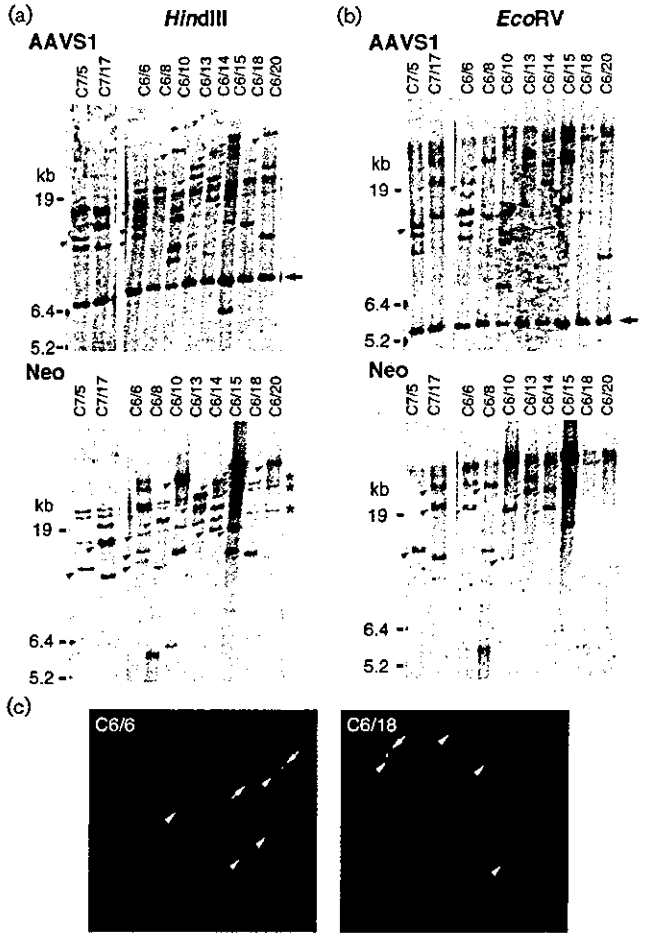

Fig. 2. Southern blot analysis of clones with the Neo gene at AAVS1. *Hin*dIII- or *Eco*RV-digested genomic DNA was hybridized initially with a ^{32}P-labelled AAVS1-specific probe [the 3·0 kb *Acc*I fragment from pRVK (a gift from K.I. Berns)]. After stripping the probe, membranes were rehybridized with a Neo-specific probe (the 2·0 kb *Not*I fragment derived from pWNeo). The presence of co-migrating bands that hybridized to both AAVS1 and Neo probes on both *Hin*dIII and *Eco*RV blots was a criterion to conclude that the Neo gene was integrated into AAVS1. (a) *Hin*dIII-digest probed with an AAVS1-specific probe (upper panel) or with a Neo probe (lower panel). (b) Blot of genomic DNA digested with *Eco*RV isolated from the same clones. Each clone presented here has an upshifted band(s) other than a basal 6·5 kb band (arrow). Common bands that hybridized to both AAVS1 and Neo probes are indicated by arrowheads. Asterisks indicate non-specific bands that cross-hybridized to the Neo probe used. Clones C7/5 and C/17 were derived from transfection with pCMVR78. Clones C6/6 to C6/20 were obtained using pCMVR68. (c) Representative FISH of clones shown to harbour the Neo gene at AAVS1 by Southern blot analysis. Note that the 293 cells used in the present study have four copies of chromosome 19 (arrowheads). Signals detected by a Neo probe (arrows) are indicated.

1994; Surosky *et al.*, 1997). A similar disruption of AAVS1 has been detected in cell lines latently infected with wild-type AAV (Kotin *et al.*, 1990). This phenomenon may be explained in three ways. First, the integrated transgene or AAV genome is disrupted during or after an integration event such that Southern blot analysis cannot detect it. The instability of the integrated AAV genome over passages in a latently infected cell line was described (Cheung *et al.*, 1980). An additional rearrangement can occur in the rearranged AAVS1 region (Shelling & Smith, 1994). Second, recombination between the AAVS1 region and other sites may cause rearrangement of AAVS1 without integration of the transgene at AAVS1. Third, the Rep protein may excise the integrated plasmid DNA or AAV genome, resulting in the loss of the preintegrated sequences.

The 293 cells used in the present study have four copies of chromosome 19. Southern blot analysis showed that some clones had more than three upshifted bands besides a basal band. We used a relatively large probe (3·0 kb) for detecting AAVS1 bands. It is possible that Rep-mediated disruption of the AAVS1 region can produce the multiple bands hybridizing to the AAVS1 probe. Another explanation is as follows: at 24 h post-transfection, we replated transfected cells to isolate clones derived from single cells. At this time-point, the Rep protein was still being expressed in cells and an additional integration event might occur in some cells after cell division.

Lamartina *et al.* (1998) reported no apparent difference between Rep78 and Rep68 in the ability to deliver foreign DNA to AAVS1 in HeLa cells. Several studies have reported the functional differences between Rep78 and Rep68. Rep68 is more efficient in processing dimers to monomer duplex DNA and possesses a stronger nicking activity (Ni *et al.*, 1994, 1998), while the helicase activity of Rep78 is stronger (Wollscheid *et al.*, 1997). The differential effects of Rep78 and Rep68 on the p5, p19 and p40 promoters were described (Weger *et al.*, 1997). In addition, Rep78 inhibits CREB-dependent transcription by interacting with protein kinase (Chiorini *et al.*, 1998; Di Pasquale & Stacey, 1998). None of these findings explains why Rep78 appears to cause more abortive integration. Rep68 may be more suitable for the AAVS1-targeted integration system. To confirm the usefulness of Rep68 in the AAVS1-targeted integration system, further analysis of a larger population of cell clones would be required. Also, the exact functions of the Rep protein in AAVS1-specific integration should be elucidated.

The results presented here have important implications for developing an AAVS1-directed integration system as well as for understanding the mechanism of AAVS1-specific integration by the Rep proteins.

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Reduction of CTLL-2 cytotoxicity by induction of apoptosis with a Fas-estrogen receptor chimera

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Allogeneic bone marrow transplantation and donor lymphocyte infusion are powerful treatments for chemotherapy-resistant leukemia. Tumor eradication is attributed to a graft-versus-leukemia reaction by the donor-derived cytotoxic T lymphocytes (CTLs), but the same cell population may cause severe graft-versus-host disease. One strategy to suppress harmful CTL activity is to incorporate a suicide gene into the donor lymphocytes prior to infusion, and to destroy these cells when they aggressively attack nonmalignant host tissues. In this study, we investigated the feasibility of using a Fas-estrogen receptor fusion protein (MfasER) to control T cell-mediated cytotoxicity, based on our previous finding that the chimera transmits a Fas-mediated death signal through activation by estrogen binding. A murine CTL line CTLL-2 was transfected with a vector encoding MfasER, and the growth, viability and cytotoxic activity of the transfected cells (CTLL/MfasER) were analyzed. The expression of apoptosis-related proteins such as Fas ligand and perforin was also investigated. In the absence of estrogen, CTLL/MfasER showed similar growth to parental CTLL-2, and the killing activity was preserved. Addition of 10^{-7} *M* estrogen induced a rapid apoptosis of CTLL/MfasER, and the cytotoxicity was severely impaired. A decrease of Fas ligand and perforin in the estrogen-treated CTLL/MfasER was seen in an immunoblot analysis. These functional and biochemical analyses showed that the estrogen-inducible apoptosis in MfasER-expressing CTLs rapidly terminated their target cell killing. The feasibility of using the MfasER-estrogen system to control graft-versus-host disease was demonstrated. (Cancer Sci 2003; 94: 639–643)

Today, allogeneic cell therapies such as bone marrow transplantation (BMT) and donor lymphocyte infusion (DLI) are indispensable means of treating hematological malignancies and achieve a life-long cure in many patients. The advantage of such therapies over conventional chemotherapy is at least two-fold. First, an intensified, myeloablative dose of chemoradiotherapy can be given during the pre-transplant period. Second, immunocompetent allogeneic donor T lymphocytes eradicate residual malignant cells. This combat reaction by the donor T cells is called a graft-versus-leukemia (GVL) effect.[1] However, GVL may be closely associated with graft-versus-host disease (GVHD), a serious complication following BMT and DLI. Currently, the dominant antigens on leukemia cells driving the GVL response are unknown, and efforts to discriminate T cell subsets responsible for GVL and GVHD have been mostly unsuccessful.

One way to avoid this dilemma is to incorporate a suicide gene into the donor lymphocytes and eliminate them when severe GVHD occurs. So far, the thymidine kinase gene from herpes simplex virus type 1 (HSVtk) has been most extensively studied for this purpose.[2] This strategy is based on the fact that the viral enzyme converts barely toxic prodrugs such as ganciclovir (GCV) into highly toxic intermediates. Although the toxic metabolites should kill only the manipulated cells expressing HSVtk, this suicide system has some limitations. Conceivable problems are as follows. (1) The rate of cell killing is relatively slow, while a rapid eradication of responsible CTLs is necessary to terminate severe GVHD. (2) Nondividing cells are refractory. The HSVtk/GCV system depends on DNA synthesis to exert cytotoxicity, but activated effector cells are not necessarily dividing at that moment. Indeed, this system was not fully functional in a patient with chronic GVHD, where responsible CTLs might be slowly cycling.[2] (3) GCV may cause nonspecific bone marrow suppression. Patients undergoing DLI are usually in a myelosuppressive state, and GCV treatment may further suppress their hematopoietic activity to cause severe marrow failure. (4) Anti-cytomegalovirus treatment may hamper GVL by the HSVtk-transduced T cells. Cytomegalovirus infection is common in immunocompromised patients, where GCV is one of the few therapeutic options. GCV administration will eradicate HSVtk-transduced cells, including the donor-derived lymphocytes contributing to GVL and graft-versus-infection. (5) HSVtk is a foreign protein to the hosts and may evoke immunological reactions against the transduced cells.[3]

To overcome these limitations, we have employed a novel suicide system using Fas and the ligand-binding domain (LBD) of a nuclear receptor.[4,5] When fused with heterologous proteins, an LBD can work as a molecular switch to control the fusion protein, and a variety of effector proteins have been converted to function in a ligand-dependent manner.[6] In fact, an apoptosis-inducing system was designed with Fas and the LBD of estrogen receptor (ER). Fas, also called CD95 or APO-1, belongs to the tumor necrosis factor receptor superfamily and regulates apoptosis upon binding to Fas ligand (FasL) or crosslinking antibodies.[7] In a previous study, the transmembrane and cytoplasmic domains of Fas (Mfas) were fused with ER, and expression of the chimeric molecule (MfasER) led to estrogen-inducible apoptosis in L929 cells both *in vitro* and *in vivo*.[4] The estrogen-induced apoptosis was rapid and extensive whether the challenged cells were proliferating or resting, unlike the HSVtk/GCV system, which was ineffective in killing nonproliferating cells.[5] We expected that the MfasER/estrogen system would also be effective in eliminating the cells involved in GVHD, because Fas-mediated apoptosis is physiologically vital in activated T lymphocytes. In addition, immunological reactions are less likely to be elicited against the fusion protein than against HSVtk, because MfasER is composed of endogenous proteins. In the present study, we investigated the feasibility of using MfasER to control T cell-mediated cytotoxicity. A murine T cell line CTLL-2,[8] with many characteristics of normal CTLs, was used as an effector to evaluate the efficacy of MfasER in controlling cytotoxic activity.

Materials and Methods

Plasmids. An MfasER expression vector was constructed as follows. MfasER cDNA was derived from pEF-BOS/MfasER (a gift from Dr. A. Kakizuka, Kyoto University, Kyoto).[4] An in-

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ternal ribosomal entry site (IRES) sequence was amplified by polymerase chain reaction (PCR) on the encephalomyocarditis virus genome (nucleotides 259–833), using primers A (5′-GCATTCCTAGGGGTCTTTCC-3′) and B (5′-CCATCTTGTTCAATCATATTATCATCGTGTTTTTCAA-3′).[9,10] The neomycin phosphotransferase gene (neo) was derived from pRSVneo by PCR with primers C (5′-TTGAAAAACACGATGATAATATGATTGAACAAGATGG-3′) and D (5′-CCGACTCGAGTCAGAAGAACTCGTC-3′).[11] Primers B and C were designed to create an overlap between the 3′-end of the IRES and the 5′-end of the neo PCR products, and the IRESneo fragment was PCR-amplified on them with primers A and D. The MfasER and IRESneo fragments were cloned into pMX retroviral vector (a gift from Dr. T. Kitamura, University of Tokyo, Tokyo).[12] The resultant bicistronic retroviral vector was designated pMX/MfasER-IRESneo. In order to increase the expression, a CAG promoter sequence (a gift from Dr. J. Miyazaki, Osaka University, Osaka; Ref. 13) was inserted into pMX/MfasER-IRESneo, at the site between the 5′-long terminal repeat (LTR) and MfasER gene. The resultant vector was designated pMX/CAGMfasER-IRESneo.

Cell culture and transfection. CTLL-2, a murine cytotoxic T cell line (a gift from Dr. K. Sugamura, Tohoku University, Sendai),[8] was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 10 U/ml of recombinant human interleukin-2 (IL-2) (Imunace; provided by Shionogi, Osaka), 2 m*M* L-glutamine (Invitrogen, Grand Island, NY) and 50 μM 2-mercaptoethanol. The expression plasmid, pMX/CAGMfasER-IRESneo, was linearized between the 5′-LTR and CAG promoter sequence and electroporated into CTLL-2 cells using a Gene Pulser (Bio-Rad, Hercules, CA). Stably transfected cells were selected by culturing in medium supplemented with 1.0 mg/ml of G418 (Invitrogen). We designated the final cell population as CTLL/MfasER. RLmale1 mouse T cell leukemia line was maintained in RPMI-1640 with 10% FBS.[14]

Induction of growth inhibition and apoptosis. To assess estrogen-dependent apoptosis, CTLL/MfasER cells and parental CTLL-2 cells were harvested from standard cultures, and recultured at an initial density of 5×10^4 cells/ml in medium with 10^{-7} *M* 17β-estradiol (E_2) (Sigma) or 0.05% ethanol (solvent for E_2). After designated periods of incubation, CTLL/MfasER and CTLL-2 cells were examined on an inverted microscope (IX-70; Olympus, Tokyo). Viable cells were counted by the trypan blue exclusion method and photographed (original magnification, 500×). The number of viable cells at each time point was standardized with the value for 0 h taken as 100%. Cytospin preparations were also made for morphological examination.

Cell-mediated cytotoxicity assays. To determine CTL activities of CTLL-2 cells, a standard ^{51}Cr release assay was performed as described,[15] using RLmale1 cells as the target. Briefly, CTLL-2 and MfasER/CTLL effector cells were deprived of IL-2 in the presence or absence of 10^{-7} *M* E_2. After 12 h, the effector cells were stimulated with 20 U/ml of IL-2 for 2 h. Meanwhile, 1×10^6 RLmale1 cells were radiolabeled with 50 μCi of $Na_2{}^{51}CrO_4$ for 2 h at 37°C, washed three times with phosphate-buffered saline (PBS) and resuspended in the culture medium at 1×10^5 cells/ml. The radiolabeled target cells were inoculated onto 96-well round-bottomed microtiter plates (1×10^4 cells in 0.1 ml per well), to which serially diluted effector cells were added. The mixed cells were incubated in a total volume of 0.2 ml for 4 h at 37°C. The ^{51}Cr released into the supernatant was measured with a γ-scintillation counter (ARC-300; Aloka, Tokyo) and designated experimental release (R_{ex}). In parallel, spontaneous release (R_{sp}) was determined by the incubation of target cells without effector cells, and maximal release (R_{max}) was obtained by lysing 1×10^4 target cells in 1% Triton X-100. The percent specific lysis was calculated from the following formula:

$$\%\ \text{specific lysis}=[(R_{ex}-R_{sp})/(R_{max}-R_{sp})]\times100.$$

The cytotoxicity assay was performed in triplicate, with varying effector/target (E/T) ratios.

Western blot analysis. CTLL-2 and CTLL/MfasER cells were harvested and washed three times with PBS. The cells were lysed with Triton X-100 buffer [1% Triton X-100, 50 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 100 m*M* EGTA, 1.5 m*M* $MgCl_2$ and a protease inhibitor cocktail tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany)] for 60 min on ice. After centrifugation, cell lysates (from 2.5×10^6 cells for each sample, counted at the beginning of E_2 exposure) were heated at 100°C for 5 min in Laemmli's sample buffer [62.5 m*M* Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol and 5% 2-mercaptoethanol] and subjected to SDS-10% polyacrylamide gel electrophoresis. Subsequently, the proteins were electroblotted onto polyvinylidene fluoride membrane (Immobilon-P; Millipore, Yonezawa). The membrane was blocked with 5% bovine serum albumin (Roche Diagnostics), then incubated with a rat anti-mouse perforin antibody (KM585; Kamiya Biomedical, Seattle, WA) or a rabbit anti-rat FasL antibody (C-178; Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated rabbit anti-rat IgG or goat anti-rabbit IgG, respectively, was used as a secondary antibody and the signal was visualized with an ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

Establishment of CTLL-2 cells expressing MfasER fusion protein. The structure of MfasER is illustrated in Fig. 1. MfasER is a chimeric protein made of the transmembrane through cytoplasmic portion of the mouse Fas (amino acids 135–305) and the carboxyl-half of the rat ER (amino acids 286–600).[16,17] The intracellular region of Fas contains a "death domain" of about 70 amino acids, which plays an essential role in apoptotic signal transduction.[18] The transmembrane domain of Fas was required for the fusion protein to anchor to the membrane and transmit a death signal.[4] The carboxyl-half of the ER contains the LBD and a ligand-induced dimerization motif,[19] which activates the chimeric receptor in an estrogen-dependent manner. CTLL-2 cells were transfected with the plasmid pMX/CAGMfasER-IRESneo, then subjected to G418 selection to produce stable cells expressing MfasER. The established line was designated CTLL/MfasER.

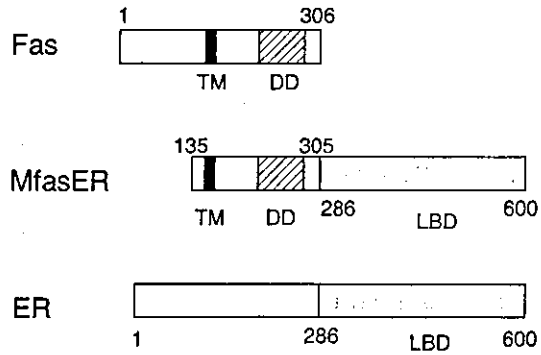

Fig. 1. Schematic structures of mouse Fas (top), rat estrogen receptor (ER; bottom), and MfasER fusion protein (middle; Ref. 4). The amino acid numbers of the original Fas and ER are shown above and below the schematics, respectively. TM (closed box), transmembrane region of Fas (amino acids 149–165); DD (hatched box), death domain of Fas (amino acids 201–286); LBD (shaded box), ligand-binding domain of ER (amino acids 286–600).

Estrogen-induced cell death of CTLL/MfasER. Without estrogen in the culture medium, both CTLL-2 and CTLL/MfasER cells showed IL-2-dependent growth. Although CTLL/MfasER cells proliferated at a slightly slower rate than the parental cells, the transfected cells exhibited continuous growth in the maintenance medium. While the addition of 10^{-7} *M* E_2 had no effect on the growth of parental CTLL-2 cells (Fig. 2A), the same dose of E_2 inhibited the growth of CTLL/MfasER cells nearly completely (Fig. 2B). Addition of the vehicle (0.05% ethanol) did not affect the proliferation of CTLL-2 or CTLL/MfasER cells (Fig. 2, A and B), compared with the cells in the maintenance medium (not shown).

The growth inhibition of CTLL/MfasER by estrogen was closely associated with the selective induction of apoptosis in these cells. Exposure to 10^{-7} *M* E_2 caused the shrinkage of CTLL/MfasER cells, with nuclear condensation and fragmentation. Finally, 80–90% of the cells were killed in 36 h (Fig. 2D). On the other hand, this concentration of E_2 did not affect the parental CTLL-2 cells (Fig. 2C). Similarly, the viabilities of CTLL-2 and CTLL/MfasER cells in 0.05% ethanol-containing medium were ~100% (Fig. 2, C and D). Fig. 3 shows the morphology of CTLL/MfasER cells treated with E_2. The cells were exposed to E_2 for the period of time indicated, and observed with a microscope using the trypan blue exclusion method (original magnification, 500×). Almost all of the treated CTLL/MfasER cells were shrunk and stained blue, having undergone the process of apoptosis, similarly to MfasER-expressing L929 fibroblasts upon E_2 treatment.[4, 5]

Inhibition of CTLL/MfasER cytotoxicity by estrogen. Crucial to the clinical application of CTLs for apoptosis induction is whether the suicide system rapidly terminates an aggressive killing event. Thus, we examined the cytotoxic activity of CTLL/MfasER cells in the absence and presence of estrogen, by means of a standard ^{51}Cr release assay. In our preliminary study, RLmale1, a radiation-induced mouse T cell leukemia line,[14] was the most appropriate target among several candidates (data not shown). Therefore we used RLmale1 as the target in the subsequent experiments.

As shown in Fig. 4A, the parent CTLL-2 cells killed up to 55% of RLmale1 cells either in the absence or presence of E_2, and the cytotoxic activity was dependent on the E/T ratio. In the absence of E_2, CTLL/MfasER cells showed an even greater cytotoxicity than the parental cells (up to 90% killing at an E/T ratio of 10; Fig. 4B, broken line). However, addition of 10^{-7} *M* E_2 dramatically reduced their killing activity. After 14 h of exposure to E_2, CTLL/MfasER showed very little cytotoxicity (up to 20% killing; Fig. 4B, solid line). These results clearly indicated that MfasER expression *per se* did not impair killing activity in CTLL-2 cells, and that the cytotoxicity was dramatically inhibited when the chimeric protein was activated by es-

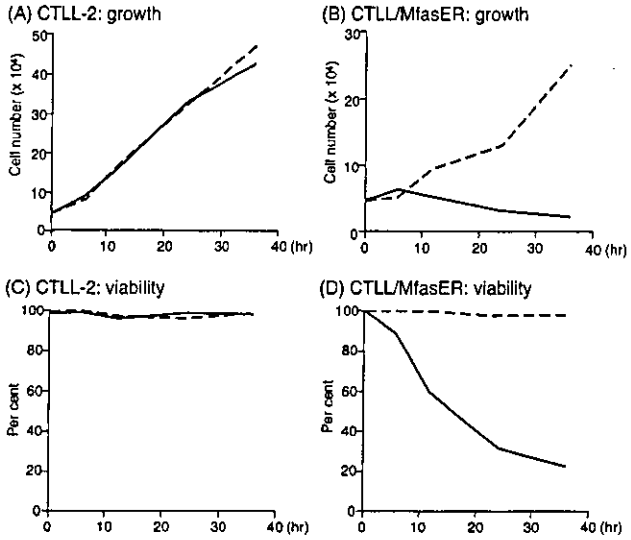

Fig. 2. Estrogen response to parental and MfasER-transduced CTLL-2 cells. The left column represents the growth (A) and viability (C) of parental CTLL-2, and the right column represents the growth (B) and viability (D) of CTLL/MfasER. Cells were cultured in IL-2-containing RPMI medium, either with 10^{-7} *M* 17β-estradiol (E_2; solid line) or vehicle (0.05% ethanol; broken line).

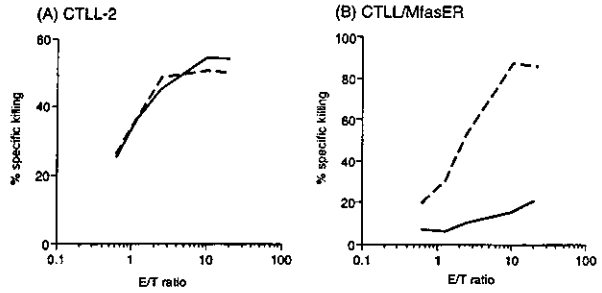

Fig. 4. ^{51}Cr release assay for cytotoxic activity of CTLL/MfasER cells. Ten thousand ^{51}Cr-labeled RLmale1 cells were plated in 96-well microtiter plates as the target. CTLL/MfasER cells were treated with 10^{-7} *M* E_2 or 0.05% ethanol (vehicle) for 14 h, then added to ^{51}Cr-labeled RLmale1 at the indicated effector/target (E/T) ratios. After 4 h of incubation, ^{51}Cr released into the supernatant was measured, and the percent specific lysis was determined as described in "Materials and Methods." Solid lines indicate specific killing by E_2-treated CTLL/MfasER, and broken lines represent specific killing by vehicle-treated cells.

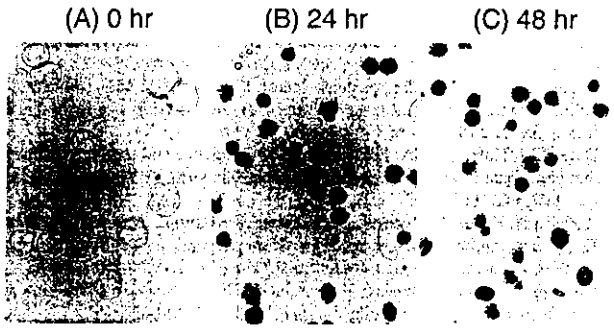

Fig. 3. Cytospin preparation of MfasER/CTLL cells stained with trypan blue dye. Cells were photographed at 0 h (A), 24 h (B), and 48 h (C) after addition of 10^{-7} *M* E_2 (original magnification, 500×). Viability decreased from ~100% to 30% in 24 h, and to <20% in 48 h.

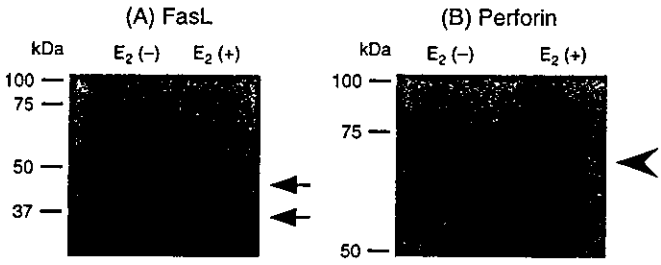

Fig. 5. Expression of Fas ligand (FasL) and perforin in E_2-treated CTLL/MfasER. Two and a half million CTLL/MfasER cells were treated with 10^{-7} *M* E_2 or 0.05% ethanol (vehicle) for 12 h. Cell lysate was electrophoresed and blotted, and the membranes were hybridized with an anti-FasL (A) or an anti-perforin (B) antibody. E_2(–), vehicle-treated CTLL/MfasER; E_2(+), E_2-treated CTLL/MfasER. Arrows indicate FasL (35 kDa and 50 kDa), and arrowhead indicates perforin (69 kDa).

trogen. Therefore, MfasER-expressing CTLs would effectively eliminate allogeneic target cells, and themselves be rapidly eradicated by estrogen when necessary.

Diminished expression of apoptosis-related proteins in estrogen-treated CTLL/MfasER. When cytotoxic T cells exert cytotoxicity, they induce apoptosis in the target cells mainly via FasL and perforin/granzyme pathways.[20-22] We investigated whether levels of these effector proteins such as FasL and perforin were also decreased upon estrogen treatment. The expression levels of FasL and perforin in CTLL/MfasER cells before and after estrogen treatment were examined by western blot analysis (Fig. 5). Without E_2, CTLL/MfasER cells contained 35 kDa and 50 kDa proteins detectable with an anti-FasL antibody (Fig. 5A, left lane). The predicted molecular weight of mouse FasL is 31 kDa, with four potential N-glycosylation sites in its extracellular domain, and several studies have shown that the apparent molecular weight of FasL in different cell lines varies from 36 to 43 kDa.[23] Therefore we considered that the 35 kDa species was the unmodified FasL and the 50 kDa protein was a glycosylated form of FasL. After 12 h of E_2 exposure, these bands were diminished in CTLL/MfasER, concomitantly with apoptosis induction in these cells (Fig. 5A, right lane). Furthermore, we obtained a similar result for perforin expression. While perforin was detected as a 69 kDa protein in the untreated CTLL/MfasER (Fig. 5B, left lane), this band was almost undetectable in the E_2-treated cells (Fig. 5B, right lane).

Discussion

Suicide gene therapy has been explored in several anti-cancer strategies. Such approaches include the direct killing of neoplastic cells, eradication of donor lymphocytes responsible for severe GVHD, and elimination of cytokine producer cells to terminate supplementary gene therapy. So far, most of the "suicide vectors" have incorporated the HSVtk gene, and several clinical studies have been performed, including some on DLI after allogeneic BMT.[2,3] In the present study, we used the MfasER/estrogen system as an alternative to the HSVtk/GCV system. We introduced the MfasER gene into the mouse CTL line CTLL-2 and evaluated the effectiveness of estrogen treatment in inducing apoptosis. MfasER induced rapid and extensive cell death in the transduced cells, accompanied with a massive reduction in cytotoxic activity. These results implied that the use of the MfasER/estrogen system is feasible for controlling CTLs involved in GVHD following DLI.

One of the concerns with the MfasER/estrogen system is that endogenous estrogen might constitutively activate ER-containing fusion molecules. It has been reported that 10^{-10} *M* or more of E_2 was required to induce apoptosis in MfasER-transduced L929 cells, a concentration considerably exceeding the physiological level of blood estrogen in female mice.[4] When transplanted into nude mice, the MfasER-expressing cells proliferated without interference from endogenous steroids, and the cells showed an apoptotic phenotype only when estrogen was administered exogenously. Thus, engineered cells similar to CTLL/MfasER are expected to survive and exert cytotoxic activity in the recipient animals, but to be eliminated by E_2 administration.

Still, it is desirable to minimize unwanted apoptosis in genetically modified cells, which may be induced by elevated levels of estrogen in females.[24] In addition, the administration of estrogen may be associated with adverse effects such as coagulopathy. One strategy to overcome these concerns is to use a mutant ER with an altered specificity. For example, a mutant receptor (TmR), with an arginine substitution for glycine 525, is unresponsive to estrogen and responds specifically to a synthetic estrogen analog, 4-hydroxytamoxifen (Tm).[25] A Fas fusion protein with TmR (MfasTmR) converted L929 cells to a Tm-induced apoptotic phenotype.[10] The pharmacological profile of tamoxifen has been well characterized through its usage as a therapeutic drug, which may be an advantage in considering the MfasTmR/tamoxifen system as a candidate clinical tool to induce apoptosis in patients. Thomis *et al.* employed yet another molecular switch to activate Fas. They fused multiple copies of FK506-binding motifs to Fas, and used a dimeric compound, AP1903, to crosslink the fusion protein.[26] Administration of AP1903 induced a rapid and extensive apoptosis in the transduced primary human T cells, regardless of the cell cycle status.

The effectiveness of Fas fusion proteins raises the possibility that other apoptosis-mediating molecules may also be incorporated in this system. For example, the recruitment of downstream molecules such as FADD/MORT1, TRAIL and caspase 9 might be considered in suicide gene therapy for CTLs.[27] Such investigations could increase the efficacy of this approach, and broaden the range of clinical applications. In any case, a better understanding of the alloreactive immune response and the identification of effector cells responsible for GVL and GVHD is required. If we can target more restricted cell populations, more sophisticated means for the control of GVHD can be explored.

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New selective amplifier genes containing c-Mpl for hematopoietic cell expansion

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Abstract

We previously developed "selective amplifier genes (SAGs)" which confer a growth advantage to transduced cells. The SAG is a chimeric gene encoding the G-CSF receptor (GCR) and the estrogen or tamoxifen (Tm) receptor and is able to expand transduced hematopoietic cells by treatment with estrogen or Tm. In the current study, we examined the in vitro efficacy of modified SAGs containing the thrombopoietin (TPO) receptor (c-Mpl) gene instead of GCR as a more potent signal generator. In addition, we constructed various mutant Mpl-type SAGs to abolish the responsiveness to endogenous TPO while retaining Tm-dependency. When Ba/F3 cells were retrovirally transduced with the Mpl-type SAGs, the cells showed Tm- and TPO-dependent growth even without IL-3. The Mpl-type SAGs induced more potent proliferation of Ba/F3 and cynomolgus $CD34^+$ cells than the GCR-type SAG. One mutant Mpl-type SAG (ΔGCRMplTmR) successfully lost the responsiveness to TPO without affecting the Tm-dependence.

Keywords: Selective amplifier gene; Hematopoietic stem cells; Mpl; Retroviral vector; Gene therapy; In vivo expansion

Hematopoietic stem cells (HSCs) are ideal targets of gene therapy for many disorders due to their self-renewal and multilineage differentiation abilities. Retroviral vectors are the most commonly used vehicles for gene transfer into HSCs. Although current retroviral vectors successfully deliver genetic materials into murine HSCs [1], the gene transfer efficiency into human HSCs is insufficient for most clinical applications [2,3]. For the achievement of clinically relevant levels of gene transfer into human HSCs with retroviral vectors, several approaches have been tried, including pseudotyping with other viral envelopes such as the gibbon ape leukemia virus envelope [4] and RD114 [5], utilizing the fibronectin fragment (CH-296) [6], and selecting and expanding successfully transduced cells in vivo [7–9]. Lentiviral vectors that can transduce nondividing cells may be more suitable gene transfer vehicles into HSCs as these cells are generally quiescent [10].

Although in vivo selection of transduced hematopoietic cells with drug-resistant genes has been studied intensively to overcome the low gene transfer efficiency into HSCs [7,8], we developed another device to directly expand transduced cells by conferring a proliferative advantage to the gene-modified cells relative to their untransduced counterparts. These genes have been designated as "selective amplifier genes (SAGs)." The prototype SAG encodes a chimeric receptor consisting of 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 signal generator and the estrogen or tamoxifen receptor moiety is a molecular switch to regulate the growth signal generated from GCR. We have previously shown

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that these prototype GCR-type SAGs can selectively expand the transduced hematopoietic cells in an estrogen- or tamoxifen-dependent manner in vitro [11–13] and in vivo in mice (unpublished). We have also reported that the GCR-type SAGs expanded transduced hematopoietic cells in a nonhuman primate (cynomolgus monkey) transplantation model. However, not all treated monkeys showed an increase in transduced cells in response to estrogen or tamoxifen in vivo, suggesting that the responses of the GCR-type SAGs to exogenous drugs may be variable among monkeys [14]. More potent SAGs would be required to achieve enough in vivo expansion in nonhuman primates.

In this respect, we paid attention to the fact that the thrombopoietin (TPO) receptor, c-Mpl, is expressed in very immature hematopoietic cells and that TPO actually stimulates the growth of these cells [15–17]. The intracellular signal from c-Mpl may be thus more appropriate than that from GCR for expansion of hematopoietic stem/progenitor cells. In the present study, we constructed another class of SAGs consisting of c-Mpl and the tamoxifen receptor (TmR) (designated as Mpl-type SAG) and examined their efficacy in vitro.

Materials and methods

Cells. IL-3 dependent mouse proB cell line Ba/F3 cells [18] were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), and 10 ng/ml recombinant mouse IL-3 (rmIL-3; Gibco-BRL). The ecotropic packaging cell line BOSC23 [19] and human embryonic kidney 293T cells were maintained in DMEM containing 10% FBS.

Bone marrow cells were harvested from femora of healthy adult cynomolgus monkeys reared in the Tsukuba Primate Center, National Institute of Health Japan (Tsukuba, Ibaraki, Japan). The 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 the red blood cells. Immunomagnetic selection of $CD34^+$ cells was conducted using the Dynabeads kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. The $CD34^+$ cells were frozen in a CellBanker (Nippon Zenyaku, Tokyo, Japan) and kept in liquid N_2 until use.

Plasmid construction. The plasmid pcDNA3.1-c-mpl (provided by Dr. M. Takatoku, Jichi Medical School, Tochigi, Japan) [20] containing the cDNA for human c-Mpl between the *Eco*RI and *Xba*I sites was digested with *Eco*RI and *Sac*I. The other part of c-Mpl cDNA between the *Sac*I site and C-terminal was generated by polymerase chain reaction (PCR) using the pcDNA-c-Mpl as a template with the primer pair 5′-CCC ACC TAC CAA GGT CCC TGG-3′ and 5′-CGG GAT CCA GAG GCT GCT GCC AAT AG-3′. The fragment containing the murine phosphoglycerate kinase (pgk) promoter and neomycin phosphotransferase gene (neo) (*Eco*RI–*Bam*HI) in the retroviral plasmid pMSCV2.2 (a gift from Dr. R.G. Hawley, University of Toronto, Canada) [21] was replaced with the *Eco*RI–*Sac*I fragment of the c-Mpl cDNA and the *Sac*I–*Bam*HI fragment of the c-Mpl cDNA by trimolecular ligation to construct pMSCV-Mpl. The tamoxifen receptor (TmR) cDNA was cloned from pMSCV-ΔGCRTmR [13] by PCR using the primer pair 5′-CTG GAT CCG GGC ACT TCA GGA GAC-3′ and 5′-CTG TCG ACC ACT AGT AGG AGC TCT CA-3′. The *Bam*HI–*Sal*I fragment of the TmR cDNA was ligated into the *Bam*HI–*Sal*I site of pMSCV-Mpl. The resultant plasmid expressing a chimeric MplTmR gene was designated as pMSCV-MplTmR.

The truncated Mpl mutants (Δ(99–267) = deletion of 99–267 amino acids, Δ(281–472), Δ(40–267), Δ(99–472), Δ(71–494), and Δ(40–472); Fig. 2A) were constructed by PCR using the primer pairs shown in Table 1. All PCR products were digested with *Eco*RI and *Bam*HI. The digested fragment (*Eco*RI-*Bam*HI) containing each truncated mutant and the TmR cDNA fragment (*Bam*HI–*Sal*I) were inserted into the *Eco*RI–*Sal*I site of pMSCV by trimolecular ligation to construct retroviral plasmids expressing the truncated Mpl mutants.

pMSCV-ΔGCRMplTmR was constructed as follows. The extracellular domain was from GCR cDNA lacking the ligand (G-CSF)-binding sequence (5–195 amino acids) [22], and was prepared by digesting pMSCV-ΔGCRTmR with *Hin*dIII and *Kpn*I. For the transmembrane fragment, the corresponding part of the GCR cDNA was used and prepared by PCR using pMSCV-ΔGCRTmR as a template with the primer pair 5′-GAG TGG GTA CCT GAG GCC CCT AGG-3′ and 5′-AAC TCG AGG CAG CAG AGC CAG GTC AC-3′. The cytoplasmic domain was from c-Mpl cDNA and again generated by PCR using the pcDNA-c-Mpl as a template with the primer pair 5′-AAC TCG AGA GGT GGC AGT TTC CTG CA-3′ and 5′-CGG GAT CCA GAG GCT GCT GCC AAT AG-3′. The extracellular fragment was cloned into the *Hin*dIII–*Kpn*I site of pEGFP-N1 (Clontech, Palo Alto, CA, USA) (pEGFP-ΔGCR) and then the transmembrane and cytoplasmic fragments were integrated into the *Kpn*I–*Bam*HI site of the obtained pEGFP-ΔGCR to form pEGFP-ΔGCRMpl. The fragment carrying pgk and neo (*Bgl*II–*Sal*I) in pMSCV was replaced with the ΔGCRMpl cDNA (*Bgl*II–*Bam*HI fragment from pEGFP-ΔGCRMpl) and TmR cDNA (*Bam*HI–*Sal*I fragment from pMSCV-MplTmR). The resultant construct was designated as pMSCV-ΔGCRMplTmR.

Table 1
Primers for construction of Mpl deletion mutants

Mutants	Primers	Sequences
Δ(99–267)	A	5′-CCGTTAACTCCTGGGGATCCTGGTCC-3′
	B	5′-CCGTTAACCTGGTCTGGAAACTGGCA-3′
Δ(40–267)	A	Described above
	C	5′-CCGTTAACCTTCAGGGGCTCTGAGTC-3′
Δ(281–472)	D	5′-CCGTTAACCCCTGGAGCTCGTGGTCG-3′
	E	5′-CCGTTAACCAGGTCCACAGTCACAGG-3′
Δ(99–472)	D	Described above
	B	Described above
Δ(40–472)	D	Described above
	C	Described above
Δ(71–494)	E	5′-CCGAATTCATGTGGAGCTCGTGGTCG-3′
	F	5′-CCGAGCTCCAGCAAGTGAGGTCCT-3′

pMSCV-ΔGCRTmR-ires-EGFP, pMSCV-MplTmR-ires-EGFP, and pMSCV-ΔGCRMplTmR-ires-EGFP were derived from pMSCV-ΔGCRTmR, pMSCV-MplTmR, and pMSCV-ΔGCRMplTmR, respectively, by inserting the *Xho*I–*Cla*I fragment containing ires-EGFP derived from the *Xho*I–*Cla*I digenst of pMSCV-ires EGFP to the *Sal*I–*Cla*I sites [13].

Retroviral vectors. To obtain ecotropic retroviral vectors, we transfected BOSC23 cells with mouse stem cell virus (MSCV)-based retroviral plasmids (derivatives from pMSCV) using the Transfection MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The supernatants harvested at 48–72 h after transfection exhibited titers of 1×10^6/ml as assessed by RNA dot-blot. To obtain amphotropic retroviral vectors, we transfected 293T cells with MSCV-based retroviral plasmids along with pCL-Ampho (Imgenex, San Diego, CA) using the Transfection MBS Mammalian Transfection Kit (Stratagene). The supernatants harvested at 48–72 h after transfection showed titers of 1×10^6/ml as assessed by RNA dot-blot.

Transduction and culture. Ba/F3 cells were suspended at a density of 1×10^5 cells/ml in 1 ml viral supernatant containing 10 ng/ml rmIL-3 and transferred to six-well plates coated with 20 μg/cm^2 of RetroNectin (Takara Bio, Shiga, Japan) [8]. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 24 h. During this period, the culture medium was replaced with fresh viral supernatant every 12 h. As an untransduced control, Ba/F3 cells were similarly cultured in medium not containing virus. Transduced or untransduced Ba/F3 cells were then cultured in DMEM containing 10% FBS in the presence or absence of 10 ng/ml rmIL-3, 10 ng/ml recombinant human (rh) TPO (provided by Kirin Brewery, Tokyo, Japan) or 10^{-7} M 4-hydroxytamoxifen (OH-Tm, an active metabolite of Tm; Sigma, St. Louis, MO).

Cynomolgus CD34$^+$ cells were placed in six-well plates coated with 20 μg/cm^2 of RetroNectin and cultured for 24 h at 37 °C with 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 50 ng/ml rhIL6 (provided by Ajinomoto, Osaka, Japan), 100 ng/ml rhSCF (provided by Amgen, Thousand Oaks, CA), 100 ng/ml rhFlt-3 ligand (Research Diagnostic, Flanders, NJ), and 100 ng/ml rhTPO. The cells were resuspended in 1 ml viral supernatant containing cytokines as described above at a density of 1×10^5 cells/ml. During the transduction, the culture medium was replaced with fresh viral supernatant every 12 h. Mock transduction was performed using medium not containing retroviral vectors.

Western blot analysis. Approximately 5×10^4 untransduced or transduced cells were suspended in 2× Tris–SDS SepraSol (Owl, Woburn, MA) and then boiled at 100 °C for 5 min. The protein samples were resolved on a 7.5% SDS–polyacrylamide gel (Multigel 7.5; Daiichi, Tokyo, Japan) followed by electroblotting to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was incubated with blocking buffer (Block Ace; Dainippon, Osaka, Japan) and then with anti-ER antibody MC-20 (Santa Cruz, Santa Cruz, CA) for 1 h at room temperature. The membrane was washed with the blocking buffer five times and incubated with goat phycoerythrin (PE)-conjugated anti-rabbit IgG antibody (Cedarlane, Ont., Canada) for 1 h at room temperature. After washing the membrane five times with the blocking buffer, the fusion proteins with TmR were visualized by an ECL system (Amersham, Little Chalfont, UK).

Flow cytometry. To assess the EGFP expression, transduced cells were washed and subjected to FACSCalibur flow cytometry (Becton–Dickinson, Palo Alto, CA) using excitation at 488 nm and fluorescence detection at 530±30 nm. Untransduced cells served as negative controls.

Results and discussion

The structures of SAGs in this study are shown in Fig. 1A. The prototype SAG is a chimeric gene encoding

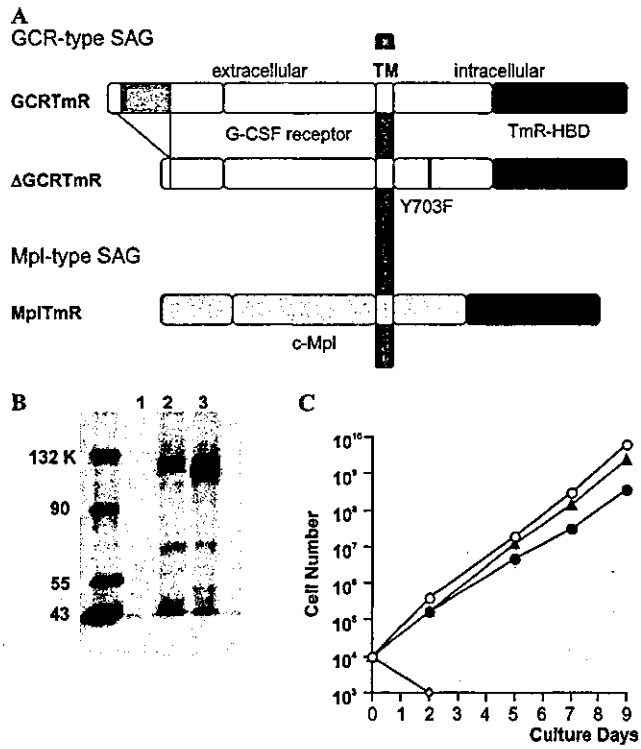

Fig. 1. Construction of modified SAGs. (A) GCRTmR is a GCR-type SAG consisting of the murine G-CSF receptor (GCR) and the tamoxifen receptor (TmR). In ΔGCRTmR, the ligand (G-CSF)-binding domain of GCR was deleted to abolish the responsiveness to G-CSF. In addition, the tyrosine residue at the 703rd amino acid was replaced with phenylalanine to hamper the differentiation signal. Mpl-TmR is an Mpl-type SAG consisting of the full-length human c-Mpl and the TmR, which responds to both TPO and Tm. TM, transmembrane. (B) Western blot analysis of transduced Ba/F3 cells. Ba/F3 cells were transduced with the retroviral vector expressing ΔGCRTmR or MplTmR. Lysates of untransduced Ba/F3 cells (lane 1), Ba/F3 cells transduced with the ΔGCRTmR vector (lane 2) or with MplTmR vector (lane 3) were immunoblotted with anti-ER antibody. (C) Growth of Ba/F3 cells expressing MplTmR in suspension culture in the presence (open circle) or absence (open diamond) of IL-3 (10 ng/ml), TPO (closed triangle) (10 ng/ml) or Tm (closed circle) (10^{-7} M). Cell counts are shown in a log scale.

GCR and the estrogen receptor hormone-binding domain [11]. In GCR, the tyrosine residue at the 703rd amino acid was replaced with phenylalanine to prevent the generation of a differentiation signal [12]. In the hormone-binding domain of the estrogen receptor, a mutation (G525R) was introduced to hamper the binding of endogenous estrogen to the SAGs retaining the responsiveness to synthetic hormones such as tamoxifen (Tm) [13,23]. The GCR-type SAG containing this mutant estrogen receptor (Tm receptor, TmR) is designated as GCRTmR. To remove the responsiveness to endogenous G-CSF from GCRTmR, the ligand (G-CSF)-binding domain was deleted (ΔGCRTmR). The new SAG in this study of Mpl-type, the Mpl-type SAG, MplTmR, is a chimeric gene in which the GCR moiety of GCRTmR is replaced by c-Mpl. Thus, MplTmR was

designed to generate growth signals in response to both TPO and Tm.

To evaluate the functions of these SAGs in vitro, we used Ba/F3 cells as targets. Murine pro-B cell growth is dependent on IL-3 and the cells enter the apoptotic cycle in the absence of the cytokine [24]. Neither TPO nor Tm supported their growth (data not shown). To generate ecotropic retroviral vectors expressing the ΔGCRTmR or MplTmR gene, we transfected BOSC23 cells with pMSCV-ΔGCRTmR or pMSCV-MplTmR. The culture supernatants were used for infection of Ba/F3 cells. The transgene expression was confirmed by Western blotting with anti-ER (lanes 2 and 3 in Fig. 1B), while endogenous ER was not detected in the parental Ba/F3 cells (lane 1). The blots indicated that the molecular weights of ΔGCRTmR and MplTmR were approximately 120 kDa (lane 2) and 115 kDa (lane 3), respectively, as predicted in their molecular design. The Ba/F3 cells expressing ΔGCRTmR (BaF3/ΔGCRTmR) showed hydroxytamoxifen (an active metabolite of Tm)-dependent growth as it has been reported [13]. The Ba/F3 cells expressing MplTmR (BaF3/MplTmR) proliferated in the presence of either TPO or Tm as well as IL-3 (Fig. 1C), suggesting that the Mpl-type SAG (MplTmR) is able to induce the proliferation of Ba/F3 cells in a TPO- or Tm-dependent manner.

To avoid responses to endogenous TPO, we deleted the extracellular domain of c-Mpl. The response of transduced cells to endogenous TPO should be avoided to strictly control the growth signal generated from the Mpl-type SAG. Since putative TPO-binding sites of Mpl have not been described yet, we generated retroviral vectors which express various mutant SAGs lacking the extracellular domain of c-Mpl; Δ(99–267), Δ(281–472), Δ(40–267), Δ(99–472), Δ(71–494), and Δ(40–472) as shown in Fig. 2A and examined which mutant most efficiently abolished the responsiveness to TPO without losing the reactivity to Tm. Ba/F3 cells were transduced with each of such truncated MplTmR-expressing vectors. The expression of each SAG was confirmed by Western blotting with anti-ER (Fig. 2B). However, the expression of these mutants in Ba/F3 cells resulted in a total loss of responsiveness not only to TPO but also to Tm (data not shown). The deletion in the extracellular domain of c-Mpl seemed to hamper the dimerization of the SAG protein.

Regarding the GCR-type SAGs, the deletion in the extracellular domain of the GCR moiety successfully lost responsiveness to G-CSF while remaining responsive to steroids [11,13]. Based on these observations we replaced the extracellular portion of MplTmR with that of the GCRTmR, which lacks the natural ligand (G-CSF)-binding ability to solely knock out the TPO-binding ability without spoiling the growth dependency on Tm, and thus obtained another mutant Mpl-type SAG designated as ΔGCRMplTmR (Fig. 3A). A retroviral vector expressing ΔGCRMplTmR was generated and Ba/F3 cells were stably transduced with the vector. Western blotting confirmed a transgene expression at the molecular weight of approximately 110 kDa (Fig. 3B). The Ba/F3 cells expressing ΔGCRMplTmR now successfully showed Tm-dependent growth, while they did not show any response to G-CSF or TPO (Fig. 3C). Thus, the new SAG can now be strictly controlled only by treatment with a synthetic hormone such as Tm.

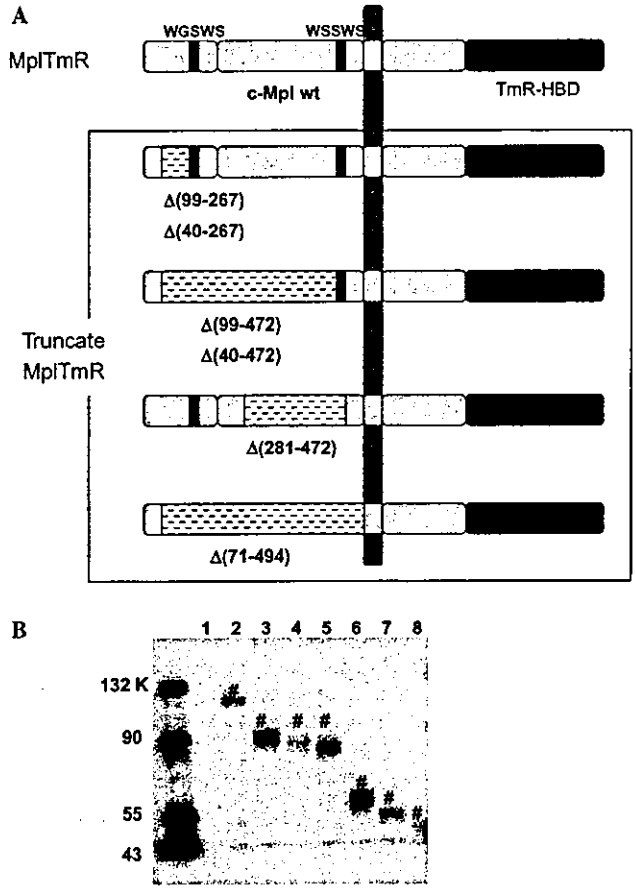

Fig. 2. Deletion of the TPO-binding sites from MplTmR. (A) A series of truncated MplTmR mutants. (B) Western blot analysis of transduced Ba/F3 cells. Ba/F3 cells were transduced with the retroviral vectors expressing truncated Mpl-TmR mutants. The cell lysates were immunoblotted with anti-ER antibody. Lane 1, untransduced Ba/F3 cells; lane 2, Ba/F3 cells transduced with the intact MplTmR gene; and lanes 3–8, Ba/F3 cells transduced with the deletion mutant genes (Δ(99–267), Δ(281–472), Δ(40–267), Δ(99–472), Δ(71–494), and Δ(40–472), respectively).

To compare the Mpl- and GCR-type SAG for their abilities to expand hematopoietic cells, we transduced Ba/F3 cells with retroviral vectors expressing these SAGs and examined the Tm-dependency of cell growth (Fig. 3D). The Mpl-type SAG (MplTmR) induced more potent proliferation of Ba/F3 cells in response to Tm than the GCR-type SAG (ΔGCRTmR). Although the proliferation rates induced by the mutant Mpl-type SAG lacking the TPO-binding ability (ΔGCRMplTmR) were lower than those induced by the parental Mpl-type

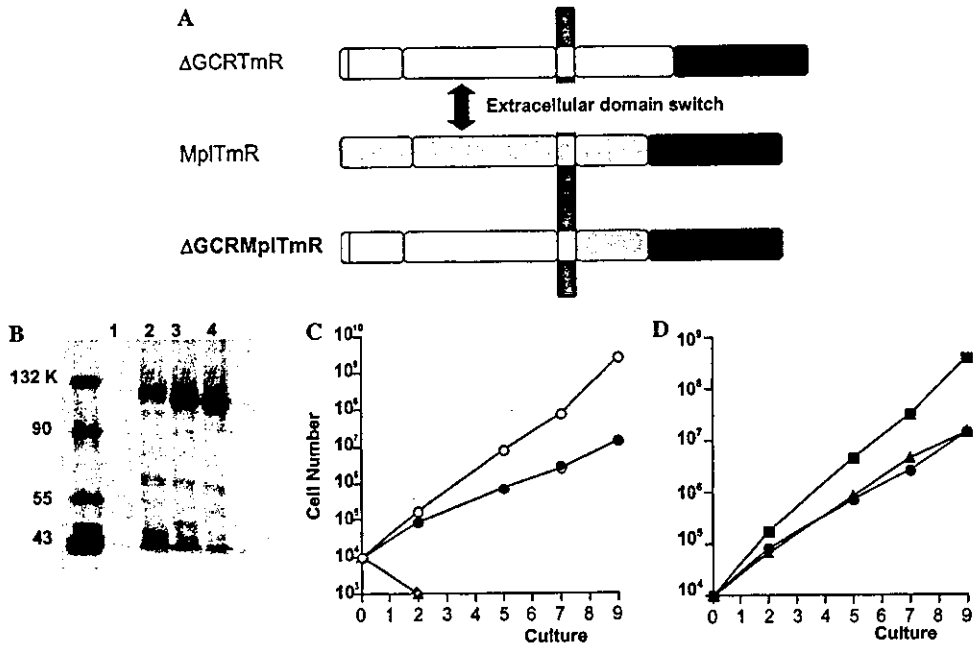

Fig. 3. Construction of a mutant MplTmR (ΔGCRMplTmR) lacking the TPO-responsiveness without affecting the Tm-responsiveness. (A) Replacing the extracellular domain of MplTmR with that of ΔGCRTmR. (B) Western blot analysis of transduced Ba/F3 cells. The cell lysates were immunoblotted with anti-ER antibody. Lane 1, untransduced Ba/F3 cells; lanes 2–4, Ba/F3 cells transduced with the ΔGCRTmR, MplTmR, and ΔGCRMplTmR genes, respectively. (C) Growth of Ba/F3 cells expressing ΔGCRMplTmR in suspension culture in the presence (open circle) or absence (open diamond) of IL-3 (10 ng/ml), in the presence of TPO (closed triangle)(10 ng/ml), or in the presence of Tm (closed circle) (10^{-7} M). (D) Growth of Ba/F3 cells transduced with the ΔGCRTmR (closed triangle), MplTmR (closed square), or ΔGCRMplTmR gene (closed circle) in the presence of Tm (10^{-7} M).

SAG (MplTmR), they were still comparable to those induced by the GCR-type SAG (ΔGCRTmR). The Ba/F3 cells expressing the Mpl-type SAGs (either MplTmR or ΔGCRMplTmR) showed long-term proliferation in the presence of Tm even without IL-3, and the cells died upon removal of Tm from the culture media (data not shown). Thus, our SAG can now be strictly controlled only by treatment with a synthetic hormone such as Tm. This finding suggests that Tm can strictly control the growth signal in the transduced Ba/F3 cells.

We constructed bicistronic retroviral plasmids which express the Mpl-type SAG (MplTmR or ΔGCRMplTmR) gene as the first cistron and the enhanced green fluorescent protein (EGFP) gene as the second cistron (designated as pMSCV-MplTmR-ires-EGFP or pMSCV-ΔGCRMplTmR-ires-EGFP). To generate amphotropic retroviral vectors, 293T cells were transfected with the retroviral plasmids along with a helper plasmid expressing the amphotropic envelope. Cynomolgus $CD34^+$ bone marrow cells were transduced with these amphotropic retroviral vectors by the supernatant transduction method. Following transduction, the cells were cultured in medium containing Flt-3, TPO or Tm. As shown in Fig. 4A, while the fractions of GFP^+ cells did not increase in the $CD34^+$ cells transduced with the GCR-type SAG retroviral vector (MSCV-ΔGCRTmR-ires-EGFP) in the presence of Flt-3 or TPO, the fraction expanded by about 6-fold (8–47%) during the first week's culture in the presence of Tm, but subsequently decreased to 5% during the second week's culture. On the other hand, as shown in Fig. 4B, the fraction of GFP^+ cells transduced with the Mpl-type SAG retroviral vector (MSCV-MplTmR-ires-EGFP) increased by 2-fold (23–50%) during the first week's culture in the presence of Tm, and the fraction did not decrease during the second week's culture. Stimulation of the transduced cells with the natural ligand TPO induced a remarkable increase in GFP^+ cells, and over 90% of cells became GFP^+ cells after a two-week culture with TPO. The fraction of GFP^+ cells transduced with the mutant Mpl-type SAG lacking the TPO-binding ability (MSCV-ΔGCRMplTmR-ires-EGFP) also increased by about 2-fold (12–26%) during the second week's culture (Fig. 4C). We repeated this experiment four times and all the results suggest that the Mpl-type SAG can expand transduced $CD34^+$ cells more efficiently than the GCR-type SAG.

In this study, we utilized the c-Mpl signal transduction domain to develop another class of SAGs (Mpl-type SAGs) and examined their ability in vitro using murine Ba/F3 cells and cynomolgus bone marrow $CD34^+$ cells. We have shown that the intracellular signal from the Mpl-type SAGs is more potent than that from the GCR-type SAGs for the expansion of these cells. Blau et al. [25] also showed that the signal generated from c-Mpl, GCR, and Flt-3

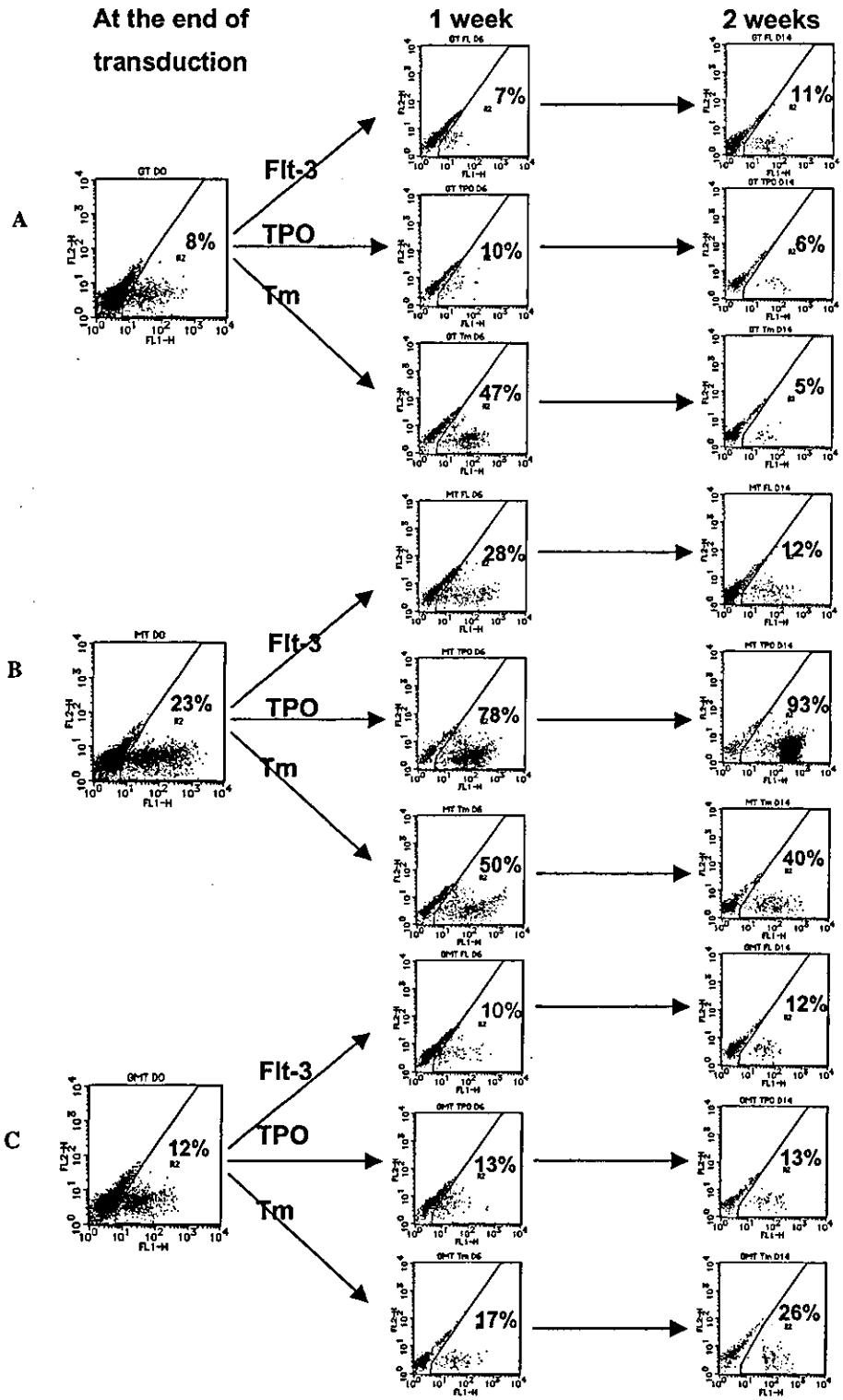

Fig. 4. Tm-induced expansion of cynomolgus bone marrow CD34$^+$ cells transduced with (A) MSCV-ΔGCRTmR-ires-EGFP, (B) MSCV-MplTmR-ires-EGFP, or (C) MSCV-ΔGCRMplTmR-ires-EGFP. The transduced CD34$^+$ cells were cultured with Flt-3 ligand (100 ng/ml), TPO (100 ng/ml), or Tm (10^{-7} M). The cells were analyzed for EGFP expression by flow cytometry on the indicated weeks after transduction. The values represent percentage of EGFP$^+$ cells.

supported the growth of Ba/F3 cells, but that only Mpl among them supported the sustained growth of transduced murine bone marrow cells. Taken together, the intracellular signal by c-Mpl was confirmed to be suitable for the expansion of immature hematopoietic cells.

However, the growth signal of the Mpl-type SAGs generated by Tm was considerably attenuated compared to that generated by the natural ligand TPO (Fig. 4B). The Tm-mediated dimerization of MplTmR may be less efficient than the TPO (natural ligand)-mediated dimerization. Since c-Mpl is a membrane receptor while the estrogen or tamoxifen receptor is a nuclear one, they may not be so compatible for the dimerization. Now, we are constructing further modified SAGs, in which a signal generator and a molecular switch are both membrane proteins.

Acknowledgments

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RESEARCH ARTICLE

In vivo expansion of transduced murine hematopoietic cells with a selective amplifier gene

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Abstract

Background Hematopoietic stem-cell-directed gene transfer has achieved limited success in transducing clinically relevant levels of target cells. The expansion of gene-modified cells is one way to circumvent the problem of inefficient transduction with current vectors. To this end, we have developed 'selective amplifier genes' (SAGs) that encode chimeric proteins that are a fusion of granulocyte colony-stimulating factor receptor and the steroid-binding domain. Prototype SAGs conferred estrogen-responsive growth on murine hematopoietic progenitors.

Methods We constructed a retroviral vector coexpressing an SAG for 4-hydroxytamoxifen (Tm)-specific proliferation and the enhanced green fluorescent protein (EGFP). Murine bone marrow cells were transduced with this vector and transplanted into myeloablated mice. Subsequently, recipients were challenged with Tm, and $EGFP^+$ cells were enumerated.

Results The challenge induced a significant increase in $EGFP^+$ leukocytes ($21 \pm 4\%$ to $27 \pm 5\%$), while $EGFP^+$ cells decreased in untreated animals ($21 \pm 5\%$ to $10 \pm 3\%$). Three months later, bone marrow cells were transplanted from the unchallenged mice to secondary hosts. Again the administration of Tm resulted in an increase of $EGFP^+$ cells ($16 \pm 4\%$ to $35 \pm 3\%$), contrasting to a decrease in controls ($22 \pm 4\%$ to $12 \pm 4\%$), and the difference was significant for more than 3 months. A detailed study of lineage showed a preferential expansion of $EGFP^+$ cells in granulocytes and monocytes following Tm administration.

Conclusions Long-term repopulating cells were transduced with the SAG, and the transduced granulocyte/monocyte precursors were most likely to be expandable *in vivo* upon Tm stimulation.

Keywords hematopoietic stem cell; *in vivo* expansion; gene therapy; retroviral vector; selective amplifier gene

Introduction

Hematopoietic stem cells (HSCs), with an indefinite self-renewal capacity and pluripotency to differentiate into all the hematopoietic lineages, are attractive targets for gene therapy. Candidate disorders include monogenic diseases such as hereditary immunodeficiencies, hemoglobinopathies and lysosomal storage diseases, as well as infectious diseases and cancer. In contrast to the efficient gene transfer to murine HSCs

and successes in treating mouse disease models by oncoretroviral vectors, gene transfer to human HSCs with the same kind of vectors has been extremely inefficient [1]. The main reason for this inefficiency is the inability of oncoretroviruses to integrate into nondividing cells such as most human HSCs [2]. Another reason is the very poor expression of the receptors for amphotropic retroviruses on HSCs [3]. To overcome these obstacles, much attention has been focused on developing vectors capable of integrating into quiescent cells. In particular, lentiviral vectors pseudotyped with vesicular stomatitis virus G-glycoprotein are most promising, with their ability to integrate into nondividing cells and ubiquitous receptor distribution for this envelope [4].

Besides vector development, an alternative strategy is to select or expand transduced stem cells after genetic manipulation and reinfusion into the patient. To this end, the transfer of a drug-resistance gene such as *mdr-1* has been extensively studied [5], but this raises the issue of giving cytotoxic compounds to patients without malignancy. In this regard, we took a less toxic approach by designing a novel system for conferring a direct growth advantage on genetically modified cells. This system is based on the ability of a steroid receptor hormone-binding domain (HBD) to reversibly activate a fusion counterpart in a steroid-dependent manner [6]. As a prototype fusion molecule, the granulocyte colony-stimulating factor (G-CSF) receptor (GCR) was linked to the HBD of the estrogen receptor (ER), and this chimeric protein (GCRER) induced an estrogen-dependent growth in transduced murine progenitors [7]. We termed the hybrid genes for the chimeric molecules comprising growth factor receptors and HBDs 'selective amplifier genes' (SAGs), and have further refined this system. The modifications of the fusion receptor include, abrogating the responsiveness to G-CSF by deleting the cytokine binding domain [7], changing the ligand specificity to a synthetic estrogen analog 4-hydroxytamoxifen (Tm) [8], and attenuating granulocyte differentiation signals [9]. Herein we investigated whether SAG-transduced murine hematopoietic cells could be expanded *in vivo*, by administering Tm specific to the refined fusion receptor.

Materials and methods

Vector construction

The construction of SAGs encoding estrogen-responsive chimeric receptors (GCRER and its G-CSF binding site-deleted form, ΔGCRER) was as described, and ΔGCR was released from pMX/ΔGCRER as a *Bam*H I-*Pme* I fragment [7,10]. Tm-specific HBD (TmR) was obtained by a polymerase chain reaction using pBS^{+}ER^{TM} (a gift from Drs. G. I. Evan and T. D. Littlewood, Imperial Cancer Research Fund, London, UK), adding a 5′-*Pme* I site and a 3′-*Sal* I site [9,11]. A retrovirus carrying the encephalomyocarditis virus-derived internal ribosome entry site (IRES) and the enhanced green fluorescent protein (EGFP) gene was also produced as described (MSCV2.2/IRES-EGFP) [12–14]. ΔGCR (*Bam*H I-*Pme* I) and TmR (*Pme* I-*Sal* I) were inserted into *Bgl* II/*Xho* I-digested MSCV2.2/IRES-EGFP by trimolecular ligation, resulting in a bicistronic retrovirus MSCV/ΔGCRTmR-IRES-EGFP. Finally, a Y703F mutation was introduced into MSCV/ΔGCRTmR-IRES-EGFP using a transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA) as described [9]. The resultant vector was designated MSCV/ΔY703FGCRTmR-IRES-EGFP (Figure 1).

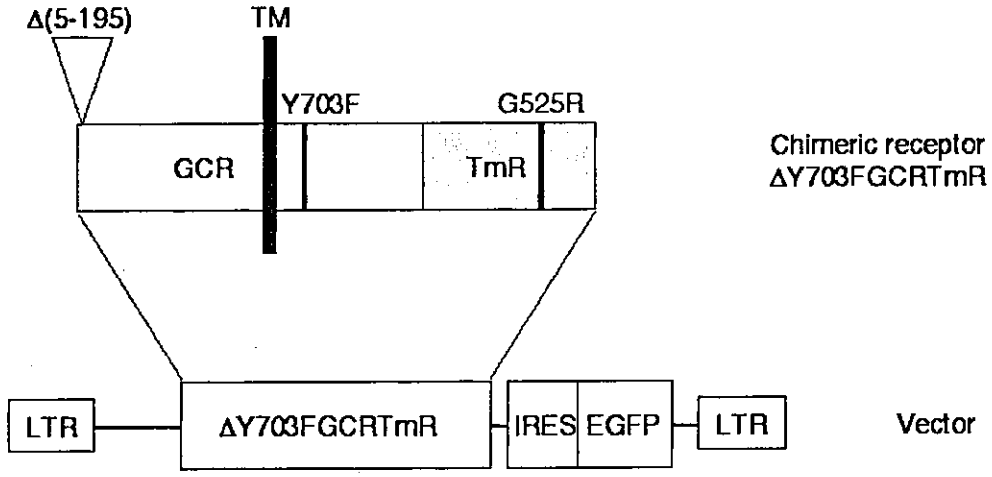

Figure 1. Schematic diagram of chimeric receptor and vector construct. Top: The chimeric protein ΔY703FGCRTmR was a fusion of the G-SCF receptor (GCR) and Tm binding domain (TmR). Δ(5–195), deletion of G-CSF binding site; TM, transmembrane domain; Y703F, amino acid substitution (Tyr703 to Phe) to minimize myeloid differentiation signal; G525R, amino acid substitution (Gly525 to Arg in estrogen receptor) to change ligand specificity. Bottom: Bicistronic retrovirus vector MSCV/ΔY703FGCRTmR-IRES-EGFP. LTR, long terminal repeat; IRES, internal ribosome entry site derived from encephalomyocarditis virus; EGFP, enhanced green fluorescent protein

Cells and animals

BOSC23 ecotropic packaging cells (American Type Culture Collection CRL-11554, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) [15]. Retroviral supernatant was prepared by transiently transfecting BOSC23 cells with MSCV/ΔY703FGCRTmR-IRES-EGFP. The transfection was carried out using LipofectAmine reagent (Invitrogen) following the manufacturer's protocol, and the viral supernatant was harvested 2 days post-lipofection. The titer of the vector supernatant was estimated by a simplified RNA dot-blot and hybridization assay with the reference vector MSCV/EGFP-neo [14,16].

C57BL/6-Ly5.2 mice were purchased from Clea Japan (Tokyo, Japan). The congenic C57BL/6-Ly5.1 mice were bred in the animal facility of the Jichi Medical School (Tochigi, Japan). The mice were maintained and treated following institutional codes for animal rights.

Bone marrow (BM) transduction and hematopoietic reconstitution

Retroviral transduction of BM cells was carried out following a fibronectin-assisted protocol [17]. C57BL/6-Ly5.2 mice (6 weeks old) were injected intraperitoneally (i.p.) with 150 mg/kg of 5-fluorouracil (Kyowa Hakko, Tokyo, Japan), and BM cells were obtained by flushing the femora 2 days post-injection. Low-density mononuclear cells were collected using Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada) and prestimulated for 2 days with 100 ng/ml of recombinant rat stem cell factor (provided by Amgen, Thousand Oaks, CA, USA) and 20 ng/ml of recombinant human interleukin (IL)-6 (provided by Ajinomoto, Kawasaki, Japan) [18]. The prestimulated cells were 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 additional days under the same growth factor conditions as for the prestimulation [17]. Supernatant infection was repeated two to four times during transduction. The manipulated cells were recovered using Cell Dissociation Buffer (Invitrogen) and injected into the hosts via the tail vein.

For primary reconstitution, C57BL/6-Ly5.1 mice (8 weeks old) were lethally irradiated (11 Gy; ^{137}Cs using a Gamma Cell 40, Nordion International, Kanata, Ontario, Canada) and intravenously given 2×10^5 transduced Ly5.2 BM cells per animal. For secondary reconstitution, fresh BM cells from the primary recipients were pooled and 2×10^6 cells were infused into lethally irradiated Ly5.1 recipients.

Tm formulation and administration

4-Hydroxytamoxifen (Tm; Sigma, St. Louis, MO, USA) was solubilized in ethanol to produce a 25 mg/ml solution, and this solution was diluted with saline containing polyethylene glycol 400 and Tween 80. The final solution for injection contained 5 mg/ml of Tm, 20% ethanol, 10% polyethylene glycol 400 and 1.4% Tween 80. For the 5 mg/day regimen, 330 μl of the solution was i.p. injected three times daily.

Hematological examinations and flow cytometry

Hematopoietic reconstitution and the impact of Tm administration were assessed by a complete blood count and fluorescence-activated cell sorting (FACS). Mouse peripheral blood (100–150 μl) was obtained by tail clipping. A complete blood count was made with a PC-608 particle counter (Erma, Tokyo, Japan), and the blood smear was Wright-Giemsa stained for leukocyte differential counts. The red blood cells in the remainder of the blood samples were lysed with ACK buffer (150 mM NH_4Cl, 12 mM $KHCO_3$, and 0.125 mM EDTA) for flow cytometry. To detect EGFP fluorescence, Ly5 congenic antigens and lineage markers, the cells were stained with antibodies specific for Ly5.1, Ly5.2, Gr1, Mac1, CD4, CD8, B220, immunoglobulin-M (IgM), T cell receptor β-chain (TCRβ) and NK1.1; (all from Pharmingen, San Diego, CA, USA), and analyzed with a FACScan (Becton-Dickinson, San Jose, CA, USA) [14].

Results

Retroviral vector

The structure of the retroviral vector used in this study is depicted in Figure 1. This vector carries two genes on a MSCV backbone, one for Tm-specific cell growth and the other for *in vivo* tracking of transduced cells. The first cistron encodes ΔY703FGCRTmR, a modified chimeric receptor for Tm-responsive growth (Figure 1, top). The prototype SAG encoded a fusion molecule (GCRER) consisting of the full-length GCR and ER [7], and three major alterations were made to this system. (1) The G-CSF binding domain of GCR was deleted (Δ) [7]. This modification abrogated an undesired activation of the chimeric receptor by the endogenous G-CSF. (2) ER was replaced with a mutant HBD (TmR) which specifically binds to Tm, to circumvent limitations with wild-type ER [8]. In our previous study, up to 10^{-7} M of estrogen, which is a 10- to 100-fold higher concentration than that in ovarian venous blood, had no influence on ΔGCRTmR-transduced Ba/F3 cells [8]. This modification greatly reduced nonspecific colony formation in murine progenitor assays (ref. 8 and unpublished observation). (3) A tyrosine residue at position 703 in the murine GCR was mutated to phenylalanine (Y703F), to diminish differentiation signals [9].

Downstream of the SAG, the IRES-controlled EGFP gene was linked for real-time identification of transduced

cells. We showed that this type of vector was a versatile tool to track the progeny of transduced stem/progenitor cells [14]. *In vivo* proliferation and differentiation of the transduced cells can be readily evaluated by multiparameter flow cytometry at desired intervals.

For gene transfer to murine BM cells, transient viral supernatant was freshly prepared by transfecting BOSC23 cells with MSCV/ΔY703FGCRTmR-IRES-EGFP plasmid, and the titer of the viral supernatant was estimated to be 1×10^6 colony-forming units/ml [14,16].

Retroviral transduction/reconstitution and Tm stimulation

In the present study, SAG-transduced cells were tracked in a murine bone marrow transplantation (BMT) model to investigate the impact of Tm stimulation. Ly5.2 BM cells were transduced by MSCV/ΔY703FGCRTmR-IRES-EGFP retrovirus following a fibronectin-assisted protocol [14,17]. After 48 h of transduction, the expression of EGFP in the BM cells was measured by flow cytometry. The frequencies of EGFP$^+$ cells were in the range 5–20% 48 h post-transduction, depending on the retroviral infection cycles. In a representative experiment, the transduction efficiency was 20% (FACS data not shown), and the *ex vivo* transduced BM cells were transplanted. Lethally irradiated Ly5.1 mice were transplanted with MSCV/ΔY703FGCRTmR-IRES-EGFP-infected BM, at 2×10^5 cells per animal. The transplants were individually numbered, and peripheral blood was periodically drawn to monitor donor cell repopulation and EGFP expression. One month after BMT, donor cell chimerism was $82 \pm 4\%$ and EGFP$^+$ cells composed $20 \pm 4\%$ of the Ly5.2 population (Figure 2C). Two months post-BMT, $93 \pm 2\%$ of the host peripheral leukocytes were donor-derived (i.e. Ly5.2$^+$) of which EGFP$^+$ cells counted for $21 \pm 3\%$. At this point, the transplants were divided into two groups, one of which was challenged with Tm.

In our previous dose-response study using Ba/F3 and murine BM cells, 10^{-7}–10^{-6} M of Tm was required to activate GCRTmR chimeric receptors, while Tm at this dose did not affect the growth of murine progenitors (ref. 8 and unpublished observation). To determine the Tm dosage for the *in vivo* study, we gave Tm to C57BL/6 mice with variable doses and regimens. Injection of Tm i.p. for 3 days at 1 mg resulted in a concentration of $1–2 \times 10^{-7}$ M in serum, while that at 5 mg achieved a concentration of $5–30 \times 10^{-7}$ M (unpublished data). In the subsequent experiments, mice were given 5 mg/day of Tm in three doses for 3 days.

Figure 2 shows the result of administering Tm to the primary BMT recipients. In the Tm-challenged group, every animal showed an increase in the frequency of EGFP$^+$ cells in 2 weeks, and the escalation was significant ($21 \pm 4\%$ to $27 \pm 5\%$, $p = 0.007$ by paired t-test; Figure 2A). In contrast, the control animals lost EGFP$^+$ cells ($21 \pm 5\%$ to $10 \pm 3\%$, $p = 0.002$ by paired t-test;

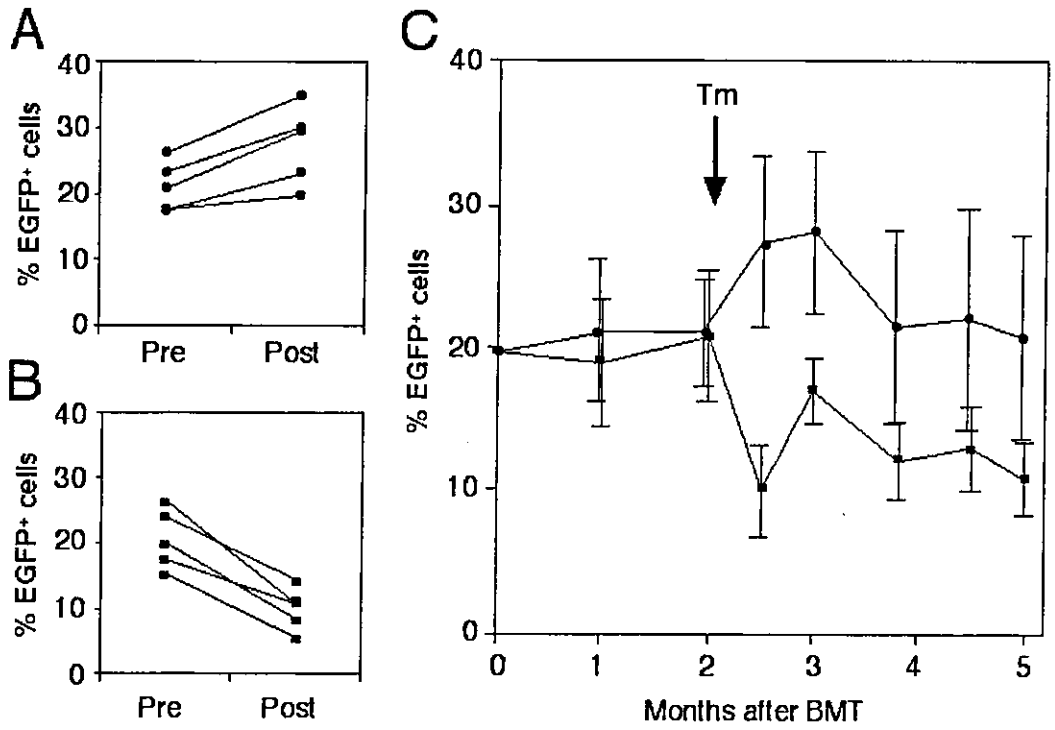

Figure 2. EGFP$^+$ cells in primary recipients. (A) EGFP$^+$ cells were counted before (Pre) and 2 weeks after (Post) Tm injection. (B) EGFP$^+$ cells in control mice were analyzed in parallel to those in Tm-challenged animals. (C) Time course of the change in EGFP$^+$ frequency in Tm-challenged (circles) and control (squares) animals. Two months after BM transduction and transplantation, Tm was administered to a subset of recipients (arrow) and EGFP$^+$ cells were tracked. Bars represent standard deviation

Figure 2B), and the frequencies stayed low (Figure 2C). The late-onset decrease of EGFP$^+$ cells in the controls was presumably because long-term repopulating cells were less actively dividing than short-term repopulating cells during *ex vivo* manipulation, and therefore relatively refractory to retroviral transduction. On the other hand, an elevated level of EGFP$^+$ cells (27–28%) in the Tm-challenged mice was maintained for 1 month, after which transduced cells returned to prestimulatory levels and stabilized at around 20% (Figure 2C). A significant difference in the frequency of EGFP$^+$ leukocytes between the Tm-stimulated and unstimulated groups was maintained for 3 months ($p < 0.05$ by Student's t-test), suggesting that MSCV/ΔY703FGCRTmR-IRES-EGFP-transduced hematopoietic progenitors proliferated more vigorously in response to Tm, i.e. SAG-carrying cells expanded *in vivo*.

We challenged several other transplants with Tm at different doses in a pilot study, and observed a similar expansion of EGFP$^+$ cells in some animals (data not shown). However, the numbers of mice in each cohort was too small for statistical evaluation; therefore, these animals were not subjected to further investigation.

Tm challenge in the secondary recipients

Observing that the administration of Tm led to a relative expansion of the population of gene-modified hematopoietic cells, we made secondary transplants to see whether the number of transduced long-term repopulating cells could be expanded by this drug. BM cells from unchallenged primary recipients were pooled, and aliquots were transplanted into myeloablated secondary hosts (Ly5.1). A flow cytometric analysis showed that the frequency of EGFP$^+$ cells in the pooled BM was 21%, and 2×10^6 cells were infused into each animal (n = 8). One month following BMT, the donor cell chimerism was 65 ± 11% and the EGFP$^+$ cells accounted for 19 ± 5% of Ly5.2$^+$ cells.

At this point, a subset of the secondary recipients (n = 4) was given Tm like the primary hosts (5 mg i.p. for 3 days). Two weeks following Tm administration, EGFP$^+$ cells showed a significant increase (16 ± 4% to 35 ± 3%, $p = 0.006$ by paired t-test; Figure 3A), in contrast to a decrease in the control animals (22 ± 4% to 12 ± 4%, $p = 0.01$ by paired t-test; Figure 3B). Subsequently, EGFP$^+$ cell numbers in the stimulated animals returned to prestimulatory levels and stabilized at 14–18% (Figure 3C). On the other hand, transduced cells in the unstimulated animals further decreased, and the difference between the two groups was significant for more than 3 months (Figure 3C). These results indicated that very immature cells, early progenitors or stem cells were transduced with the SAG and maintained for at least 6 months through serial transplantation. In the presence of Tm, the transduced cells appeared to have a proliferative advantage over untransduced cells, and, therefore, gave rise to more EGFP$^+$ leukocytes in the

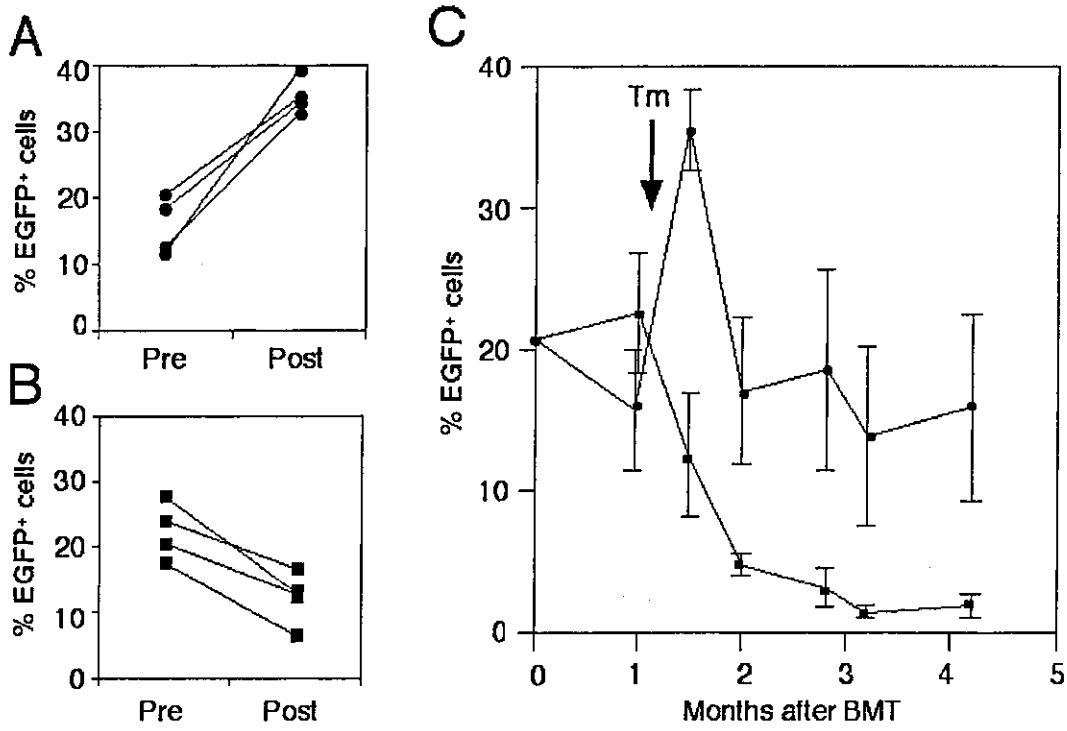

Figure 3. EGFP$^+$ cells in secondary recipients. (A) EGFP$^+$ cells were counted before (Pre) and 2 weeks after (Post) Tm injection. (B) EGFP$^+$ cells in control mice were analyzed in parallel to those in Tm-challenged animals. (C) Time course of the change in EGFP$^+$ frequency in Tm-challenged (circles) and control (squares) animals. One month after BMT, Tm was administered to a subset of recipients (arrow) and EGFP$^+$ cells were tracked. Bars represent standard deviation