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Selection of genetically modified cell population using hapten-specific antibody/receptor chimera

Masahiro Kawahara,* Hiroko Kimura, Hiroshi Ueda,* and Teruyuki Nagamune

Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

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Abstract

Efficient selection of the genetically modified cell population is a critical step to obtain the cells with desired properties. In this study, we propose an antigen-mediated genetically modified cell amplification (AMEGA) system employing an antibody/receptor chimera that triggers a growth signal in response to a non-toxic hapten dimer. An anti-fluorescein single-chain Fv fused to the extracellular D2 domain of erythropoietin receptor and transmembrane/intracellular domains of gp130 was expressed together with a model transgene, enhanced green fluorescent protein (EGFP) downstream of IRES sequence, by retroviral infection to IL-3-dependent Ba/F3 cells. Addition of fluorescein dimers connected by various oligo-DNA linkers induced selective growth of transfectants, thus leading to efficient expansion of EGFP-positive cell population. Also, digestion of the oligonucleotides by specific restriction endonuclease completely suppressed cell growth. Because these hapten dimers are not harmful for normal cells, the approach will be especially useful for reversible in vitro or in vivo expansion of genetically modified cell population employed for cell therapy and tissue engineering.

Keywords: Erythropoietin receptor; gp130; Single-chain Fv; Chimeric receptor; Transgene selection; Hapten; Fluorescein dimer; Hematopoietic cell; Cell therapy; Tissue engineering

Selection of genetically modified cells is crucial to obtain the cells with desired properties. Drug-resistance selection has been widely used for selection of genetically modified cells. However, cytotoxic drugs often lead to deleterious effects such as inhibition of cell growth and morphological change [1–3]. Especially, the prolonged selection period due to growth inhibition is undesirable for the cells in which transduction efficiency is low and/or cell differentiation readily occurs [4].

To avoid these problems associated with drug-resistance selection, attempts have been made to develop a positive selection method, where a growth advantage is conferred exclusively on genetically modified cells. To this end, a number of chimeric receptor constructs were investigated, where an exogenous ligand-binding domain was fused with a signaling domain of a cytokine receptor, and they could mimic signaling of the cognate cytokine with exogenous ligands [5–8]. At least in theory, the co-expression of such a chimeric receptor and a gene of interest is expected to result in a growth advantage only for genetically modified cells by adding the cognate ligand in the culture medium. However, although these methods can be used for the reversible expansion of a single transduced cell population, reversible expansion of multiple cell populations transduced by different transgenes that can be individually controlled by a specific ligand will further enhance the therapeutic effects in cell therapies or tissue engineering. In such case, while a specific binding domain is required for the specific control of each cell population, alteration of the binding specificity was not readily attained in those previous methods. In addition, almost all the ligands employed therein needed special organic synthesis and are still not readily available in market.

To overcome such limitations, here we adopt the use of antigen–antibody system, which has an unlimited

* Corresponding authors: Fax: +81-3-5841-8657.
E-mail addresses: kawahara@bio.t.u-tokyo.ac.jp (M. Kawahara), hueda@chembio.t.u-tokyo.ac.jp (H. Ueda).

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number of ligand–receptor combinations with high specificity. In this study, we chose a small molecular weight hapten as an antigen without any toxicity or known immunogenicity, unless conjugated to proteins. Since a phage display-based selection method to obtain high-affinity single-chain Fv (ScFv) has already been established, here we attempted to take advantage of using a ScFv-based antibody–receptor chimera that is expected to be dimerized by a hapten dimer. The administration of haptens dimerized by an oligo-DNA linker could reversibly control dimerization/activation of the constructed chimeric receptor, and also amplification of transfected cell populations. In this study, we examined the efficacy of the ScFv-based AMEGA approach using fluorescein dimer as the ligand.

Materials and methods

Ligand preparation. Fluorescein-conjugated BSA was prepared by the mixture of 0.75 mg/ml fluorescein isothiocyanate (FITC) (Wako, Tokyo, Japan) and 15 mg/ml BSA (Sigma, St. Louis, MO) in 0.25 M sodium bicarbonate buffer (pH 9.0) containing 0.1 M NaCl at 4 °C overnight. BSA–Fl and free FITC were separated by NAP-5 column (Amersham–Pharmacia, Little Chalfont, UK). In this procedure, the fluorescein/protein ratio was around 5, which was determined by measuring absorbance at 280 and 490 nm.

Fluorescein dimer was prepared by annealing of 5′-Fl-labeled palindromic DNA with heating at 70 °C for 5 min and gradual cooling to 20 °C with a temperature slope at 0.01 °C/s using a thermal cycler. The sequences of the palindromic DNAs were as follows: 8 mer, Fl-GCGG CCsGsC; 9 mer, Fl-TGCGGCCsGsC; 10 mer, Fl-GGCGGCCGsCsC; 11 mer, Fl-TGGCGGCCGsCsC; 12 mer, Fl-GGGCGGCCGCsCsC; 13 mer, Fl-TGGGCGGCCGCsCsC; 14mer, Fl-CGGGCGGCCGCCs CsG; and 12 mer-B, Fl-CGCGGATCCGsCsG. The 3′-terminal two bases in each Fl-labeled DNA were made with s-oligo to prevent degradation by exonucleases. These Fl-labeled oligo DNAs were purchased from Proligo (La Jolla, CA).

Phage ELISA. To prepare 31IJ3 ScFv-displayed phage, *Escherichia coli* strain TG-1 transformed with a phagemid containing 31IJ3 was cultured in 5 ml YT medium containing 100 μg/ml ampicillin and 2% glucose at 30 °C for 2 h and subsequently at 37 °C for 2.5 h. M13KO7 helper phage was added to the medium and shaken at 37 °C for 30 min. The cells were transferred to 100 ml of 2× YT medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. After vigorous shaking at 30 °C overnight, cells were pelleted at 8000*g* for 13 min and the supernatant was precipitated with 3.3% polyethylene glycol/0.42 M NaCl. After 1 h incubation on ice, the sample was centrifuged at 8000*g* for 13 min and the pellet was resuspended with 10 mM Tris–HCl and 1 mM EDTA (pH 7.5). The phage solution was centrifuged at 12,000*g* for 10 min and the supernatant containing the phage particle displaying 31IJ3 ScFv was recovered.

To perform phage ELISA, 100 μl of 5 μg/ml BSA or BSA–Fl was applied to each well of a microtiter plate and incubated at 4 °C for 24 h. After blocking with 200 μl/well of 2% skimmed milk at 4 °C for 2 h, the plate was washed three times with 0.1% Tween 20 in PBS (PBS-T), and 100 μl/well of 31IJ3 ScFv-displayed phage was applied and incubated at 37 °C for 1 h. After washing six times with PBS-T, 100 μl/well of 1:1000 diluted HRP-labeled anti-M13 phage antibody (Amersham–Pharmacia) was added and incubated for 1 h at room temperature. The plate was washed three times with PBS-T and developed with 100 μl/well of 100 μg/ml 3,3′,5,5′-tetramethylbenzidine (TMB). After incubation for 5–30 min, the reaction was stopped with 50 μl/well of 1 M sulfuric acid and the absorbance at 450 nm was measured by a Bio-Rad Model 550 microplate reader (Hercules, CA).

Vector construction. The ecotropic retroviral vector pMX [9] was kindly provided by Dr. T. Kitamura, Institute of Medical Science, University of Tokyo. The construction of pMX-Hg encoding HyHEL-10 V_H, a GSG linker, extracellular D2 domain of EpoR, and transmembrane/cytoplasmic domains of gp130 was described previously [10]. The anti-fluorescein ScFv sequence was amplified with two primers (31IJ3 sense: CCCCTGCAGGAGTCTGGGGGAGGCTTGGTA, 31IJ3 anti: CCCAAGCTTAAGACTTACCTTTGATTTCCACCTTG GTCCC), having *Pst*I and *Hin*dIII sites (underlined), respectively. The amplified ScFv fragment was digested with *Pst*I and *Hin*dIII, and inserted into *Pst*I and *Hin*dIII-digested pTVSig, which is a variant of pTV118N (Takara, Kyoto, Japan) with a 574 bp *Nco*I fragment of pRSVVμERCA [11] containing mouse IgH signal sequence. After the sequence of the resultant plasmid (pTVSig-ScFv) was confirmed with SQ-5500 Sequencer (Hitachi, Tokyo, Japan), the signal sequence-ScFv fragment was digested with *Nco*I and *Hin*dIII, and inserted into *Nco*I and *Hin*dIII-digested pMX-Hg to create pMX-ScFvg. The ScFvg fragment in pMX-ScFvg was digested with *Eco*RI and inserted into *Eco*RI-digested pMX-LgIGFP-Xho, which is a variant of pMX-LgIGFP [10] made by digesting and blunting at a *Xho*I site, to create pMX-ScFvgIGFP.

Cell culture. A murine IL-3-dependent pro-B cell line, Ba/F3 [12], was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Iwaki, Tokyo, Japan) and 2 ng/ml of murine IL-3 (Genzyme/Techne, Cambridge, MA). A retroviral packaging cell line, Plat-E [13], was cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical) supplemented with 10% FBS, 1 μg/ml puromycin (Sigma), and 10 μg/ml blasticidin (Kaken Pharmaceutical, Tokyo, Japan).

Retroviral infection. Plat-E cells were inoculated into a ∅ 60 mm dish at 5×10^5 cells/ml in 4 ml DMEM and cultured for 20 h. Nine microliters of Fugene6 (Roche Diagnostics, Basel, Switzerland) was diluted with 100 μl serum-free DMEM, and added to 3 μg of the retroviral vector solubilized in 6 μl sterile water. After 15 min incubation at room temperature, the vector-Fugene6 mixture was added to the Plat-E cells. After 24 h incubation, the culture medium was refreshed with 3 ml DMEM, followed by additional 24 h incubation. After the supernatant was centrifuged at 1000*g* for 5 min at 20 °C, Ba/F3 cells (10^5 cells) were infected with 500 μl of the viral supernatant in the presence of 10 μg/ml polybrene (Sigma) and 4 ng/ml IL-3 in 12-well plates. After 5 h incubation, 0.6 ml RPMI1640 was added to reduce the toxicity of polybrene.

Flow cytometric analysis and sorting. Cells were washed once and resuspended with PBS. Green fluorescence intensity was measured using FACS Calibur flow cytometer (Becton–Dickinson, Lexington, KY) at 488 nm excitation and fluorescence detection at 530 ± 15 nm. The sorting was performed according to the manufacturer's protocol.

Western blotting. The cells (10^6 cells) were washed with PBS, lysed with 100 μl lysis buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin], and incubated on ice for 10 min. After centrifugation at 16,000 G for 5 min, the supernatant was mixed with Laemmli's sample buffer and boiled. The lysate was resolved by SDS–PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). After the membrane was blocked with 5% skimmed milk, the blot was probed with 200 ng/ml of rabbit anti-mouse gp130 antibody (Santa Cruz Biotechnology) followed by 600 ng/ml of HRP-conjugated anti-rabbit IgG (Biosource, Camarillo, CA), and detection was performed using the ECL system (Amersham–Pharmacia).

Selection of the transfectant and cell proliferation assay. For selection of the transfectants with ligand addition, the cells were washed with PBS three times and inoculated into 24-well plates in the medium containing either no factor, 70 μg/ml BSA–Fl, 1 μM fluorescein dimer or 2 ng/ml IL-3.

For cell proliferation assay, the selected cells were washed three times with PBS and seeded in 24-well plates containing various concentrations of ligands. Cell number and viability were determined using a hemocytometer and the trypan blue exclusion assay. Free fluorescein (fluorescein sodium salt) used as a negative control ligand was purchased from Wako.

Results

Specific binding of anti-fluorescein ScFv

We first designed a chimeric receptor, in which an anti-fluorescein (Fl) ScFv was fused to extracellular D2 domain of EpoR and transmembrane/cytoplasmic domains of gp130 (Fig. 1A). Cell surface expression of the chimeric receptor would result in preferential cell growth of genetically modified cells in response to a Fl dimer (Fig. 1B). As for ScFv, a clone named 31IJ3, selected from a phage antibody library Tomlinson J having a single human framework [14] by 3 rounds of selection with Fl-conjugated BSA, was employed. A phage ELISA was performed to confirm specific binding of 31IJ3 to Fl. Immobilization of Fl-conjugated BSA (BSA–Fl) on a microplate resulted in binding of the 31IJ3-displaying phage in a titer-dependent manner, whereas immobilization of BSA resulted in very low non-specific binding, indicating specific recognition of Fl by the ScFv (Fig. 2A).

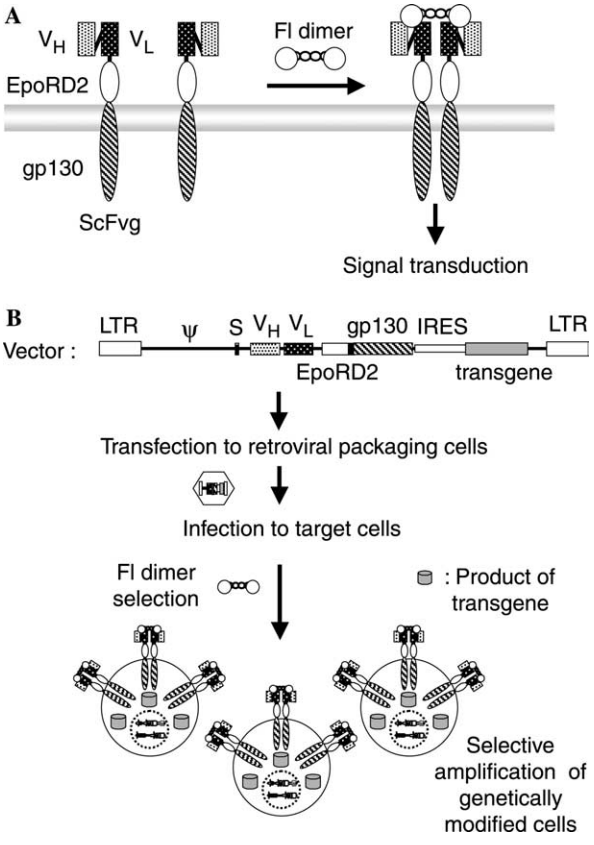

Fig. 1. Scheme of the antigen-mediated genetically modified cell amplification (AMEGA) system. (A) The construct and the activation scheme of the chimeric receptor. The extracellular N-terminal half of EpoR was replaced with ScFv of anti-fluorescein antibody 31IJ3 with a GSG tripeptide linker between ScFv and Val 118 of EpoR D2 domain to create ScFvg. V_H and V_L regions were linked with a $S(G_4S)_3$ flexible linker. Fluorescein dimer addition brings two ScFvg chains into close proximity, leading to signal transduction. (B) Schematic diagram of the selection procedure. Retroviral vectors with long-terminal repeats (LTRs) and a packaging signal (ψ) are used for the efficient transcription of ScFvg-IRES-transgene. An immunoglobulin heavy chain secretion signal sequence (S) is located upstream of the chimeric receptor gene to enable their cell surface expression.

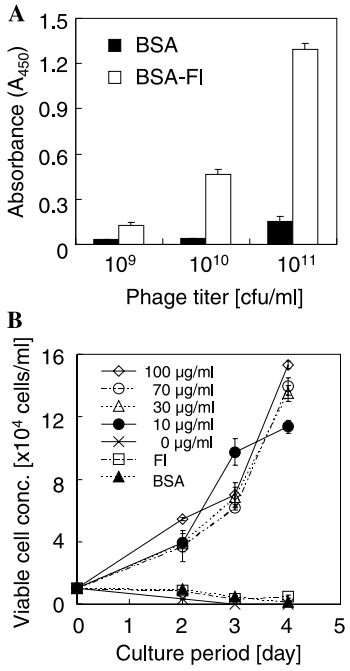

Fig. 2. Functional analysis of ScFv and ScFvg using BSA–Fl. (A) Phage ELISA. 31IJ3 ScFv displayed on phage coat protein pIII was incubated with BSA or BSA–Fl immobilized on a microplate. The phage binding was probed with HRP-labeled anti-M13 antibody and developed with TMB. Absorbance at 450 nm was plotted against the phage titer. (B) BSA–Fl-dependent cell growth of the transfectant. Ba/F3 transfectant expressing ScFvg was sorted by FACS and cultured with BSA–Fl. The selected cells (10^4 cells/ml) were inoculated into 24-well plates at day 0 with either 10 μg/ml BSA, 100 μM fluorescein sodium salt (Fl) or indicated concentrations of BSA–Fl (10 μg/ml corresponds to 0.76 μM as a Fl concentration). Viable cell concentrations of triplicates are plotted with average and 1 SD.

Chimeric receptor transduces a growth signal in response to BSA–Fl

With the use of 31IJ3 ScFv, a retroviral chimeric receptor expression vector pMX-ScFvgIGFP was constructed, in which an IRES-EGFP cassette was placed downstream of the chimeric receptor gene to facilitate scoring and sorting of chimeric receptor-expressing cells. After transfecting Plat-E packaging cells to yield

retrovirus having packaged pMX-ScFvgIGFP, IL-3-dependent Ba/F3 cells were infected with the virions to create Ba/ScFvgIGFP cells. Flow cytometric analysis revealed the transduction efficiency of around 6%, as estimated by EGFP-positive cell ratio of the transduced cell population (data not shown). To enrich the cell population with chimeric receptor expression, EGFP-positive cells were sorted by FACS, resulting in positive cell population of 94% (data not shown).

To confirm that the sorted cells express the chimeric receptor, cell lysate was prepared to perform Western blotting using anti-gp130 antibody. A specific band for the chimeric receptor was clearly observed around 70 kDa, suggesting that the chimeric receptor was correctly expressed in the sorted cells (data not shown).

To examine whether the sorted cells could proliferate in response to Fl dimer, first the cells were cultured in a medium containing BSA–Fl but without IL-3. Since the prepared BSA–Fl had multiple Fl per protein, the existence of a number of different Fl pairs with different Fl–Fl distances was expected to enhance the probability of the chimeric receptor taking functionally active conformations. As expected, BSA–Fl induced cell growth without any IL-3. Thus, the BSA–Fl-selected cells were expanded and subjected to cell proliferation assay. As shown in Fig. 2B, cells grew in a BSA–Fl-dependent manner, while no cell growth was observed with either adding BSA or Fl alone, or without adding any factors. These results demonstrate that the chimeric receptor transduces a growth signal in response to polyvalent Fl such as BSA–Fl.

Fl dimer induced cell growth

Because the chimeric receptor was confirmed to be functional as a polyvalent Fl receptor, next we designed DNA-based Fl dimers, which could be easily prepared by self-annealing of each 5′-Fl-labeled oligo-DNA with a palindromic sequence. To attain higher melting temperature T_m than 37 °C to keep sufficiently stable duplex during cell culture period, GC-rich sequences were incorporated (Fig. 3A). According to the X-ray crystallographic analysis of the peptide agonist–EpoR complex [15], the distance between two amino-terminal residues (Val 118) of EpoR D2 domains was 35.9 Å. Based on this value, a series of Fl dimers with different DNA linker lengths, ranging from 8 mer (27.2 Å) to 14 mer (47.6 Å), were designed, where the 3′-terminal two bases were made with s-oligo to prevent degradation by 3′-exonuclease that reportedly exists in serum [16]. BSA–Fl-selected cells were washed and cultured in the medium containing each Fl dimer at a fixed concentration of 1 μM to determine which length was most effective for cell growth. As shown in Fig. 3B, the optimal linker length was 12 mer (40.8 Å) or 13 mer (44.2 Å), although all Fl dimers induced cell growth. To

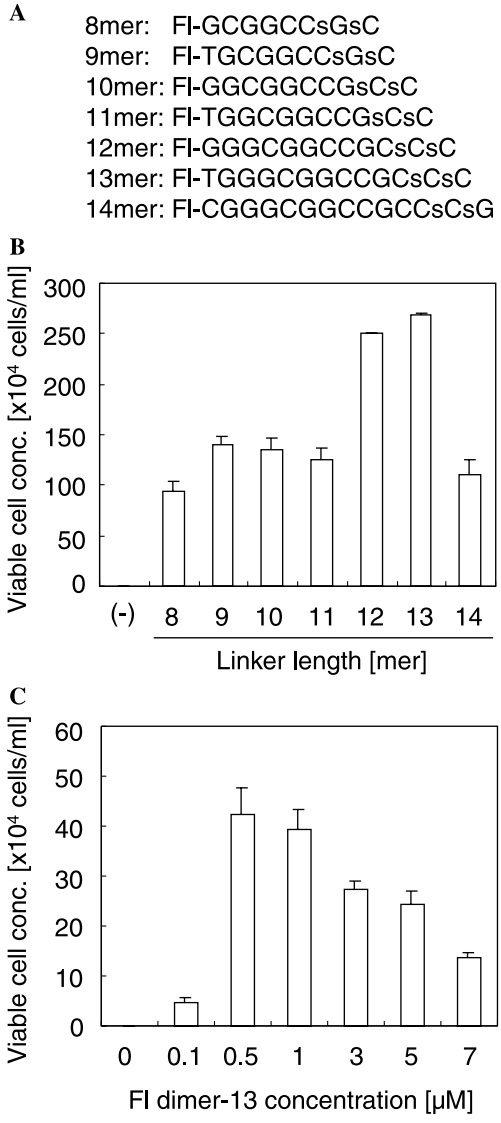

Fig. 3. Fl dimer-dependent cell growth of the transfectant. (A) The sequences for a series of Fl-labeled oligo-DNAs. Fl dimer was prepared by annealing of each Fl-labeled palindromic DNA with heating and gradual cooling. (B) Linker length-dependent cell growth. The BSA–Fl-selected cells (10^4 cells/ml) were inoculated into 24-well plates at day 0 without any growth factors (–) or with 1 μM of each Fl dimer. Viable cell concentrations of triplicates on day 5 are plotted with average and 1 SD. (C) Dose-dependent cell growth with Fl dimer-13. The BSA–Fl-selected cells (10^4 cells/ml) were inoculated into 24-well plates at day 0 without any growth factors (–) or with indicated concentrations of Fl dimer-13. Viable cell concentrations of triplicates on day 4 are plotted with average and 1 SD.

investigate Fl dimer concentration dependency on cell growth, Fl dimer with a 13 mer linker (Fl dimer-13) was added as a representative ligand with various concentrations (Fig. 3C). There appeared an optimal concentration from 0.5 to 1 μM, with a lower limit of 0.1 μM. Since free fluorescein exhibited no toxicity to Ba/F3 cells at the concentration less than 1 mM (data not shown), the decline of growth-stimulating activity at higher Fl dimer concentrations might be due to the formation of

1:1 Fl dimer–receptor complexes rather than 1:2, which is a generally observed phenomenon for receptors activated by polymeric ligands [11]. These results clearly demonstrate that the chimeric receptor can be activated by small molecular weight Fl dimers as well as by BSA–Fl.

Cell growth control with restriction enzyme

Since any DNA sequences with higher T_m than 37 °C could be used as a Fl dimer linker, a Fl dimer with a 12 mer DNA linker including a *Bam*HI endonuclease recognition sequence (named Fl dimer-12B) was designed to investigate whether cell growth could be controlled by the addition of *Bam*HI through the digestion of the Fl dimer at the specific sequence (Fig. 4A). BSA–Fl-selected cells were washed and cultured in the medium containing Fl dimer-12B or a Fl dimer with a 12 mer DNA linker without a *Bam*HI recognition sequence (Fl dimer-12) on day 0, and *Bam*HI was added to the culture medium on day 2. Cell proliferation assay on day 4 revealed that *Bam*HI addition clearly suppressed the growth of the cells cultured with Fl dimer-12B but not of those cultured with Fl dimer-12 (Fig. 4B). The result confirmed the crucial role of DNA-mediated Fl dimerization in cell growth promotion, and indicates that cell growth can be reversed by adding dimerization inhibitors in the culture medium.

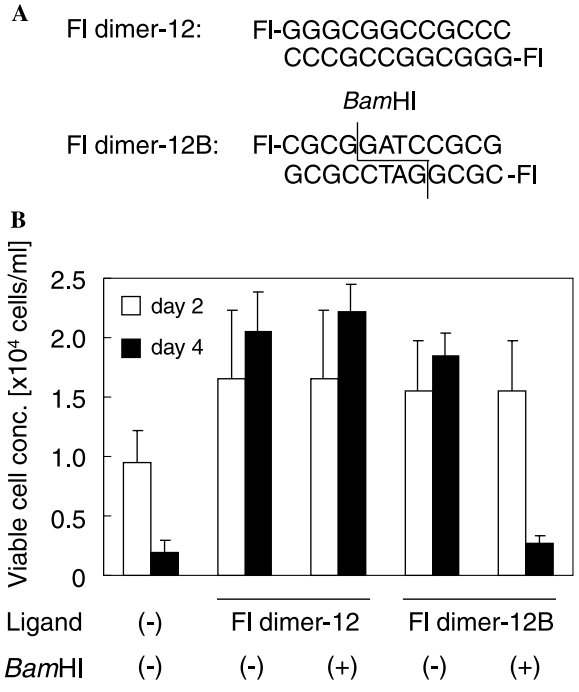

Fig. 4. Cell growth control with restriction enzyme. (A) Comparison of the sequences for Fl dimer-12 and Fl dimer-12B. *Bam*HI recognition site is indicated. (B) The BSA–Fl-selected cells (1.5×10^4 cells/ml) were inoculated into 24-well plates at day 0 without any growth factors (−) or with 1 μM of Fl dimer-12 or Fl dimer-12B. Forty units per milliliter of *Bam*HI was added on day 2. Viable cell concentrations of triplicates are plotted with average and 1 SD.

Direct selection of transfectants with Fl dimers

To show the applicability of our approach to genetically modified cell amplification, the virus-infected cells before FACS sorting were washed and directly selected in the medium containing either BSA–Fl, Fl dimer-12 or Fl dimer-13, followed by monitoring EGFP-positive cell ratio by flow cytometric analysis. After a single round of selection, addition of either BSA–Fl, Fl dimer-12 or Fl dimer-13 resulted in almost 100% EGFP-positive cells, while IL-3 selection did not amplify the transfectant cells (Fig. 5). In addition, cell lysates after the selection with each ligand were prepared and amounts of expressed chimeric receptor were compared by Western blotting using an anti-gp130 antibody (Fig. 6). Fl dimer or

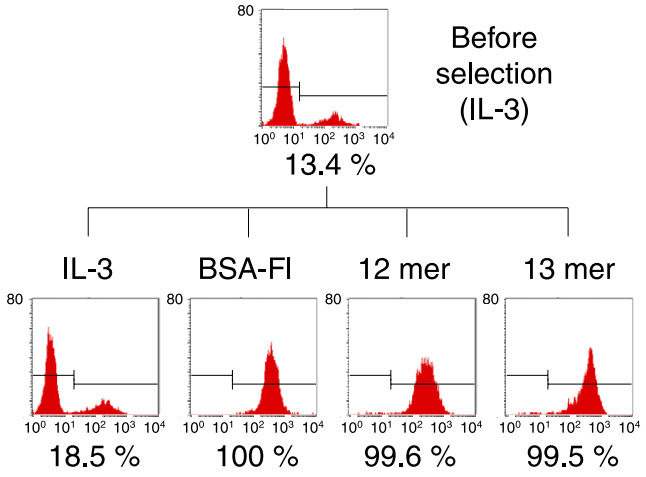

Fig. 5. Demonstration of antigen-mediated genetically modified cell amplification (AMEGA). Retrovirally infected cells (9×10^4 cells/ml) were washed with PBS once and directly selected in 24-well plates with either 2 ng/ml IL-3, 26 μg/ml BSA–Fl (2.0 μM as a Fl concentration), 1 μM Fl dimer-12 or 1 μM Fl dimer-13. Flow cytometric analysis was performed before and after selection (day 18). Cell number is plotted against log green fluorescent intensity. EGFP-negative and EGFP-positive regions were determined by taking parental Ba/F3 cells as a negative control. EGFP-positive cell ratios are indicated.

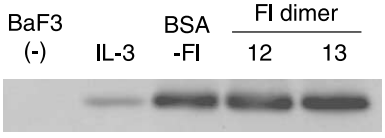

Fig. 6. Antigen-mediated expansion of the transfectants with chimeric receptor expression. The expression levels of the chimeric receptor after selection (day 31) were compared with Western blot using anti-gp130 C-terminus antibody. Parental Ba/F3 cell lysate was used as a negative control. The selection conditions were the same as those in Fig. 5.

BSA–Fl-selected cells expressed higher amounts of the chimeric receptor than IL-3-selected cells, confirming that the cell amplification was induced by the expressed chimeric receptor. These results demonstrate the utility of our system as a positive screening method of genetically modified cells.

Discussion

In this study, we demonstrated a rational design of a pair of specific receptors (ScFv–receptor chimera) and its ligand (fluorescein dimer), for the specific amplification of genetically modified hematopoietic cells. In addition, as far as we know, this may be the first demonstration of restriction enzyme-based cell growth control. Either before or after the selection, the clonal variability in EGFP expression levels in our system was similar to that in the conventional drug-resistance selection using pMX-neoIGFP vector (data not shown). Furthermore, the clones with apparently distinct EGFP expression levels showed similar growth rates in response to BSA–Fl (data not shown). Therefore, our method is almost comparable to the conventional drug-resistance selection in both clonal difference and maintenance of original complexity of the transfectants. While we demonstrated the feasibility of AMEGA using a human ScFv–murine receptor combination, expression of fully humanized ScFv–receptor chimera also induced Fl dimer-mediated genetically modified cell amplification (data not shown). Since hapten-specific high-affinity ScFv can be relatively easily obtained by phage display-based selection, the establishment of selection system using the ScFv-based chimeric receptor would open a way to individual growth control of multiple transgenic populations by administration of a series of cognate hapten dimers. Another merit of the present system is that it requires only one ScFv–receptor construct per transgene, which significantly reduces the vector size and simplifies the construct, compared with our previous AMEGA systems requiring co-infection of two vectors encoding each receptor subunit [10,17].

We used oligo-DNA linkers to make fluorescein dimers partly because of their relatively rigid structure of double helix predictable enough to estimate the appropriate linker length. Although the use of specific DNA sequence enables facile homo- or heterodimerization of multiple pairs of haptens, other materials such as methylene, polyethylene glycol, and peptide might be used as the linker, as Fl dimers used in this study also contained a hexamethylene linker between Fl and 5′-end of the DNA. Recent X-ray crystallographic analyses revealed the importance of the conformation of the extracellular domain in EpoR signaling [15,18–21]. Although such a detailed study including agonist/antagonist–receptor complexes has not been reported in the case of gp130, the linker length-dependent growth characteristic in this study strongly suggests that activation of gp130 would depend on distance and conformation of two receptor chains as well as that of EpoR.

Although the ScFv–receptor chimera would be most effective when used with strictly factor-dependent cells such as Ba/F3, recently we also succeeded in AMEGA of non-strictly IL-6-dependent hybridoma by employing continuous subculture in the presence of BSA–Fl without IL-6 (M.K., manuscript in preparation). Further modification of selection conditions to either the selection in serum limitation or gradual adaptation to serum-free medium may enable amplification of completely factor-independent cells. Also, choosing the most appropriate signaling domains for the target cell would realize AMEGA of many other cell types with various signaling properties. In case the optimal receptor conformation for the particular cell is different from the one observed here, in our system the optimal conformation could be attained by fine-tuning of the inter-hapten distance.

While we adopted retroviral infection with relatively high-transduction efficiency as a gene delivery method, it is not absolute necessity for AMEGA. Indeed, direct electroporation of pMX-ScFvgIGFP vector with as low transfection efficiency as $\sim 10^{-5}$ per cell also resulted in almost 100% EGFP-positive cells after 15-day selection with BSA–Fl (data not shown). Therefore, our growth-based selection works equally well to, or even better than other selection methods like panning or sorting, in the point that a small fraction of transfectant population can be easily recovered and amplified. However, while the transfectant was successfully amplified from a single cell with BSA–Fl, cell cloning with Fl dimer was found to be difficult at this moment (data not shown), indicating that the growth signal generated by current Fl dimers is not strong enough to counteract the strong selective pressure in such a limiting-diluted condition. Considering the power of BSA–Fl, future development of more potent Fl dimer or oligomers, such as covalently linked multimers that elicit a much stronger growth signal, is thought to be promising, since such molecules are small and potentially non-immunogenic when used in vivo in future.

We have focused on the antigen–antibody system because of its virtually unlimited combinations. Although Fl was chosen in this study, use of other hapten/ScFv pairs will further enhance the merit of this approach. Almost all other artificial dimerization systems reported to date started the receptor design based on the natural receptor or its mutant, followed by a rational screening of the artificial ligand [5–8]. Here we proposed a reverse approach to start with an appropriate ligand followed by rational screening of its cognate binding domain. Since many cellular processes are regulated through dimerization/oligomerization of protein

domains, further extension of our approach by the selection of appropriate hapten–antibody pairs and fusion with many other cellular signaling domains will not be difficult. Eventually, combinations of these artificial switches will realize very precise mimicry of complicated cellular events without any adverse effects.

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