hum. Gene Ther.*, **9**: 2629–2640.

Hagglund, H., Ringden, O., Agren, B., Wennberg, L., Remberger, M., Rundquist, L., Svahn, B.M. and Aspelin, P. (1998). Intraosseous compared to intravenous infusion of allogeneic bone marrow. *Bone Marrow Transplant.*, **21**: 331–335.

Harper, W.M., Barnes, M.R. and Gregg, P.J. (1991). Femoral head blood flow in femoral neck fractures: an analysis using intra-osseous pressure measurement. *J. Bone Joint Surg.*, **73-B**: 73–75.

Heim, D.A., Hanazono, Y., Giri, N., Wu, T., Childs, R., Sellers, S.E., Muul, L., Agricola, B.A., Metzger, M.E., Donahue, R.E., Tisdale, J.F. and Dunbar, C.E. (2000). Introduction of a xenogeneic gene via hematopoietic stem cells leads to specific tolerance in a rhesus monkey model. *Mol. Ther.*, **1**: 533–544.

Huhn, R.D., Tisdale, J.F., Agricola, B., Metzger, M.E., Donahue, R.E. and Dunbar, C.E. (1999). Retroviral marking and transplantation of rhesus hematopoietic cells by nonmyeloablative conditioning. *Hum. Gene Ther.*, **10**: 1783–1790.

Kushida, T., Inaba, M., Ikebukuro, K., Ichioka, N., Esumi, T., Oyaizu, H., Yoshimura, T., Nagahama, T., Nakamura, K., Ito, T., Hisha, H., Sugiura, K., Yasumizu, R., Iida, H. and Ikehara, S. (2002). Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys at various ages by perfusion or aspiration methods: a preclinical study for human BMT. *Stem Cells*, **20**: 155–162.

VSP 2001/04/20 Prn:28/10/2004; 12:59 {RA} F:gtr033.tex; VTeX/Olga p. 12 (657-737)

Lucht, U., Djurhuus, C., Sorensen, S., Bunger, C. and Sneppen, O. (1981). The relationship between increasing intraarticular pressures and intraosseous pressures in the juxtaarticular bones. *Acta Orthop. Scand.*, **52**: 491–495.

Malech, H.L., Maples, P.B., Whiting-Theobald, N., Linton, G.F., Sekhsaria, S., Vowells, S.J., Li, F., Miller, J.A., DeCarlo, E., Holland, S.M., Leitman, S.F., Carter, C.S., Butz, R.E., Read, E.J., Fleisher, T.A., Schneiderman, R.D., Van Epps, D.E., Spratt, S.K., Maack, C.A., Rokovich, J.A., Cohen, L.K. and Gallin, J.I. (1997). Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. *Proc. Natl. Acad. Sci. USA*, **94**: 12133–12138.

Mazurier, F., Doedens, M., Gan, O.I. and Dick, J.E. (2003). Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. *Nat. Med.*, **9**: 959–963.

Nakamura, K., Inaba, M., Sugiura, K., Yoshimura, T., Kwon, A.H., Kamiyama, Y. and Ikehara, S. (2004). Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of GVHD by intra-bone marrow bone marrow transplantation plus donor lymphocyte infusion. *Stem Cells*, **22**: 125–134.

Plett, P.A., Frankovitz, S.M. and Orschell-Traycoff, C.M. (2002). *In vivo* trafficking, cell cycle activity, and engraftment potential of phenotypically defined primitive hematopoietic cells after transplantation into irradiated or nonirradiated recipients. *Blood*, **100**: 3545–3552.

Rosenzweig, M., MacVittie, T.J., Harper, D., Hempel, D., Glickman, R.L., Johnson, R.P., Farese, A.M., Whiting-Theobald, N., Linton, G.F., Yamasaki, G., Jordan, C.T. and Malech, H.L. (1999). Efficient and durable gene marking of hematopoietic progenitor cells in non-human primates after nonablative conditioning. *Blood*, **94**: 2271–2286.

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

Takatoku, M., Sellers, S., Agricola, B.A., Metzger, M.E., Kato, I., Donahue, R.E. and Dunbar, C.E. (2001). Avoidance of stimulation improves engraftment of cultured and retrovirally transduced hematopoietic cells in primates. *J. Clin. Invest.*, **108**: 447–455.

Ueda, K., Hanazono, Y., Shibata, H., Ageyama, N., Ueda, Y., Ogata, S., Tabata, T., Nagashima, T., Takatoku, M., Kume, A., Ikehara, S., Taniwaki, M., Terao, K., Hasegawa, M. and Ozawa, K. (2004). High-level *in vivo* gene marking after gene-modified autologous hematopoietic stem cell transplantation without marrow conditioning in non-human primates. *Mol. Ther.*, **10**: 469–477.

Wang, J., Kimura, T., Asada, R., Harada, S., Yokota, S., Kawamoto, Y., Fujimura, Y., Tsuji, T., Ikehara, S. and Sonoda, Y. (2003). SCID-repopulating cell activity of human cord blood-derived $CD34^-$ cells assured by intra-bone marrow injection. *Blood*, **101**: 2924–2931.

Wynn, R.F., Hart, C.A., Corradi-Perini, C., O'Neill, L., Evans, C.A., Wraith, J.E., Fairbairn, L.J. and Bellantuono, I. (2004). A small proportion of mesenchymal stem cells strongly express functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood*, prepublished 10.1182/blood-2004-02-0526.

Yahata, T., Ando, K., Sato, T., Miyatake, H., Nakamura, Y., Muguruma, Y., Kato, S. and Hotta, T. (2003). A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. *Blood*, **101**: 2905–2913.

Zhong, J.F., Zhan, Y., Anderson, W.F. and Zhao, Y. (2002). Murine hematopoietic stem cell distribution and proliferation in ablated and nonablated bone marrow transplantation. *Blood*, **100**: 3521–3526.

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High-Level *in Vivo* Gene Marking after Gene-Modified Autologous Hematopoietic Stem Cell Transplantation without Marrow Conditioning in Nonhuman Primates

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The successful engraftment of genetically modified hematopoietic stem cells (HSCs) without toxic conditioning is a desired goal for HSC gene therapy. To this end, we have examined the combination of intrabone marrow transplantation (iBMT) and *in vivo* expansion by a selective amplifier gene (SAG) in a nonhuman primate model. The SAG is a chimeric gene consisting of the erythropoietin (EPO) receptor gene (as a molecular switch) and c-Mpl gene (as a signal generator). Cynomolgus CD34⁺ cells were retrovirally transduced with or without SAG and returned into the femur and humerus following irrigation with saline without prior conditioning. After iBMT without SAG, 2–30% of colony-forming cells were gene marked over 1 year. The marking levels in the peripheral blood, however, remained low (<0.1%). These results indicate that transplanted cells can engraft without conditioning after iBMT, but *in vivo* expansion is limited. On the other hand, after iBMT with SAG, the peripheral marking levels increased more than 20-fold (up to 8–9%) in response to EPO even at 1 year posttransplant. The increase was EPO-dependent, multilineage, polyclonal, and repeatable. Our results suggest that the combination of iBMT and SAG allows efficient *in vivo* gene transduction without marrow conditioning.

Key Words: gene therapy, hematopoietic stem cell, intrabone marrow transplantation, nonconditioning, *in vivo* expansion, selective amplifier gene, nonhuman primate

INTRODUCTION

The ability to expand selectively cells containing potentially therapeutic genes *in vivo* would represent an important tool for the clinical application of hematopoietic stem cell (HSC)-based gene transfer. This would circumvent low gene transfer efficiency into HSCs, which is one of the current limitations of this promising technology. Furthermore, the ability to expand genetically modified cells *in vivo* would circumvent another major problem of HSC gene therapy; myeloablative conditioning is necessary unless gene-modified cells have clear growth advantage [1]. Current myeloablative conditioning regimens are associated with high systemic toxicity and potential damage to marrow stroma, possibly resulting in impaired engraftment [2]. With the *in vivo* selection method using a drug-resistance gene, engraftment of transduced cells at low levels may allow successful expansion to clinically relevant levels even without marrow conditioning, although the administration of cytotoxic agents is required for the selection [3]. It has recently been reported that bone marrow cells can efficiently engraft mice without marrow conditioning when implanted directly into the

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bone marrow cavity (intrabone marrow transplantation, iBMT) [4,5]. Using the iBMT method, human cord blood cells are also able to engraft efficiently in bone marrow of sublethally irradiated immunodeficient mice [6–8]. Although the iBMT method has been successful in mice, the efficacy in primates remains to be examined.

We have previously developed a selective amplifier gene (SAG) consisting of a chimeric gene encoding the granulocyte colony-stimulating factor (G-CSF) receptor (as a growth-signal generator) and the hormone-binding domain of the steroid receptor (as a molecular switch) [9]. Hematopoietic cells genetically engineered to express this SAG can be expanded in a steroid-dependent manner *in vitro* and *in vivo* in mice and nonhuman primates [10,11]. Here we have examined such expansion in the setting of nonhuman primate iBMT without marrow conditioning using a new SAG encoding the erythropoietin (EPO) receptor (as a molecular switch) and thrombopoietin receptor (c-Mpl; as a signal generator) [12].

RESULTS

Engraftment after iBMT

First, we examined whether gene-marked CD34$^+$ cells engraft after iBMT using two cynomolgus macaques. Cynomolgus CD34$^+$ cells were transduced with the nonexpression retroviral vector PLI (which contains untranslated sequence) [13]. The transduction results are summarized in Table 1. We injected the transduced CD34$^+$ cells directly into the bone marrow cavity of four proximal limb bones (the femurs and humeri) after gently irrigating the cavity with saline (Fig. 1). This transplant procedure was safely performed without pulmonary embolism or infection of bone marrow. Conditioning treatment such as irradiation was not conducted prior to transplantation. In addition, we returned the transduced CD34$^+$ cells into two monkeys by the conventional transplantation method without prior conditioning.

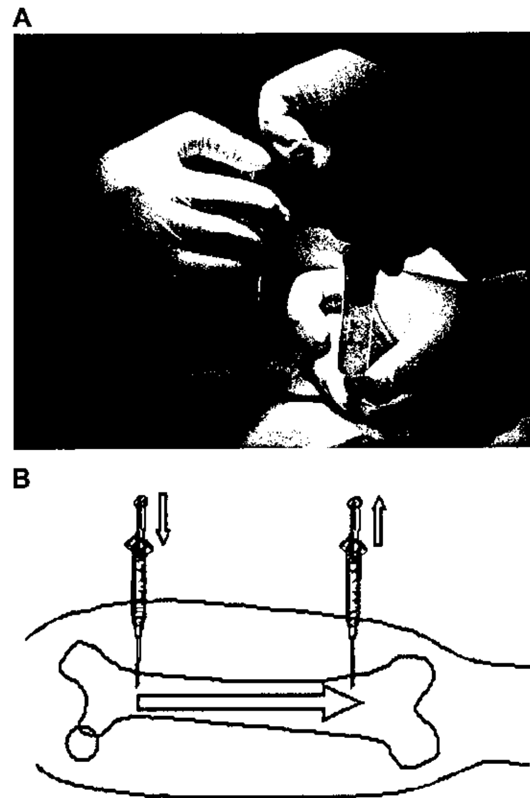

FIG. 1. The iBMT method. We inserted needles at both ends of limb bones (femurs and humeri) and irrigated the bone marrow cavity gently with saline without inflicting extra pressure (A, photo; B, schematic diagram). Gene-modified CD34$^+$ cells were then injected directly into the bone marrow through the needle on one side.

After iBMT, we plated cells from the nonimplanted iliac marrow in methylcellulose medium. We examined the resulting colonies (colony-forming units, CFU) for the provirus by PCR (Fig. 2A and 2B). Two to 30% of colonies (overall 14.2% (74/522)) were positive for the

TABLE 1: *Ex vivo* transduction

Animal	Target cell source	Vector	No. of infused CD34$^+$ cells/kg	Fraction of provirus-positive CFUs in infused CD34$^+$ cells
		Intrabone marrow transplantation		
IB3048	Bone marrow	PLI	4.5×10^7	34/46 (73.9%)
IB3053	Peripheral blood	PLI	8.1×10^6	49/78 (62.8%)
S9042	Peripheral blood	SAG	2.6×10^7	20/35 (57.1%)
S3047	Peripheral blood	SAG	8.1×10^6	11/21 (52.4%)
D8058	Peripheral blood	SAG	7.8×10^5	11/43 (25.6%)
		PLI	5.7×10^5	9/42 (21.4%)
		Intravenous transplantation		
V0065	Peripheral blood	PLI	1.2×10^7	3/45 (6.7%)
V1007	Peripheral blood	PLI	1.5×10^6	14/41 (34.1%)

PLI, nonexpression vector; SAG, selective amplifier gene vector.

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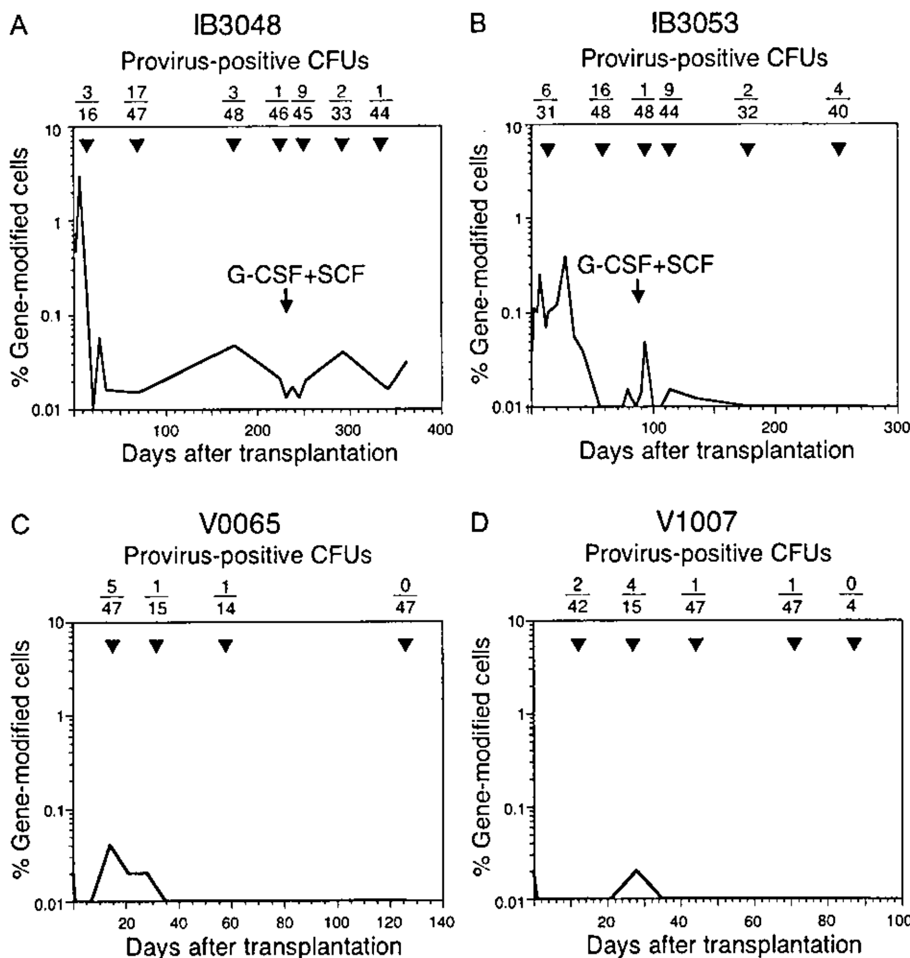

FIG. 2. *In vivo* marking after iBMT and intravenous transplantation without marrow conditioning. CD34$^+$ cells were transduced with nonexpression retroviral vector PLI and returned by iBMT (A, IB3048, and B, IB3053) or by intravenous transplantation (C, V0065, and D, V1007) without conditioning. The upper row shows ratios of provirus-positive CFUs to β-actin-positive CFUs taken from the nonimplanted marrow at time points indicated by arrows. Overall number of provirus-positive CFUs versus overall number of β-actin-positive CFUs was 74/522 (14.2%) for iBMT (A and B) and 15/274 (5.5%) for the intravenous transplantation (C and D). The lower diagram shows percentages of gene-modified cells in the peripheral blood as assessed by quantitative PCR.

provirus and this high marking level persisted for over 1 year posttransplantation. On the other hand, after the conventional intravenous transplantation, generally fewer CFU contained the provirus (overall 5.5% (15/274)) in the bone marrow (Fig. 2C and 2D). Interestingly, the provirus in CFU from the nonimplanted marrow was detectable within 2 weeks after iBMT. Thus, transplanted cells relocated from an implanted bone to another at early time points. A similarly early translocation posttransplantation has also been reported in mouse syngeneic iBMT and human-mouse xeno-iBMT models [4,6–8]. We also examined peripheral blood cells for the provirus by quantitative PCR (Fig. 2A and 2B). The marking levels were, however, very low (<0.1%) in the peripheral blood.

Taken together, these results suggest that transplanted cells can engraft nonconditioned recipients after iBMT but their contribution to the peripheral blood is minimal compared to myeloablated recipients. The cells stay at a resting state in bone marrow without proliferation. In an attempt to proliferate and mobilize iBMT-engrafted resting progenitor cells, we administered G-CSF and stem cell factor (SCF) for 5 consecutive days [14]; however, no obvious increase in the vector-containing cells was observed in the peripheral blood (Fig. 2A and 2B).

EPO-dependent expansion with SAG

We constructed a retroviral vector expressing an SAG that is a chimeric gene of the human EPO receptor gene (extra- plus transmembrane region as a molecular switch) and the human c-Mpl gene (cytoplasmic region as a signal generator) [12]. Cells genetically engineered to express this SAG will proliferate in an EPO-dependent manner. We transduced cynomolgus CD34$^+$ cells with the SAG retroviral vector and introduced them into nonconditioned autologous recipients by iBMT (Table 1). *In vivo* results after transplantation are summarized in Table 2.

In one animal (Fig. 3A), EPO administration triggered a striking elevation in marking levels (7.4% at day 105 posttransplantation) in the peripheral blood. The level of marking in the periphery stayed high for the duration of EPO administration. After cessation of EPO, the level fell to <0.1%. Resumption of EPO administration produced a

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TABLE 2: *In vivo* expansion with SAG after iBMT

Animal	Treatment course	EPO treatment: Period (days posttransplant)	EPO treatment: Dosage	Marked leukocytes (%)[a]: Basal marking before treatment	Marked leukocytes (%)[a]: Peak marking after treatment (day posttransplant)
S9042	1	1–40	200 IU/kg once daily	NA	7.36% (day 105)
		41–100	200 IU/kg twice daily	NA	7.36% (day 105)
	2	132–210	200 IU/kg twice daily	0.02%	7.72% (day 188)
	3	246–367	200 IU/kg twice daily	0.41%	8.90% (day 348)
S3047	1	75–134	200 IU/kg once daily	0.01%	0.23% (day 145)
		135–166	200 IU/kg twice daily	0.01%	0.23% (day 145)
	2	210–289	200 IU/kg twice daily	0.02%	0.00% (day 289)
D8058	1	1–86	200 IU/kg twice daily	NA	2.30% (day 14)

[a] As assessed by quantitative PCR (see Materials and Methods). NA, not applicable.

similar elevation in the marking levels. The third EPO administration again resulted in the increased marking levels to 8.9% at day 348 posttransplantation. EPO administration was associated with a mild increase in hematocrit (up to 63.5%), which was manageable by occasional phlebotomy. No other adverse effects were observed.

In another animal (Fig. 3B), the SAG-transduced cells increased following transplantation even without exogenous EPO administration. The increase may have been due to increased endogenous EPO elevation resulting from anemia present in the second animal. Overall marking fell with resolution of the anemia. Following resolution, EPO was administered, resulting in an increase in marking levels by more than 20-fold. Marking levels declined to the basal level after discontinuation of EPO. A second attempt to increase marking levels failed, with clearance of SAG-positive cells from the periphery within a month after the second administration, most likely due to cellular immune responses to the xenogeneic SAG (see below).

Multilineage and Polyclonal Expansion

In situ PCR for the proviral sequence showed many transduced cells in the peripheral blood taken from animal S9042 receiving EPO at day 89 posttransplantation (Fig. 4A). We subjected granulocytes and T and B lymphocytes sorted from the peripheral blood of this animal at day 91 posttransplantation to semiquantitative PCR for the provirus. The provirus-containing fraction in granulocytes was 6% and that in B and T lymphocytes was 2% (Fig. 4B), thus indicating that multilineage expansion had occurred. The persistence of marked, short-lived granulocytes for the long term is also another evidence of the successful engraftment of gene-modified HSCs after iBMT. The integration site analysis using the linear amplification-mediated (LAM) PCR method [15]

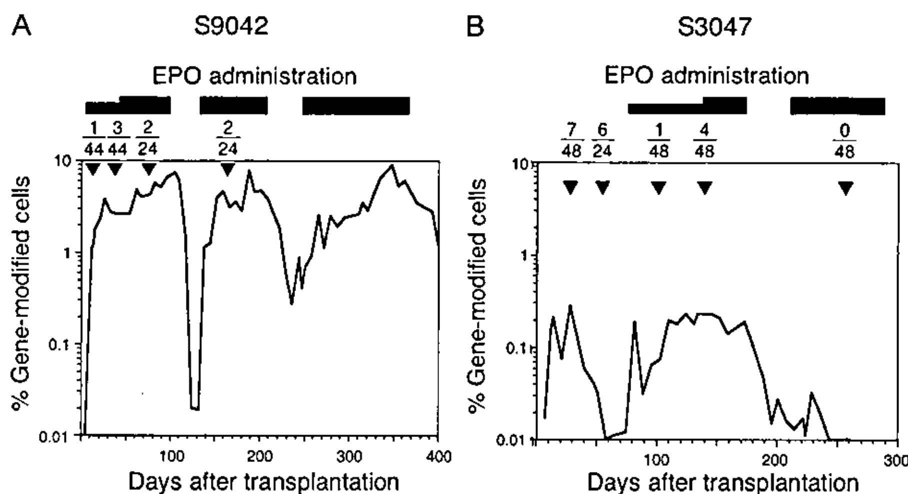

FIG. 3. Expansion of SAG-transduced cells by treatment with EPO after iBMT. CD34⁺ cells transduced with SAG were returned to each animal by iBMT without conditioning. The animals (A) S9042 and (B) S3047 received EPO at 200 IU/kg once or twice daily (indicated by closed bars). The upper row shows ratios of provirus-positive CFUs to β-actin-positive CFUs taken from the nonimplanted marrow at time points indicated by arrows. The lower diagram shows percentages of gene-modified cells in the peripheral blood as assessed by quantitative PCR.

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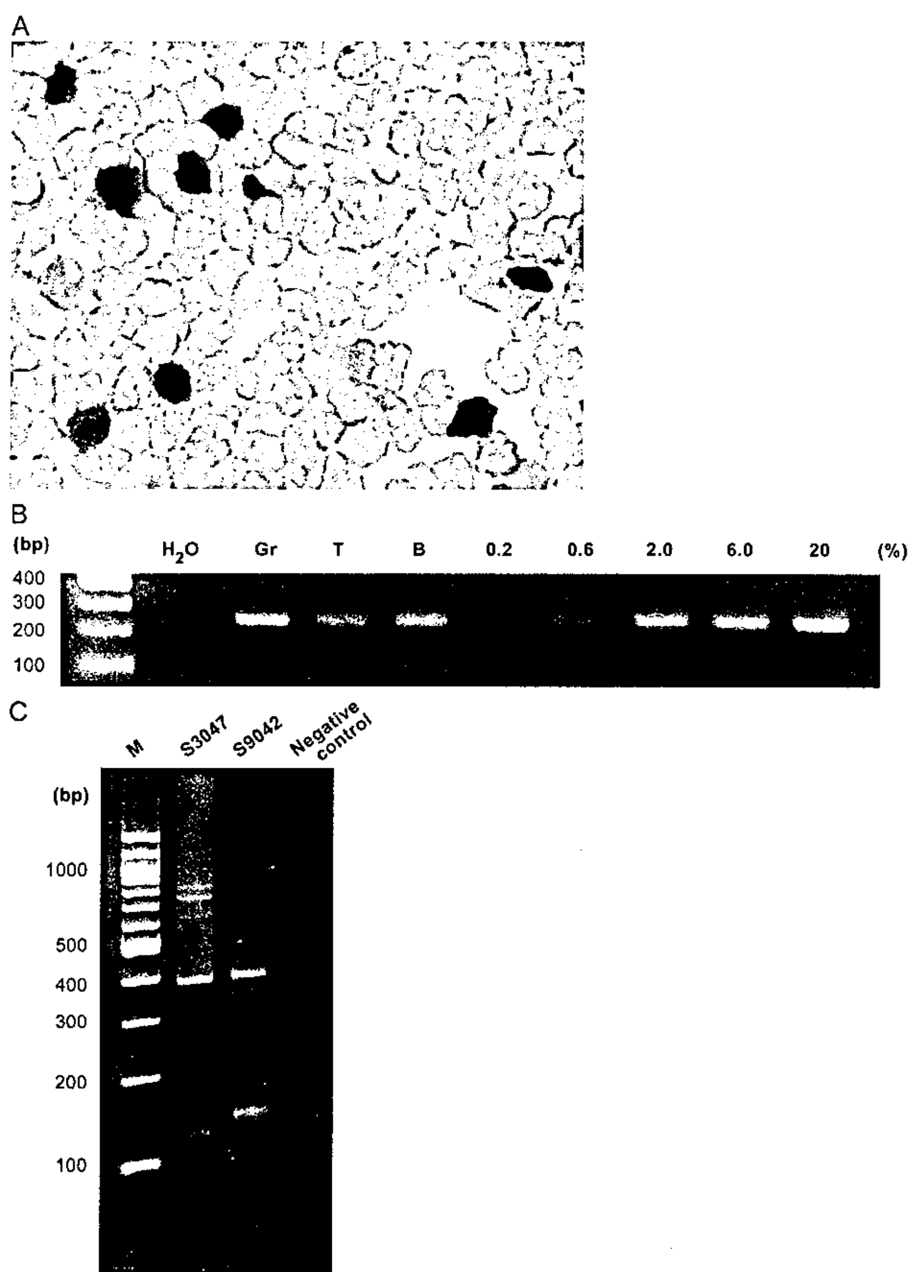

FIG. 4. High-level, multilineage, and polyclonal expansion of gene-modified cells in the peripheral blood after iBMT with SAG in nonconditioned recipients. (A) *In situ* PCR for the provirus. Peripheral blood nucleated cells were collected from animal S9042 receiving EPO at day 89 posttransplantation. Many SAG-transduced cells (stained in black) were detected by *in situ* PCR. (B) Lineage analysis by semiquantitative PCR. DNA from granulocytes (Gr) and T and B lymphocytes sorted from animal S9042 receiving EPO at day 91 posttransplantation was examined for the provirus by semiquantitative PCR. Positive controls corresponding to 0.2, 0.6, 2.0, 6.0, and 20% of transduced cells in peripheral blood were included. (C) Clonal analysis by LAM-PCR. Genomic DNA from peripheral blood of the animals receiving EPO (S9042 at day 90 and S3047 at day 150 posttransplantation) was analyzed by LAM-PCR. Each band indicates different integrants. Negative control was genomic DNA from a naive monkey. M, molecular weight marker.

indicates that the expansion of transduced cells in response to EPO was polyclonal, not mono- or oligoclonal (Fig. 4C).

Dual-Marking Study

We then compared the effects of the SAG vector to a non-SAG vector within, rather than between, individual animals. We harvested cytokine-mobilized peripheral blood $CD34^+$ cells and split them into two equal aliquots. We transduced one aliquot with the SAG vector and the other with the control nonexpression vector (PLI). We mixed both aliquots and returned them by iBMT without marrow conditioning. The animal received EPO from the day after transplantation, and we examined *in vivo* marking levels derived from the two populations by quantitative PCR.

Cells containing the SAG vector increased by 2 logs in the peripheral blood in response to EPO, although cells containing the nonexpression vector remained at low levels (Fig. 5). However, SAG-containing cells were rapidly cleared within 1 month posttransplantation from the periphery and overall SAG-vector marking

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levels became even lower than those from the nonexpression vector-marked fraction. Since cyclosporin A was concomitantly administered to prevent immune responses to human EPO, human EPO concentrations were maintained within an effective range. Thus, it is unlikely that the clearance of xenogeneic human EPO due to immune responses turned off the molecular switch of SAG, resulting in the decrease in SAG-transduced cells.

Immune Responses

The current SAG is a chimeric gene of human origin (the human EPO receptor and human c-Mpl). We collected peripheral lymphocytes from the animal receiving both SAG and nonexpressing PLI (D8058, Fig. 5) at day 169 posttransplantation and examined whether the lymphocytes responded to the xenogeneic SAG *in vitro* (Fig. 6). The response to SAG-transduced target cells was stronger than that to nontransduced target cells (P = 0.05), while the response to PLI-transduced target cells did not differ significantly from that to nontransduced target cells (P = 0.13). The cellular immune response is, therefore, the most likely reason for the clearance of SAG-transduced cells in this animal. This is not novel, but it has been reported that immune responses against transgene products recognized as foreign can indeed be a major obstacle to long-term persistence of gene-modified cells *in vivo* [13,16,17]. In the human clinical setting, however, immune responses should not occur against SAG, because the SAG is made of human genes.

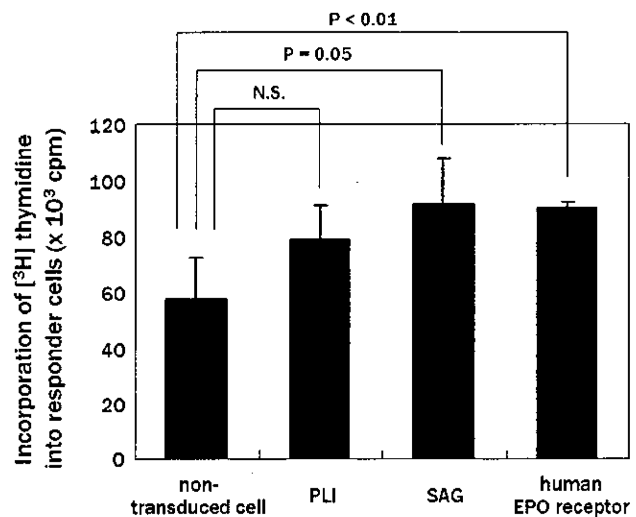

FIG. 6. Positive blastogenic response of lymphocytes to SAG. Peripheral blood mononuclear cells (responder cells) were isolated from monkey D8058 at day 169 posttransplantation (Fig. 5) and cocultured with stimulator cells. The stimulator cells were autologous stromal cells untransduced or transduced retrovirally with PLI, SAG, or human EPO receptor cDNA followed by irradiation with 4000 cGy. After 5 days in culture, the blastogenesis of responder cells was assessed by counting the [^{3}H]thymidine incorporation into responder cells. The averages ± SD of triplicate experiments are shown. N.S., not significant.

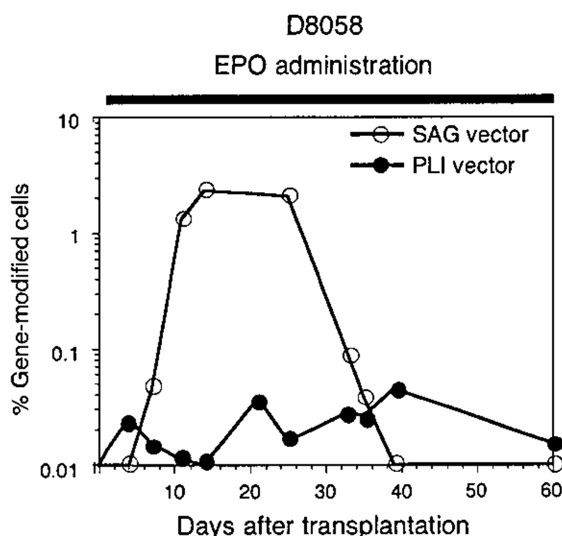

FIG. 5. Dual genetic marking study. CD34$^+$ cells from monkey D8058 were split into two equal aliquots; one aliquot was transduced with SAG vector and the other with nonexpression PLI vector. Both aliquots were together returned to the bone marrow cavity by iBMT without conditioning. EPO (200 IU/kg, twice daily) was administered from the day after transplantation (indicated by a closed bar).

Discussion

Previous papers documented that, without marrow conditioning, very low levels (much less than 0.1%) of cells were marked (or corrected) after CD34$^+$ cell gene therapy of chronic granulomatous disease and Gaucher disease [18,19]. This clinical observation has formed the foundation for the contention that myeloablation (or at least conditioning of reduced intensity) is necessary for successful engraftment of transplanted, genetically modified cells. Our results, however, suggest that nonconditioned iBMT results in much higher gene marking levels (up to 8–9%) through the utilization of an SAG. The physical elimination of endogenous marrow with saline before injection might increase gene marking. In the current study, the marrow of four proximal limb bones (femurs and humeri) was replaced with transplanted cells. If other bones such as the iliac bone (which contains more marrow) are similarly used for iBMT, even higher *in vivo* marking levels may be achieved using an SAG.

Expansion of SAG-transduced cells was seen in three lineages: granulocytes, B lymphocytes, and T lymphocytes. The c-Mpl signal generated by the SAG may work even in lymphocytes. In fact, B lymphocytes were shown to be increased by the activated c-Mpl in a canine trans-

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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 $\mu g/cm^2$ 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′-TTGGACAGCAAGAAAGT-GAGCTT-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′-CCACCCC-TAGCCCTAAATCTTATG-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 *Tas*I (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′-GATCTGAATTCAGTGG-CACAG-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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References

1. Cavazzana-Calvo, M., *et al.* (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288:** 669–672.
2. Plett, P. A., Frankovitz, S. M., and Orschell-Traycoff, C. M. (2002). In vivo trafficking, cell cycle activity, and engraftment potential of phenotypically defined primitive hematopoietic cells after transplantation into irradiated or nonirradiated recipients. *Blood* **100:** 3545–3552.
3. Bowman, J. E., Reese, J. S., Lingas, K. T., and Gerson, S. L. (2003). Myeloablation is not required to select and maintain expression of the drug-resistance gene, mutant MGMT, in primary and secondary recipients. *Mol. Ther.* **8:** 42–50.

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4. Zhong, J. F., Zhan, Y., Anderson, W. F., and Zhao, Y. (2002). Murine hematopoietic stem cell distribution and proliferation in ablated and nonablated bone marrow transplantation. *Blood* **100:** 3521–3526.
5. Nakamura, K., *et al.* (2004). Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of GVHD by intra-bone marrow transplantation plus donor lymphocyte infusion. *Stem Cells* **22:** 125–134.
6. Wang, J., *et al.* (2003). SCID-repopulating cell activity of human cord blood-derived $CD34^-$ cells assured by intra-bone marrow injection. *Blood* **101:** 2924–2931.
7. Mazurier, F., Doedens, M., Gan, O. I., and Dick, J. E. (2003). Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. *Nat. Med.* **9:** 959–963.
8. Yahata, T., *et al.* (2003). A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. *Blood* **101:** 2905–2913.
9. Ito, K., *et al.* (1997). Development of a novel selective amplifier gene for controllable expansion of transduced hematopoietic cells. *Blood* **90:** 3884–3892.
10. Kume, A., *et al.* (2003). In vivo expansion of transduced murine hematopoietic cells with a selective amplifier gene. *J. Gene Med.* **5:** 175–181.
11. Hanazono, Y., *et al.* (2002). In vivo selective expansion of gene-modified hematopoietic cells in a nonhuman primate model. *Gene Ther.* **9:** 1055–1064.
12. Nagashima, T., *et al.* (2004). In vivo expansion of gene-modified cells by a novel selective amplifier gene utilizing the erythropoietin receptor as a molecular switch. *J. Gene Med.* **6:** 22–31.
13. Heim, D. A., *et al.* (2000). Introduction of a xenogeneic gene via hematopoietic stem cells leads to specific tolerance in a rhesus monkey model. *Mol. Ther.* **1:** 533–544.
14. Horsfall, M. J., Hui, C. H., To, L. B., Begley, C. G., Basser, R. L., and Simmons, P. J. (2000). Combination of stem cell factor and granulocyte colony-stimulating factor mobilizes the highest number of primitive haemopoietic progenitors as shown by pre-colony-forming unit (pre-CFU) assay. *Br. J. Haematol.* **109:** 751–758.
15. Schmidt, M., *et al.* (2003). Clonality analysis after retroviral-mediated gene transfer to $CD34^+$ cells from the cord blood of ADA-deficient SCID neonates. *Nat. Med.* **9:** 463–468.
16. Riddell, S. R., *et al.* (1996). T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nat. Med.* **2:** 216–223.
17. Rosenzweig, M., *et al.* (2001). Induction of cytotoxic T lymphocyte and antibody responses to enhanced green fluorescent protein following transplantation of transduced CD34(+) hematopoietic cells. *Blood* **97:** 1951–1959.
18. Malech, H. L., *et al.* (1997). Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. *Proc. Natl. Acad. Sci. USA* **94:** 12133–12138.
19. Dunbar, C. E., *et al.* (1998). Retroviral transfer of the glucocerebrosidase gene into CD34+ cells from patients with Gaucher disease: in vivo detection of transduced cells without myeloablation. *Hum. Gene Ther.* **9:** 2629–2640.
20. Neff, T., *et al.* (2002). Pharmacologically regulated in vivo selection in a large animal. *Blood* **100:** 2026–2031.
21. Hacein-Bey-Abina, S., *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302:** 415–419.
22. Wrighton, N. C., *et al.* (1996). Small peptides as potent mimetics of the protein hormone erythropoietin. *Science* **273:** 458–464.
23. Macdougall, I. C. (2000). Novel erythropoiesis stimulating protein. *Semin. Nephrol.* **20:** 375–381.
24. Shibata, H., *et al.* (2003). Collection and analysis of hematopoietic progenitor cells from cynomolgus macaques (Macaca fascicularis): assessment of cross-reacting monoclonal antibodies. *Am. J. Primatol.* **61:** 3–12.
25. Takatoku, M., *et al.* (2001). Avoidance of stimulation improves engraftment of cultured and retrovirally transduced hematopoietic cells in primates. *J. Clin. Invest.* **108:** 447–455.
26. Kushida, T., *et al.* (2002). Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys at various ages by perfusion or aspiration methods: a preclinical study for human BMT. *Stem Cells* **20:** 155–162.
27. Schuurman, H. J., *et al.* (2001). Pharmacokinetics of cyclosporine in monkeys after oral and intramuscular administration: relation to efficacy in kidney allografting. *Transplant. Int.* **14:** 320–328.
28. Haase, A. T., Retzel, E. F., and Staskus, K. A. (1990). Amplifications and detection of lentiviral DNA inside cells. *Proc. Natl. Acad. Sci. USA* **87:** 4971–4975.

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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.

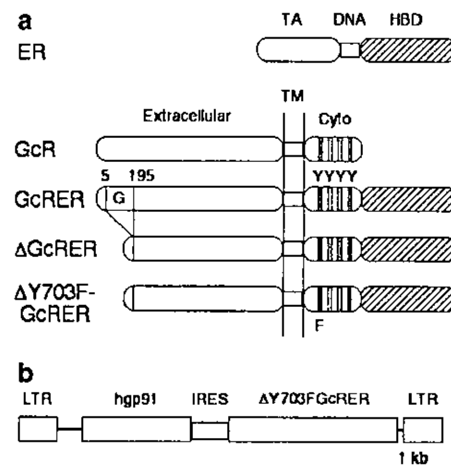

Figure 1 Structure of selective amplifier gene-encoded proteins and gene transfer vector. (a) GcRER is a fusion protein comprising the full-length mouse G-CSF receptor (GcR) and the hormone-binding domain of the rat estrogen receptor (ER). ΔGcRER is deleted of the G-CSF binding domain of GcR (amino acids 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.

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 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