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A G-CSF Receptor–Gyrase B Fusion Gene: A New Type of Molecular Switch for Expansion of Genetically Modified Hematopoietic Cells

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We have developed a novel system for expansion of transduced hematopoietic cells. This system involves "selective amplifier genes" encoding fusion proteins between the granulocyte colony-stimulating factor receptor (Gcr) and the estrogen receptor (Er). The GcrEr chimeric gene conferred estrogen-dependent growth ability on murine hematopoietic cells. Here, we constructed a modified "selective amplifier gene" to circumvent possible concerns with the Er/estrogen switching system. The bacterial gyrase B (Gyr) gene was fused to the Gcr gene, and the GcrGyr fusion construct was introduced into interleukin-3 (IL-3)-dependent Ba/F3 cells. The dimeric antibiotic coumermycin induced IL-3-independent growth in Ba/F3 cells expressing GcrGyr. This stimulatory effect was antagonized by an excess amount of novobiocin, a monomeric form of coumermycin. These results suggest the feasibility of using Gyr as a molecular switch to regulate a growth signal in hematopoietic cells.

Hematopoietic stem cells (HSCs) are attractive targets of gene transfer to treat a variety of disorders such as hereditary diseases, cancer and acquired immunodeficiency syndrome (1). To date, human HSC gene therapy trials have resulted in minimal clinical efficacy, primarily due to inadequate gene transfer efficiency by currently available vectors (2). In cases such as adenosine deaminase deficiency and Fanconi's anemia, inefficient gene transfer may be complemented by survival advantage of the cells expressing therapeutic genes. To treat most other disorders in which therapeutic genes do not confer a selective advantage, it is necessary to increase the gene transfer efficiency and/or expand transduced cells to achieve clinical benefits. As one of the possible ways to tackle this problem, we have developed a novel system for expansion of transduced hematopoietic cells. This system involves "selective amplifier genes" encoding fusion proteins of granulocyte colony-stimulating factor (G-CSF) receptor (Gcr) and the hormone-binding domain (HBD) of estrogen receptor (Er) (3, 4). Similarly to previous reports that fused HBDs converted heterologous proteins to function in a hormone-dependent manner (5), the fusion genes between Gcr and Er conferred an estrogen-inducible proliferation on transduced hematopoietic cells (3, 4). However, the Er/estrogen switching system has raised several practical concerns. For instance, elevated endogenous estrogen in women may evoke undesired activation of the Gcr-mediated signals and lead to uncontrollable cell growth.

Recently, the bacterial gyrase B (Gyr) has been introduced as a dimerization motif, which binds to a dimeric coumarin drug coumermycin (6). Development of selective amplifier genes with this molecular switch may lead to a safer system for expansion of transduced cells *in vivo.* In this study, we investigated the feasibility of using Gyr to control Gcr-mediated proliferation.

MATERIALS AND METHODS

Plasmid construction. The Gyr cDNA (encoding amino acids 2-220 of the *Escherichia coli* DNA gyrase B) was cleaved from pBluescript/GyrB (a gift from Dr. M. A. Farrar, University of Washington, Seattle, WA; ref. 6, 7) as an *Xba* I-*Bam*H I fragment, and a stop codon-*Eco*R I linker (Nippon Gene, Toyama, Japan) was attached to its 3′-end. The murine Gcr coding sequence was obtained from pBluescript/Gcr (a gift from Dr. S. Nagata, Osaka University,

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Suita, Japan; ref. 8) as a *Hin*d III-*Xba* I fragment. Fusion constructs were obtained by trimolecular ligation of Gcr (*Hin*d III-*Xba* I), Gyr (*Xba* I-*Eco*R I), and pcDNA3 (*Hin*d III-*Eco*R I; Invitrogen, Groningen, The Netherlands) or pCMX (*Hin*d III-*Eco*R I; a gift from Dr. K. Umesono, Kyoto University, Kyoto, Japan; ref. 9). The resultant plasmids were designated as pcDNA/GcrGyr and pCMX/GcrGyr, respectively, and the fusion gene (GcrGyr) fragment was transferred from pCMX/GcrGyr to pCAGnPolyA (a gift from Dr. Naruse, Kaketsuken, Kumamoto, Japan; ref. 10) to make pCAG/GcrGyr.

For murine IL-3 production in cultured cells, pBMG-hph-IL3 was constructed. This plasmid had a Rous sarcoma virus promoter-driven IL-3 cDNA, and a hygromycin B resistance gene (from pY3 plasmid) in the pBMGNeo backbone (4).

Cells and reagents. Ba/F3 cells (Riken Gene Bank RCB0805, Tsukuba, Japan) were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Bioserum, Victoria, Australia), and 0.5% conditioned medium of C3H10T1/2 cells (Riken Gene Bank RCB0247) transfected with a murine IL-3 expression vector pBMG-hph-IL3 (4). Titration studies on IL-3-dependent 32D cells (Riken Gene Bank RCB1145) showed that this dose of the conditioned medium had a titer equivalent to 100 units/ml (U/ml) recombinant murine IL-3 (Dr. A. Okano, Ajinomoto Co., Yokohama, Japan). Ba/F3 cells were switched to AIM-V medium (Life Technologies) on the day prior to coumermycin stimulation (see below). 293 cells (American Type Culture Collection CRL-1573, Manassas, VA) were maintained in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% FCS. Coumermycin and novobiocin were purchased from Sigma (St. Louis, MO).

Transfection of 293 and Ba/F3 cells. GcrGyr expression vectors were transfected into 293 cells using Lipofectamine reagent (Life Technologies) according to the manufacturer's protocol. Briefly, 3 μg of each plasmid was mixed with 18 μl of Lipofectamine and added to 293 cells inoculated on the previous day at 1×10^6 cells per 60 mm dish. Ba/F3 cells were transfected with pCAG/GcrGyr by electroporation. The cells were extensively washed with ice-cold OPTI-MEM I (Life Technologies) and resuspended at 2×10^7 cells/ml in the buffer. Aliquots of 1×10^7 Ba/F3 cells were mixed with 50 μg of the plasmid DNA, and electroporation was carried out at 960 μF $\times$ 290 V with a Gene Pulser (BioRad, Hercules, CA). G418 (Life Technologies) selection was started on the following day at 1 mg/ml, and G418-resistant Ba/F3 cells were cloned by limiting dilution.

Immunoprecipitation and Western blotting. Transfected 293 cells were directly lysed with RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40, 500 U/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride) on ice 2 days post-lipofection, and the protein concentrations of the lysates were determined by BCA Protein Assay (Pierce, Rockford, IL). The lysates (10 μg per lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Yonezawa, Japan). The blots were probed with anti-Gcr antibody (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Gyr monoclonal antibody (7D3; a gift from Dr. A. Maxwell, University of Leicester, Leicester, UK), and the fusion protein was visualized by an ECL system (Amersham, Little Chalfont, UK).

Tyrosine phosphorylation of GcrGyr in the transfected Ba/F3 clones was determined by immunoprecipitation and western blotting. Control and GcrGyr-expressing Ba/F3 cells were washed and incubated in AIM-V medium without IL-3 and serum for 8 hours. Aliquots of 1×10^7 starved cells were resuspended in 1 ml of AIM-V medium containing 2 mM $NaVO_4$, and stimulated by 10 ng/ml human G-CSF (provided by Chugai Pharmaceuticals, Tokyo, Japan) or 100 nM coumermycin for 10 minutes at 37°C. The cells were lysed in 1 ml of RIPA buffer containing 2 mM $NaVO_4$ and 100 mM NaF, and the lysates were immunoprecipitated with M-20 antibody and protein A-sepharose beads (Sigma). Immunoprecipitated proteins were recovered in 20 μl of loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue) by boiling for 2 minutes, from which aliquots of 10 μl were subjected to SDS-PAGE and electroblotting onto PVDF membranes. The membranes were probed with an anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology, Lake Placid, NY), and reprobed with M-20 antibody.

Growth stimulation of transfected Ba/F3 cells. Parental and derivative Ba/F3 cells were washed and incubated in AIM-V medium without serum (but containing 0.5% 10T1/2 conditioned medium as an IL-3 source) for 8 hours. After serum starvation, the cells were extensively washed with plain AIM-V, and resuspended at 1×10^5 cells/ml in the medium. IL-3 (0.5% 10T1/2 conditioned medium), 10 ng/ml G-CSF or 100 nM coumermycin was added to the culture, and viable cells were counted daily by the trypan blue exclusion method. To determine the effects of novobiocin, an excess amount (50 μM) of the reagent was added to the IL-3 and coumermycin cultures, and the viable cells were counted after three days of incubation.

RESULTS AND DISCUSSION

Construction of a modified selective amplifier gene. The fusion gene encoding the full-length Gcr and the N-terminal subdomain of *E. coli* gyrase B (6, 7) was cloned into three mammalian expression vectors. In pcDNA/GcrGyr, the chimeric gene was placed 3′ to the human cytomegalovirus (CMV) promoter without an intron. In the second construct, pCMX/GcrGyr, the GcrGyr gene was also driven by the CMV promoter, and incorporation of the SV40 small t intron may lead to better transgene expression in mammalian cells (9). Finally, pCAG/GcrGyr had the potent CAG promoter, containing the CMV immediate early enhancer, the chicken β-actin promoter, and a chicken β-actin/rabbit β-globin hybrid intron (10). In addition to the GcrGyr fusion gene, pcDNA/GcrGyr and pCAG/GcrGyr had neomycin-resistance cassettes, which allowed G418 selection of the transfected cells. To determine the relative expression levels of GcrGyr, human embryonic kidney-derived 293 cells were transfected with these plasmids. Western blotting with anti-Gcr antibody (M-20) and anti-Gyr antibody (7D3) revealed that pCAG/GcrGyr-transfected 293 cells expressed the GcrGyr fusion protein most abundantly (data not shown). Therefore, pCAG/GcrGyr was used in the following experiments for GcrGyr expression in hematopoietic cells.

Coumermycin-induced growth of GcrGyr-expressing Ba/F3. IL-3-dependent Ba/F3 cells (11) were used to investigate the growth-promoting activity of the newly constructed selective amplifier gene. Ba/F3 cells were transfected with pCAG/GcrGyr by electroporation and selected with 1 mg/ml G418. Selected Ba/F3 pool was incubated with coumermycin at various concentrations between 10 nM and 1 μM, and the cells showed the most active proliferation with 100 nM coumermycin (data not shown). Based on this finding, G418-resistant Ba/F3 transfectants were cloned by limiting dilution, and the isolates were screened for responsiveness to

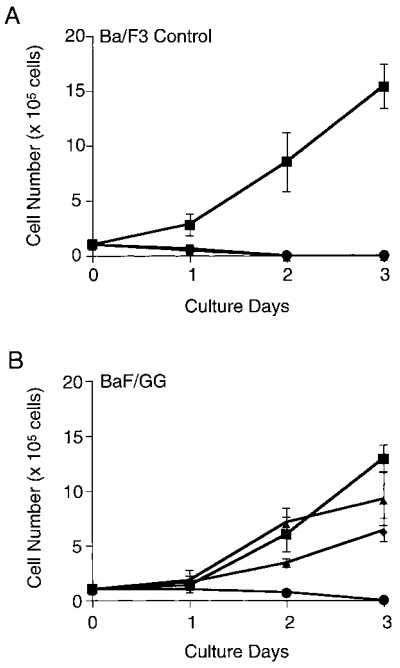

FIG. 1. Coumermycin-induced proliferation of GcrGyr-expressing Ba/F3 cells.Untransfected Ba/F3 control cells (A) and BaF/GG clones (B) were stimulated by IL-3 (squares), G-CSF (triangles), or 100 nM coumermycin (diamonds), and numbers of viable cells were counted daily. Circles represent unstimulated cells. Graphs represent means ± SD in triplicate determinants (A) or means ± SD of three independent BaF/GG clones (B).

100 nM coumermycin. Three clones were chosen and designated as BaF/GG-1, 2 and 3. Expression of GcrGyr protein in BaF/GG clones was confirmed by immunoblotting with anti-Gcr (M-20) and anti-Gyr (7D3) antibodies, and the GcrGyr expression levels were similar in these clones (data not shown).

To characterize GcrGyr-mediated growth, parental Ba/F3 cells and the BaF/GG clones were stimulated by several reagents, and the results are illustrated in Fig. 1. Untransfected Ba/F3 proliferated in the presence of IL-3, but neither G-CSF nor 100 nM coumermycin supported these cells (Fig. 1A). With IL-3, BaF/GG cells proliferated exponentially in a similar fashion to the parental Ba/F3 (Fig. 1B, squares), and all the cells died without growth factor support (Fig. 1B, circles). In contrast to parental Ba/F3, BaF/GG clones proliferated well with G-CSF (Fig. 1B, triangles). This difference resulted from the fact that parental Ba/F3 cells did not express Gcr endogenously, whereas BaF/GG clones expressed the fusion protein containing the full-length Gcr capable of transmitting a G-CSF-mediated growth signal. The BaF/GG clones also responded to 100 nM coumermycin (Fig. 1B, diamonds), in contrast to the parental Ba/F3 which showed no response to the reagent. The coumermycin-induced growth rates of the BaF/GG clones were about half of those induced by

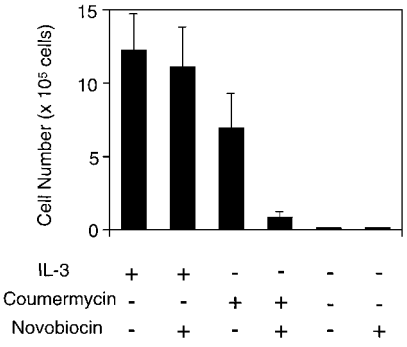

FIG. 2. Effects of novobiocin on cultured BaF/GG clones. BaF/GG clones were inoculated at 1×10^5 cells/ml on day 0 and stimulated with IL-3 or 100 nM coumermycin. An excess amount (50 μM) of novobiocin was added to each culture, and viable cells were counted on day 3. Bars represent means ± SD of three independent clones.

IL-3 and G-CSF, comparable to the growth induction via the fusion proteins between Gcr and Er (3).

The stimulatory effect of coumermycin was antagonized by an excess amount of novobiocin, a monomeric form of coumermycin (Fig. 2). When 50 μM novobiocin was added to the BaF/GG cultures containing 100 nM coumermycin, growth of these cells was almost completely blocked. Toxicity of novobiocin at this concentration was minimal, since IL-3-stimulated growth of parental Ba/F3 and BaF/GG cells was not affected by 50 μM novobiocin. Therefore, novobiocin specifically blocked the stimulatory effect of coumermycin on BaF/GG cells, presumably because the excess monomeric reagent inhibited GcrGyr dimerization.

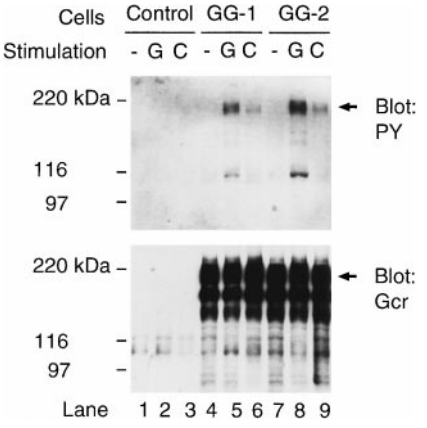

FIG. 3. Tyrosine phosphorylation of GcrGyr following G-CSF or coumermycin stimulation. Control Ba/F3 cells, BaF/GG clone 1 (GG-1) and clone 2 (GG-2) were stimulated with G-CSF (G) or 100 nM coumermycin (C) for 10 min. GcrGyr protein was immunoprecipitated with anti-Gcr antibody (M-20) and probed with anti-phosphotyrosine monoclonal antibody (4G10; upper panel). The blot was subsequently reprobed with M-20 for detection of GcrGyr fusion protein (lower panel). Arrows indicate tyrosine-phosphorylated GcrGyr. Lanes 1, 4, and 7 show unstimulated cells.

Tyrosine phosphorylation of GcrGyr upon coumermycin stimulation. BaF/GG clones were incubated with G-CSF or coumermycin for 10 minutes, to determine whether GcrGyr was tyrosine-phosphorylated upon stimulation. Lysates of parental Ba/F3 and BaF/GG cells were immunoprecipitated with an anti-Gcr antibody (M-20), and probed with an anti-phosphotyrosine antibody (4G10). As shown in Fig. 3, the fusion receptor was tyrosine-phosphorylated following G-CSF or coumermycin stimulation (arrow). Thus, ectopically expressed GcrGyr fusion receptor can be activated by coumermycin to transmit a growth signal analogous to the native Gcr, and eventually lead to proliferation of hematopoietic cells. Recently, Blau et al. reported a similar cell expansion system involving fusion proteins between cytokine receptors (e.g., erythropoietin receptor; EpoR) and FKBP (12, 13). They showed that cross-linking of the EpoR-FKBP fusion protein by FK1012 (a dimeric ligand capable of binding to FKBP) resulted in phosphorylation of JAK2 and STAT5 in transfected Ba/F3. Taken together, these chimeras (including ours) are likely to transmit growth signals when dimerized by exogenously added chemicals, and selective expansion of transduced hematopoietic cells may be generally applicable. We found that the coumermycin-induced tyrosine phosphorylation of GcrGyr was somewhat weaker than that in G-CSF-treated cells (Fig. 3), and the weaker phosphorylation may account for the reduced growth-promoting activity of coumermycin compared to G-CSF (Fig. 1B). We are currently investigating the detailed signal transduction properties downstream of our fusion molecules, to achieve more potent and selective expansion of transduced hematopoietic cells.

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