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Top-down motif engineering of a cytokine receptor for directing ex vivo expansion of hematopoietic stem cells

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ABSTRACT

The technique to expand hematopoietic stem cells (HSCs) ex vivo is eagerly anticipated to secure an enough amount of HSCs for clinical applications. Previously we developed a scFv-thrombopoietin receptor (c-Mpl) chimera, named S-Mpl, which can transduce a proliferation signal in HSCs in response to a cognate antigen. However, a remaining concern of the S-Mpl chimera may be the magnitude of the cellular expansion level driven by this molecule, which was significantly less than that mediated by endogenous wild-type c-Mpl. In this study, we engineered a tyrosine motif located in the intracellular domain of S-Mpl based on a top-down approach in order to change the signaling properties of the chimera. The truncated mutant (trunc.) and an amino-acid substitution mutant (Q to L) of S-Mpl were constructed to investigate the ability of these mutants to expand HSCs. The result showed that the truncated and Q to L mutants gave higher and considerably lower number of the cells than unmodified S-Mpl, respectively. The proliferation level through the truncated mutant was even higher than that of non-transduced HSCs with the stimulation of a native cytokine, thrombopoietin. Moreover, we analyzed the signaling properties of the S-Mpl mutants in detail using a pro-B cell line Ba/F3. The data indicated that the STAT3 and STAT5 activation levels through the truncated mutant increased, whereas activation of the Q to L mutant was inhibited by a negative regulator of intracellular signaling, SHP-1. This is the first demonstration that a non-natural artificial mutant of a cytokine receptor is effective for ex vivo expansion of hematopoietic cells compared with a native cytokine receptor.

1. Introduction

Since blood plays essential roles in maintaining homeostasis of our body, various blood disorders including myeloma, leukemia and anemia are life-threatening. Today, hematopoietic stem cell (HSC) transplantation is an effective treatment for these diseases. However, HSCs are rare population of blood cells, representing less than 0.01% of the bone marrow cells. Therefore, the technique to expand HSCs ex vivo is eagerly anticipated to secure an enough amount of HSCs for clinical applications.

The HSC expansion has been regulated by intrinsic and extrinsic regulators, such as cytokines and small molecules (Walasek et al., 2012). Cytokines, for example, thrombopoietin (TPO) (Ohmizono et al., 1997; Piacibello et al., 1998) and stem cell factor (SCF) (Abboud et al., 1994), together with the corresponding receptors, c-Mpl and c-Kit, respectively, are important for the maintenance of HSCs. Signal transduction from these receptors are initiated by binding of the cognate ligand, which triggers receptor oligomerization. Consequently, the intracellular kinase domain of receptors or Janus kinase (JAK), a tyrosine kinase which constitutively associates with the receptor, is activated. Accordingly, the activated kinase phosphorylates tyrosine residues located in the intracellular domain of the receptor. Signaling molecules bind to the phosphorylated tyrosine residues, and are phosphorylated by the kinase. Phosphorylated signaling molecules dissociate from the receptor, resulting in signal transduction to intracellular second messengers (Ihle, 1995; Kaushansky, 2005). The amino acid sequence surrounding the tyrosine residue determines whether a signaling molecule can bind to the tyrosine residue (Songyang et al., 1993).

Therefore, activation of these intracellular signaling molecules is crucial for the control of cellular fates through c-Mpl and c-Kit signalings. In fact, the PTEN/PI3K/Akt signaling (Datta et al., 1997; Rossi and Weissman, 2006; Salmena et al., 2008), which is activated through both TPO and SCF signalings, are known as the key regulators for ex vivo expansion and maintenance of HSCs. However, the natural cytokine-mediated expansion methods generate only

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moderate increases in progenitor cell numbers with at best modest improvements in clinically relevant outcomes (Dahlberg et al., 2011). The robust ex vivo expansion techniques that are useful for clinical applications have yet to be accomplished.

To overcome these problems, we previously developed scFv-receptor chimeras that can transduce a signal in response to a cognate inexpensive antigen (Kaneko et al., 2012; Kawahara et al., 2011). An anti-fluorescein single-chain Fv (scFv) fused to the extracellular D2 domain of erythropoietin receptor (EpoR) was joined to the transmembrane and whole cytoplasmic domains of c-Mpl or c-Kit, resulting in the S-Mpl or S-Kit chimera, respectively. When the chimeras were expressed in interleukin (IL)-3-dependent pro-B cell line Ba/F3, genetically modified cells were selectively expanded in the presence of fluorescein-conjugated BSA (BSA-FL) as a specific antigen. Furthermore, highly purified mouse HSCs transduced with the retrovirus carrying the S-Mpl chimera gene proliferated in vitro in response to BSA-FL, and the cells retained in vivo long-term repopulating abilities (Kawahara et al., 2011). However, a remaining concern of the S-Mpl chimera may be the magnitude of the cellular expansion level driven by this molecule, which was significantly less than that mediated by endogenous wild-type c-Mpl.

In this study, to enhance the expansion levels of hematopoietic cells by S-Mpl, we engineered the intracellular domain in S-Mpl and changed the signaling properties of the chimera. Focusing on the tyrosine motif (YWQQ) located at the C-terminus of S-Mpl, we constructed the truncated mutant (trunc.) and the amino-acid substitution mutant (Q to L) of S-Mpl. We compared the ability of HSC proliferation among the original and the mutants of S-Mpl. Moreover, Ba/F3 cells were transduced with the retroviral vector carrying each chimeric receptor to examine the signaling properties of the receptors in detail by western blot.

2. Materials and methods

2.1. Plasmid construction

pBS-EMpl-IG, which encodes the extracellular D2 domain of erythropoietin receptor (EpoR), the cytoplasmic/transmembrane domains of c-Mpl and an IRES-EGFP cassette, was as described (Kawahara et al., 2011). To engineer the intracellular domain of c-Mpl, pBS-EMpl-IG was mutated by PCR using two primer sets (trunc sense: 5′-CCTACCACTAAGCTAGTGGCAGCAGCCTTG-3′, trunc antisense: 5′-CAAGGCTGCTGCCACTAGCTTAGTGGTAGG-3′ and Q to L sense: 5′-GCTATTGGCAGCTGCCTTGAGGATCCGCC-3′, Q to L antisense: 5′-GGCGGATCCTCAAGGCAGCTGCCAATAGC-3′), resulting in pBS-EMpl(trunc.)-IG and pBS-EMpl(Q to L)-IG, respectively. To utilize the GCDNsam retroviral expression system and to append a HA tag at the N-terminus of the chimeric receptors, pBS-EMpl-IG, pBS-EMpl(trunc.)-IG and pBS-EMpl(Q to L)-IG were digested by *Bsp*EI and *Bam*HI, and the fragments were inserted into *Bsp*EI- and *Bam*HI-digested pGCDNsam-HA-SD2g-I/E (manuscript in preparation) to obtain pGCDNsam-HA-S-Mpl(WT)-I/E, pGCDNsam-HA-S-Mpl(trunc.)-I/E and pGCDNsam-HA-S-Mpl(Q to L)- I/E, respectively.

2.2. Animals and cell lines

C57BL/6-Ly5.1 mice were purchased from Japan SLC (Shizuoka, Japan). The Animal Experiment Committee of the Institute of Medical Science, The University of Tokyo, approved all animal care and use in this study.

A murine IL-3-dependent pro-B cell line, Ba/F3 (RCB0805, RIKEN Cell Bank, Tsukuba, Japan), was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Biowest, Paris, France) and 1 ng/ml murine IL-3 (R&D systems, Cambridge, MA). Three retroviral packaging cell line were used; Plat-E (Morita et al., 2000) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical) supplemented with 10% FBS, 1 μg/ml puromycin (Sigma, St Louis, MO) and 10 μg/ml blasticidin (Kaken Pharmaceutical, Tokyo, Japan); 293GP (Burns et al., 1993) was cultured in DMEM supplemented with 10% FBS, and 293GPG (Ory et al., 1996) was cultured in DMEM supplemented with 10% FBS, 2 μg/ml puromycin, 300 μg/ml G418 (Calbiochem, Darmstadt, Germany) and 1 μg/ml tetracycline (Sigma).

2.3. Purification of murine CD34⁻KSL HSCs

$CD34^{-/low}c\text{-}Kit^{+}Sca\text{-}1^{+}Lin^{-}$ ($CD34^{-}$KSL) HSCs were purified from bone marrow of C57BL/6-Ly5.1 mice, as previously described (Osawa et al., 1996). Briefly, bone marrow cells were stained with a lineage antibody mixture consisting of anti-Gr-1, Mac-1, B220, CD4, CD8, IL-7R and Ter-119 monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ), and magnetic beads-conjugated anti-rat IgG secondary antibody (Miltenyi, Auburn, CA), followed by depletion of lineage-positive cells using magnetic cell sorting. The cells were subsequently stained with phycoerythrin (PE)-conjugated anti–Sca-1, allophycocyanin (APC)-conjugated anti–c-Kit, fluorescein isothiocyanate (FITC)-conjugated anti-CD34 and biotin-conjugated lineage antibodies (all from BD Biosciences) and APC-Cy7-conjugated streptavidin (Molecular Probes, Eugene, OR). $CD34^{-}$KSL HSCs were sorted at 400 cells/well into a 96-well plate containing α-MEM with 1% FBS, 100 ng/ml TPO and 100 ng/ml stem cell factor (SCF) (Peprotech, Rocky Hill, NJ) using a MoFlo Cell Sorter (Beckman Coulter, Fullerton, CA).

2.4. Vector transduction

Plat-E cells were transfected with the constructed plasmids using Lipofectamine LTX (Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol. The culture supernatant on day 2 was used for retroviral transduction of Ba/F3 cells in the presence of 1 ng/ml IL-3 in a 24-well plate, using RetroNectin (Takara Bio, Otsu, Shiga, Japan) according to the manufacturer's instructions.

For the transduction of mouse $CD34^{-}$KSL HSCs, a stable virus producer cell line based on 293GPG cells was established. A retroviral packaging cell line 293GP was co-transfected with pGCDNsam variants encoding the chimeric receptors and pcDNA3.1-VSV-G encoding a VSV-G envelope gene by lipofection for a transient production of VSV-G pseudotyped retroviruses. The culture medium of the transfected 293GP was collected and subsequently used for transduction of 293GPG, which had been engineered to express the VSV-G protein under control of a tetracycline-inducible system (Ory et al., 1996). 293GPG cells transduced with GCDNsam-HA-S-Mpl-I/E, GCDNsam-HA-S-Mpl(trunc.)-I/E, GCDNsam-HA-S-Mpl(Q to L)-I/E or GCDNsam-I/E stably produced retroviruses encoding the respective genes. The culture supernatant of the transduced 293GPG was collected and centrifuged at 6000 × *g* for 16 h at 4 °C, followed by resuspension of the viral pellet in α-MEM to obtain a 100-fold concentrated virus. $CD34^{-}$KSL HSCs cultured overnight after sorting were transduced with the virus at multiplicity of infection (MOI) of 500 in the presence of SCF and TPO (50 ng/ml each) in a 96-well plate, using RetroNectin.

2.5. In vitro growth assay of HSCs

The medium of the transduced $CD34^{-}$KSL HSCs was exchanged into S-clone SF-03 (Sanko Junyaku Inc., Tokyo, Japan) containing 50 ng/ml SCF and 50 ng/ml TPO on the day after transduction, and the cells were cultured for 2 days. The cells were washed with