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Erythroblast differentiation at spleen in Q137E mutant ribosomal protein S19 gene knock-in C57BL/6J mice

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ABSTRACT

We recently found that erythroblast-like cells derived from human leukaemia K562 cells express C5a receptor (C5aR) and produce its antagonistic and agonistic ligand ribosomal protein S19 (RP S19) polymer, which is cross-linked between K122 and Q137 by tissue transglutaminases. RP S19 polymer binds to the reciprocal C5aRs on erythroblast-like cells and macrophage-like cells derived from human monocytic THP-1 cells and promotes differentiation into reticulocyte-like cells through enucleation *in vitro*. To examine the roles of RP S19 polymer in mouse erythropoiesis, we prepared *Q137E mutant RP S19* gene knock-in C57BL/6J mice. In contrast to wild-type mice, erythroblast numbers at the preliminary stage ($CD71^{high}/TER119^{low}$) in spleen based on transferrin receptor (CD71) and glycophorin A (TER119) values and erythrocyte numbers in orbital artery bloods were not largely changed in knock-in mice. Conversely, erythroblast numbers at the early stage ($CD71^{high}/TER119^{high}$) were significantly decreased in spleen by knock-in mice. The reduction of early erythroblast numbers in spleen was enhanced by the phenylhydrazine-induced pernicious anemia model knock-in mice and was rescued by a functional analogue of RP S19 dimer S-tagged C5a/RP S19. These data indicated that RP S19 polymer plays the roles in the early erythroblast differentiation of C57BL/6J mouse spleen.

1. Introduction

Blood cell linage-specific maintenance, proliferation, differentiation, and maturation in cell homeostatic system are partially regulated by a stage-specific balance between pro-apoptotic signals and anti-apoptotic signals through the direct pathways via receptors and the indirect pathways via the cell–cell interaction with stromal cells or macrophages in an autocrine/paracrine manner (Singh et al., 2012; Fontenay-Roupie et al., 1999). In the erythrocyte homeostatic system, erythroid burst-forming units (BFU-E) are maintained by a precursor stage-specific anti-apoptotic signal through an indirect pathway via cell–cell interaction with stromal cells (Arkin et al., 1991). Lymphocyte function antigen-1 (CD11a) on stromal cells binds to intracellular adhesion molecule-1 (CD54) on BFU-E and raises an anti-apoptotic extracellular signal-regulated kinase 1/2 (ERK1/2) signal. In addition, another erythroid precursor stage-specific anti-apoptotic signal through a direct pathway via the ribosomal protein S19 (RP S19) monomer-dependent ribosome formation is found in patients with an inherited erythroblastopenia, Diamond-Blackfan anemia (DBA) (Da Costa et al., 2013; Hamaguchi et al., 2003). Differentiation of BFU-E to erythroblasts through erythroid colony-forming units (CFU-E) is undergone due to the dissociation with stromal cells in a CD54 expression-dependent manner. Proliferation, differentiation, and maturation of erythroblasts are regulated by a stage-specific anti-apoptotic ERK1/2 signal through an indirect pathway via the cell–cell interaction with macrophages depending on 30-kD heparin-binding protein and/or integrin α4β1 (Hanspal and Hanspal, 1994; Spring et al., 2013; Kapur et al., 2001). In addition, there is another erythroblast stage-specific balance between the erythropoietin-mediated anti-apoptotic signal through Raf-1 activation and Fas-mediated pro-apoptotic signal through caspase activation in proliferation and differentiation (Liu et al., 2006; Rubiolo et al., 2006; Koulnis et al., 2011; Kumkhaek et al., 2013).

Erythroblast stages are sometimes classified by fluorescence activated cell sorting (FACS) analysis based on glycophorin A (TER119) and transferring receptor (CD71) values. We need further examinations

Abbreviations: $TER119^{high}/CD71^{high}$, basophilic-erythroblasts; $TER119^{high}/CD71^{middle}$, chromatophilic-erythroblasts to polychromatophilic-erythroblasts through orthochromatophilic-erythroblasts; C5aR, C5a receptor; DBA, Diamond-Blackfan anemia; ES, embryonic stem; BFU-E, erythroid burst-forming units; CFU-E, erythroid colony-forming units; ERK1/2, extracellular signal-regulated kinase 1/2; FACS, fluorescence activated cell sorting; GPCRs, G protein-coupled receptors; TER119, glycophorin A; CD54, intracellular adhesion molecule-1; hemin, iron-containing porphyrin; CD11a, lymphocyte function antigen-1; PHZ, phenylhydrazine; $TER119^{middle}/CD71^{high}$, pro-erythroblasts; RGS3, regulator of G protein signaling 3; $TER119^{high}/CD71^{low}$, reticulocytes; RP S19, ribosomal protein S19; CD71, transferrin receptor

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to understand about the erythroblast stage-specific balance between pro-apoptotic and anti-apoptotic signals in an autocrine/paracrine manner from the preliminary stage (pro-erythroblasts; TER119^{middle}/CD71^{high}) to the mature stage (reticulocytes; TER119^{high}/CD71^{low}) through the early stage (basophilic-erythroblasts; TER119^{high}/CD71^{high}) and the late stage (chromatophilic-erythroblasts to polychromatophilic-erythroblasts through orthochromatophilic-erythroblasts; TER119^{high}/CD71^{middle}) (Zhang et al., 2003).

Conversely, we have demonstrated that cell lifespan is partially regulated by a balance between survival ERK1/2 signals via constitutively active G protein-coupled receptors (GPCRs) and death signals via death receptors (Miura et al., 2005; Dittmer et al., 2008; Akazawa et al., 2009; Qin et al., 2011). An intensity of survival signals via constitutively active GPCRs is lower than the ligand-activated one. We are now interested in an environment-specific expression of GPCRs and their antagonistic or agonistic ligands. In a case of neutrophils including apoptotic cells, cells express G protein-coupled C5a receptor (C5aR) and its alternative ligand RP S19 polymer other than the natural ligand C5a (Nishiura et al., 2009). C5a arises survival ERK1/2 signal, resulting in a lengthen cell lifespan. In contrast to C5a, RP S19 polymer initiates a promoter of *regulator of G protein signaling 3 (RGS3)* gene through an activation of delta type-lactoferrin (Nishiura et al., 2015). The increment of GTPase activity of Gα subset causes a decrement of survival ERK1/2 signals via constitutively active GPCRs, resulting in a shorten cell lifespan. We are now very interested in acute inflammation, we suggested that C5a induces pro-inflammatory and/or anti-apoptotic signals at the acute inflammation stage, while RP S19 polymer oppositely induces anti-inflammatory and/or pro-apoptotic signals at the acute inflammation stage (Yamanegi et al., 2017).

We recently found that erythroblast-like cells derived from human erythroleukemia K562 cells (BFU-E-like cells) by iron-containing porphyrin (hemin) also expressed C5aR and produced RP S19 polymer (Nishiura et al., 2012). A sensitivity of K562 cells against the hemin-induced proliferation and differentiation to erythroblast-like cells was increased by overexpression of either RP S19 or RGS3 under a single cell culture condition, indicating a participation of pro-apoptotic signal through the direct pathway via C5aR. In addition, the sensitivity of K562 cells was more efficient by co-culture with macrophage-like cells derived from human monocytic THP-1 cells by phorbol-12-myristate-13-acetate. The sensitivity of K562 cells by the cell–cell interaction was suppressed by anti-RP S19 rabbit IgG or a C5aR antagonistic peptide PMX-53, indicating a participation of anti-apoptotic signal through an indirect pathway via cell–cell interaction with macrophages (Lappas et al., 2012) (Supplemental Fig. 1).

We have demonstrated that RP S19 monomer dissociated from ribosome under an apoptotic condition concentrates on phosphatidylserine and cross-links between K121 and Q137 by an activation of tissue type transglutaminases (Nishiura et al., 1999; Nishiura, 2013). K121 in one RP S19 molecule and Q137 in another RP S19 molecule cross-linked by an isopeptide bond makes an adequate distance in the polymer conformation to the distance between the first and second binding sites in C5aR (Nishimura et al., 2001; Nishiura et al., 2010). Therefore, Q137N mutant RP S19 polymers do not have any potencies to induce C5aR-mediated outputs. Mouse Q137 locates at the end of exon 5 of the *RP S19* genomic fragment in BAC#24 158H15 clone (BACPAC Resources Children's Hospital Oakland, Oakland, USA) (Supplemental Fig. 2). To examine the different roles of RP S19 polymer in erythropoiesis from those of RP S19 monomer, we here replaced original *RP S19* gene in C57BL/6J mice to Q(CAG)137E(GAG) mutant *RP S19* gene by homologous recombination (knock-in mice).

2. Materials and methods

2.1. Animals

Pathogen-free Crij:CD1 (ICR) and C57BL/6J mice (15–20 g body weight range) were purchased from Charies River (Yokohama, Japan). Mice were maintained in the Center for Animal Resources and Development, Kumamoto University under the control of the Ethical Committee for Animal Experiment, Kumamoto University School of Medicine (Grant number: B23-165). Mouse *RP S19* genomic fragment including exon 5 in BAC#24 158H15 clone cut by restriction enzyme PstI was inserted into pBS vector (Promega, Tokyo, Japan). Conversely, polymerase chain reaction (PCR) product of exon 5 was inserted into pGEM®-T Easy vector (Promega). CAG codon (Q137) was exchanged to GAG codon (E137) by PCR. Mutant exon 5 with a marker cassette (Lox71-SA-IRES-neomysin-pA-Lox71) was replaced to the wild-type exon 5 at *RP S19* genomic fragment in pBS vector (Araki et al., 1999). Mutant *RP S19* genomic fragment cut by restriction enzyme SacI was homogonously replaced to the same position in wild-type mouse *RP S19* gene of embryonic stem (ES) cells of TT2-KTPU8 F1 mice by electroporation. ES cells containing mutant *RP S19* gene in G418 medium were selected by PCR-southern blotting. Aggregated mutant RP S19 ES cells with germinal cells of ICR mice were implanted to endometrium of pseudopregnance male mice. ICR mice with 100% chimerism were mated with Cre C57BL/6J transgenic mice. We prepared tail DNAs to confirm a deletion of the marker cassette from the heterozygous mice with a deficient of the RP S19 polymer-dependent functions by PCR-southern blotting. Heterozygous mice back-crossed with wild-type C57BL/6J mice at 6 times were mated and homozygous mice were again confirmed by PCR-southern blotting (data not shown).

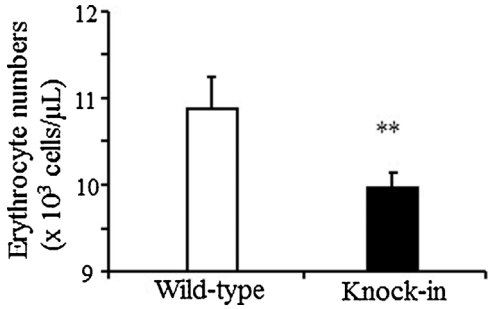

Fig. 1. Erythrocyte linage-specific defect in knock-in mice. Wild-type and knock-in mice (n = 20) were exsanguinated under Somnopentyl anaesthesia and erythrocyte numbers in orbital artery bloods were automatically measured by Siemens Advia 2120 Hematology Analyzer. P values of less than 0.05 were considered statistically significant (no difference: N.D., $P < 0.05$:* or $P < 0.01$:**).

The growth rate was not big difference between wild-type and knock-in mice at least until 12 weeks. We know that plasma RP S19 molecules are cross-linked by an activation of factor XIII during coagulation (Semba et al., 2010; Ota et al., 2011). RP S19 conserves a high homology among species, indicating a high affinity of mouse plasma RP S19 polymer to human C5aR. We here confirmed that wild-type mouse serum at least has a chemotactic potency to human monocytes/macrophages. The chemotactic potency is suppressed by either a neutralization of mouse RP S19 polymer with anti-human RP S19 rabbit IgG or a competitive inhibition of human C5aR on monocytes/macrophages with PMX-53 (data not shown). In our experimental conditions, knock-in mouse serum at least lost a chemotactic potency to human monocytes/macrophages (Chen et al., 2014). Moreover, we prepared side population cells by fluorescence activated cell sorting (FACS) with Hoechst33342 dye efflux assay and confirmed that phosphatidylserine was exposed on the cell surface of side population cells at 48 h after an induction of apoptosis with $MnCl_2$-loading (data not shown) (Nishiura et al., 2005). In our experimental conditions, the apoptosis-induced phosphatidylserine exposure was suppressed by knock-in mice (data not shown). From all above data, we suggested that Q137E RP S19 monomer functions as one of members in ribosome. Conversely, Q137E RP S19 polymer released from apoptotic cells in knock-in mice lost various potencies, such as monocyte chemotaxis, pro-apoptosis and so on.