Growth control of hybridoma cells with an artificially induced EpoR-gp130 heterodimer

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Abstract IL-6 has been known to modulate the growth of many hybridoma cells and also promote resultant antibody productivity. However, IL-6 is so expensive that the use of IL-6-dependent hybridomas for industrial antibody production is not practical. In this study, we aimed at designing antibody/gp130 and antibody/EpoR chimeras which could tightly control cell growth in response to more affordable cognate antigen. Retroviral vectors encoding V_H or V_L region of anti-hen egg lysozyme (HEL) antibody HyHEL-10 tethered to a pair of extracellular D2/transmembrane domains of erythropoietin receptor (EpoR) and cytoplasmic domains of either EpoR or gp130, were constructed, and a homodimeric or a heterodimeric pair of chimeric

receptor combinations (V_H-gp130 and V_L-gp130 or V_H-gp130 and V_L-EpoR) were expressed in an IL-6-dependent hybridoma 7TD1. The chimeric receptor-derived growth signal was observed in both combinations, while some residual growth signal was observed in the absence of HEL. To reduce interchain interaction between the two receptor chains, we introduced mutations to the transmembrane domain of both chimera combinations. Consequently, the heterodimeric combination of V_H-gp130 and V_L-EpoR showed clear HEL-dependent cell growth, while the homodimeric combination of V_H-gp130 and V_L-gp130 showed reduced cell growth in the absence of HEL. This is the first report that an EpoR-gp130 cytoplasmic domain heterodimer could transduce a growth signal in hybridoma cells, indicating tight and economical growth control of hybridoma cells via our chimeric receptors.

Keywords Antibody · Chimeric receptor · Erythropoietin · Growth control · Hybridoma · Interleukin-6

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Introduction

Monoclonal antibodies (MAbs) are widely used for a range of biological assays and clinical

therapies (Chapman et al. 1999). The production of large amounts of MAbs at low cost is required for industrial application (Fussenegger et al. 1999). Today, MAbs are predominantly produced by hybridoma cell culture, which needs to be supplemented with cytokines and growth factors for controlling cell growth (Doverskog et al. 1997). One of the key cytokines is IL-6, which was originally identified as B-cell stimulatory factor-2 and was shown to affect hybridoma growth and also stimulate antibody productivity (Makishima et al. 1992). In the proposed mechanism, IL-6 binds to IL-6R and gp130 (Bravo et al. 1998; Hibi et al. 1990; Muller-Newen et al. 2000) to form hexameric complex composed of two molecules of each component (Paonessa et al. 1995; Skiniotis et al. 2005; Ward et al. 1996). This in turn triggers homodimerization and activation of gp130, leading to activation of the Janus kinase family (JAK1, JAK2, TYK2) of signal transducers, which further activate transcription activators (STAT1, STAT3) and mitogen-activated protein kinases (MAP-KKK, MAPKK, MAPK) (Matsuda et al. 1999).

To control the hybridoma cell growth with an inexpensive substance instead of expensive cytokines, we designed several antibody/cytokine receptor chimeras that transduced a growth signal in response to a cognate antigen. V_H or V_L region of anti-hen egg lysozyme (HEL) antibody HyHEL-10 tethered to a pair of extracellular D2 domains of erythropoietin receptor (EpoR) and transmembrane/cytoplasmic domains of either EpoR or gp130, induced a HEL-dependent growth signal in IL-3-dependent pro-B cell line Ba/F3 (Ueda et al. 2000) or IL-6-dependent hybridoma cell line 7TD1 (Kawahara et al. 2001a), respectively. In addition, both types of chimeric receptors could be employed to specifically amplify gene-transduced cells in an antigen-dependent manner in pro-B cell line Ba/F3 (antigen-mediated genetically modified cell amplification, AMEGA) (Kawahara et al. 2003). However, in the case of 7TD1 cells, we detected HEL-dependent cell growth only after drug-resistance selection, and the possibility of direct HEL selection (AMEGA) was not pursued (Kawahara et al. 2001a). Furthermore, in the previous study for 7TD1 cells, we tried only one combination of chimera (V_H -gp130 and

V_L -gp130), while we also found that heterodimeric combination (V_H -gp130 and V_L -EpoR) exhibited the best properties for cell growth in Ba/F3 cells transfected with plasmids (Kawahara et al. 2001b) or transduced with retroviral vectors (Kawahara et al. 2003). These reports indicate that combination of chimeric receptors affects signal transduction properties, and that efficient cell growth is attained by the heterodimeric combination (V_H -gp130 and V_L -EpoR), which transduces both gp130- and EpoR-derived signals with strict on/off regulation in response to HEL. Therefore, we speculated that the chimeric receptors could be applied to more efficient antibody production in hybridoma in industrial applications, where the strict control of cell growth is desirable in both growth and production phases.

In this study, we aimed to investigate whether we could bypass the drug-resistance selection in hybridoma 7TD1 cells, and whether the heterodimeric combination (V_H -gp130 and V_L -EpoR) could transduce a growth signal in 7TD1 cells. We employed retroviral vector constructs encoding a pair of chimeric receptors (either V_H -gp130 and V_L -gp130, or V_H -gp130 and V_L -EpoR) to attain direct HEL selection without drug-resistance selection. We also examined which combination of chimeric receptors could be better for AMEGA with strict on/off regulation of cell growth with/without HEL.

Materials and methods

Vector construction

Strategy of vector construction is shown in Fig. 1. The gene constructs for V_H -gp130 (Hg), V_L -gp130 (Lg) and V_L -EpoR (LE), were as described (Kawahara et al. 2003; Kawahara et al. 2001b). A plasmid FLJ00153 encoding human EpoR was obtained from Kazusa DNA Research Institute. The intracellular domain of human EpoR was amplified with PCR using a set of primers (hEpoRi forward: 5'-CCCAAGCTTCGTACGTCCCACC GCCGGGCTCTGAAG-3' and hEpoRi reverse: 5'-CCCGGATCCATCGATCTAAGAGCAAG CCACATAGCTG-3') and FLJ00153 as a template. The amplified fragment was digested

Fig. 1 Strategy of vector construction. The use of HgLE chimeric receptor is depicted in the scheme as a representative. **(A)** EpoR transmembrane (TM) domain, either with or without mutation, was linked with EpoR intracellular (i) domain, followed by ligation with EpoRD2 domain. The resultant EpoRD2-TM-i was inserted after V_L with a signal sequence (S) to create

IRES-V_L-EpoRD2-TM-i-IRES. **(B)** TM domain of V_H-gp130 was replaced by EpoR TM either with or without mutation, and IRES-EGFP cassette was incorporated, resulting in V_H-EpoRD2-TM-gp130i-IRES-EGFP. **(C)** IRES-V_L-EpoRD2-TM-i-IRES was inserted into IRES site in V_H-EpoRD2-TM-gp130i-IRES-EGFP, leading to the final construct

with *Hind*III (underlined) and *Bam*HI (double underlined), and inserted into pBluescript II SK- (Stratagene, La Jolla, CA) digested with the same restriction enzymes to create pBS-Ei. pBS-Ei was digested with *Bsi*WI and *Bam*HI, and inserted into pBS-EmTM (Kawahara et al. 2004b) digested with the same restriction enzymes to produce pBS-EmE. To get the transmembrane and intracellular domains, pBS-EmE was digested with *Eco*RV and *Bam*HI, and inserted into pBS-ED2-IG (Kawahara et al. 2004b) digested with the same restriction enzymes to produce pBS-ED2-EmE-IG.

pMK-Hg (Kawahara et al. 2003) was digested with *Eco*RI, and the fragment encoding Hg was inserted into *Eco*RI-digested retroviral vector pMK (Kawahara et al. 2004b) to make pMK-Hg. pBS-ED2-EmE-IG (Kawahara et al. 2004b) was digested with *Bsp*EI and *Not*I, and the inserted fragment encoding ED2-EmE-IG was ligated with pMK-Hg digested with the same restriction enzymes to produce pMK-Hgm-IG. pBS-ED2-EmE-IG and pBS-ED2-EmE-IG were digested with *Bsp*EI and *Not*I, and the inserted fragments were ligated with pBS-ILgIGFP (Kaneko et al. 2004) digested with the same restriction enzymes to generate pBS-ILEm-IG and pBS-ILgm-IG, respectively. These vectors

were digested with *Bln*I, and ligated into *Bln*I-digested pMK-Hgm-IG to produce pMK-Hgm-ILEm-IG and pMK-Hgm-ILgm-IG, respectively.

The control constructs with wild-type EpoR TM (pMK-Hg-ILE-IG and pMK-Hg-ILg-IG) were created by the same procedure as described above except using pBS-ETM (Kawahara et al. 2004b) instead of pBS-EmTM.

Cell culture

A murine IL-6-dependent hybridoma cell line, 7TD1 (Van Snick et al. 1986), was cultured in RPMI 1640 medium (Nissui Pharma, Tokyo, Japan) supplemented with 10% FBS (Iwaki, Tokyo, Japan) and 2 ng/ml of murine IL-6 (Genzyme/Techne, Cambridge, MA). A retroviral packaging cell line, Plat-E (Morita et al. 2000), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 µg/ml puromycin (Sigma, St. Louis, MO) and 10 µg/ml blasticidin (Kaken Pharmaceutical, Tokyo, Japan).

Transfection and selection

Plat-E cells were inoculated into a 60 mm diameter dish at 5×10^5 cells/ml in 4 ml DMEM

containing 10% FBS, and cultured for 20 h. After dilution of 9 μ l Fugene6 (Roche Diagnostics, Basel, Switzerland) in 100 μ l of serum-free DMEM, the solution was added to 3 μ g of each vector dissolved in 5 μ l of sterile water. After 15 min incubation at room temperature, the vector-Fugene6 mixture was added to the Plat-E cells. After 24 h incubation at 37°C, the culture medium was refreshed with 3 ml DMEM containing 10% FBS, followed by 24 h incubation at 37°C. After recovering the viral supernatant by centrifugation at 1,000g for 5 min at 20°C, 7TD1 cells (1×10^5 cells) were infected with 500 μ l of the viral supernatant in the presence of 10 μ g/ml polybrene (Sigma) and 2 ng/ml IL-6 in a 24-well plate. After 5 h incubation at 37°C, 500 μ l RPMI1640 containing 10% FBS was added to each well to reduce the toxicity of polybrene. After incubation for 4 days at 37°C, the cells were washed twice with 10 ml PBS, and inoculated into 24-well plates at 1×10^4 cells/ml (1 ml/well). Selection was performed in the medium containing no factor, 1 μ g/ml HEL (Seikagaku Corporation, Tokyo, Japan), or 2 ng/ml IL-6 with periodical passages.

Western blotting

The cells (10^6 cells) were washed with PBS, lysed with 100 μ l of lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, pH 7.5) and incubated on ice for 10 min. After centrifugation at 16,000g 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 1:1000 diluted rabbit anti-mouse EpoR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-mouse gp130 antibody (Santa Cruz Biotechnology) followed by 1:1000 diluted HRP-conjugated anti-rabbit IgG (Biosource, Camarillo, CA), and detection was performed using ECL system (Amersham-Pharmacia).

Flow cytometric analysis

Cells were washed once and resuspended in 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.

Cell growth assay

The HEL-selected cells were washed twice with PBS and seeded in 24-well plates containing various concentrations of HEL. The initial cell concentration was adjusted to 10^4 cells/ml. Viable cell concentrations were determined using a hemocytometer and the trypan blue exclusion assay.

Results and discussion

AMEGA detection by flow cytometric analysis

We first designed an assay scheme to compare the signal intensities from chimeric receptors based on a flow cytometric analysis (Fig. 2). The expression vectors were constructed by inter-connecting chimeric receptors and EGFP with IRES sequences for the co-expression, followed by the retroviral transduction to a non-strictly IL-6-dependent hybridoma 7TD1. Since the hybridoma exhibited some residual cell growth even in an IL-6-deficient medium, an increase in the transduced cell population was expected to be observed only if the chimeric receptors gave additional growth advantage. Since the three cistrons placed upstream and downstream of the IRES sequence are expected to be efficiently coexpressed (Kaneko et al. 2004; Persons et al. 1997; Sugimoto et al. 1995), the speed of EGFP-positive cell amplification is expected to correlate with the signaling intensity from the chimeric receptors.

Since the IL-6 signaling was successfully mimicked by the combination of V_H-gp130 (Hg) and V_L-gp130 (Lg) chimeric receptor chains in our previous studies (Kawahara et al. 2001a; Kawahara et al. 2003; Kawahara et al. 2001b), 7TD1 cells were

Fig. 2 Scheme of antigen-mediated genetically modified cell amplification assay. The use of HgLE chimeric receptor is depicted in the scheme as a representative. IRES-EGFP cassette was located downstream of a chimeric receptor gene with an immunoglobulin heavy chain signal sequence (S) to facilitate scoring of genetically modified cells. Retroviral transduction of the vector into non-strictly IL-6-dependent 7TD1 cells resulted in some transduced cell populations, followed by the selections either without any additional factors (-), with HEL or with IL-6. The growth signal generated by the chimeric receptor confers growth advantage on genetically modified cells, leading to the increase of EGFP-positive cell ratio in a time-dependent manner. The growth signal intensity generated by the chimeric receptor is expected to correlate with the increasing rate of EGFP-positive cell amplification, which can be detected by occasional flow cytometric analyses

retrovirally transduced with the vector encoding the Hg and Lg chimeras with a wild-type EpoR TM sequence (pMK-Hg-ILg-IG) to create 7TD/HgLE cells. The cells were washed to remove IL-6 and selected in the medium containing no additional factor, 1 μ g/ml HEL or 2 ng/ml IL-6. According to flow cytometric analyses, the transduced cells showed similar time-dependent increase in EGFP-positive cell ratio irrespective of HEL addition (Fig. 3A), while addition of IL-6 did not amplify EGFP-positive cells (data not shown). This result indicates that HgLE chimeras showed a significant background growth signal without antigen in spite of successful replacement of drug-resistance selection.

Fig. 3 The time course of EGFP-positive cell amplification when 7TD1 cells were transduced with chimeras bearing wild-type EpoR TM domain. Gene-transduced cells (1×10^4 cells/ml) were washed with PBS twice and directly selected in 24-well plates with or without 1 μ g/ml HEL. At each time point, viable cells (10^4) were analyzed with flow cytometry. EGFP-negative and positive regions were determined by taking parental 7TD1 cells as a negative control. The average of the positive cell ratios in duplicates was plotted. **(A)** 7TD/HgLE cells, **(B)** 7TD/HgLE cells

To investigate whether other combinations of chimeras could attain tighter growth control in the hybridoma, we tried the gp130-EpoR heterodimeric combination, which had transduced an efficient growth signal in mouse pro-B cell line Ba/F3 (Kawahara et al. 2004a; Kawahara et al. 2003; Kawahara et al. 2001b). 7TD1 cells were retrovirally transduced with the co-expression vectors for V_H-gp130 (Hg) and V_L-EpoR (LE) with a wild-type EpoR TM (pMK-Hg-ILE-IG) to yield 7TD/HgLE cells. Again, 7TD/HgLE cells showed efficient EGFP-positive cell amplification even in the absence of HEL (Fig. 3B), while addition of IL-6 did not amplify EGFP-positive cells (data not shown). Although

this result suggests that HgLE combination also transduces a growth signal in 7TD1 cells, further engineering of the chimeric receptors would be required to attain stricter antigen-dependent cell amplification.

A previous report revealed that simultaneous two mutations (L241G and L242P) to introduce a kink into the leucine zipper motif in the transmembrane domain resulted in a marked decrease of interchain interaction of EpoR, thereby leading to decrease of the growth signal intensity (Kubatzky et al. 2001). Therefore, we tried to engineer HgLg and HgLE chimeras by introducing the mutations in their TM domains. 7TD1 cells were retrovirally transduced with the vectors encoding the chimeric receptors with a mutant EpoR TM domain (pMK-Hgm-ILgm-IG and pMK-Hgm-ILEm-IG) to create 7TD/HgmLgm and 7TD/HgmLEm cells, respectively. Flow cytometric analysis revealed that HgmLgm chimeras still showed significant background growth advantage in the absence of the ligand (Fig. 4A). However, it is worth noting that 7TD/HgmLgm cells exhibited slower increase in EGFP-positive cell ratio than 7TD/HgLg cells, suggesting that the mutations did lower the growth signal intensity. On the other hand, 7TD/HgmLEm cells exhibited significant HEL-dependent amplification of EGFP-positive cells (Fig. 4B), with much slower amplification due to background growth in the absence of HEL. Again, addition of IL-6 did not amplify EGFP-positive cells (data not shown), and the TM mutation lowered overall growth signal intensity. These results indicate that the TM mutation for the HgLE combination was indeed effective to attain stricter antigen-dependent genetically modified cell amplification.

HEL-dependent cell growth of 7TD/HgmLEm cells

To investigate whether the amplification of EGFP-positive cells surely corresponds to the growth signal intensity, cell growth assay was performed. The cells after HEL selection were washed and cultured in various concentrations of HEL, followed by measurement of viable cell concentrations and viability on day 6. As expected from flow cytometric analyses, 7TD/HgLg and 7TD/HgLE

Fig. 4 The time course of EGFP-positive cell amplification when transduced with chimeras bearing mutant EpoR TM domain. Gene-transduced cells (1×10^4 cells/ml) were washed with PBS twice and directly selected in 24-well plates with or without 1 μ g/ml HEL. At each time point, viable cells (10^4) were analyzed with flow cytometry. EGFP-negative and positive regions were determined by taking parental 7TD1 cells as a negative control. The average of the positive cell ratios in duplicates was plotted. (A) 7TD/HgmLgm cells, (B) 7TD/HgmLEm cells

cells showed strong background cell growth without HEL (Fig. 5). 7TD/HgmLgm also showed a background growth signal, although the signal intensity was lowered (Fig. 6). On the other hand, 7TD/HgmLEm cells exhibited a clearer HEL-dependent cell growth with a marked reduction in background growth compared to other transduced cells (Fig. 6). These results demonstrate that HgmLEm chimeric receptor was the best among engineered receptors this time to yield a stricter growth switch.

Comparison of chimeric receptor expression levels

To compare the expression levels of the chimeric receptors, Western blot analysis was performed.

Fig. 5 Cell growth properties of 7TD1, 7TD/HgLg and 7TD/HgLE cells. Cells (10^4 cells/ml) were inoculated into 24-well plates at day 0, and cultured in the presence of several HEL concentrations. Viable cell concentration (**A**) and viability (**B**) on day 6 in triplicates are plotted with average and 1 SD

The observed expression levels after selection with (and without) HEL were almost comparable between the wild-type and TM-mutants in both HgLg and HgLE chimeras (Fig. 7). No expression (i.e. amplification) of the chimeric receptor chains were observed for the cells selected with IL-6.

Fig. 6 Cell growth properties of 7TD1, 7TD/HgmLgm and 7TD/HgmLEm cells. Cells (10^4 cells/ml) were inoculated into 24-well plates at day 0, and cultured in the presence of several HEL concentrations. Viable cell concentration (**A**) and viability (**B**) on day 6 in triplicates are plotted with average and 1 SD

The results indicate that the reduction in the amplification efficiency of the TM-mutated chimeras was not due to the reduction in the expression level of the receptors, but probably due to the decreased interchain interaction of the chimeric receptor chains.

Fig. 7 Comparison of the expression levels of the chimeric receptors. The cell lysates after selection were subjected to Western blot analysis using anti-gp130 or anti-EpoR C-terminus antibody. The bands for wild-type gp130 were indicated as a loading control. Parental 7TD1 cell lysate was used as a negative control

In this study, we demonstrated antigen-mediated genetically modified cell amplification (AMEGA) without drug-resistance selection in 7TD1 cells. This is the first report of EpoR-gp130 cytoplasmic domain heterodimer combination as functional receptors in IL-6-dependent hybridoma. While the HgLg chimeric receptor combinations did not show HEL-dependent enrichment of transduced cell population irrespective of the TM mutation, a clear HEL-dependent AMEGA was observed in the HgLE combination with TM mutation (HgmLEm). This may be because of the difference in the antigen-dependent conformational change, where preformed HgmLEm dimer in the absence of HEL

was a less competent signal transducer than HgmLgm dimer.

Although HgmLEm transduced a stricter antigen-dependent growth signal, a few concerns remain to be resolved: the residual growth signal in the absence of HEL and weak signal intensity and relatively low viability (around 60%) even in the presence of HEL, as suggested from EGFP positive cell amplification assay and from growth assay. Further molecular design of the chimeric receptors including the engineering of extracellular domain or the use of other intracellular domain combinations could realize more precise mimicry of natural receptors with higher dynamic range in signaling. Since the growth control of hybridoma cells is proven effective for increasing antibody productivity, our AMEGA system with stricter on/off regulation could be a new tool for industrial antibody production.

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