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Full Length Research Paper

Quercetin 3-*O*-*β*-(2"-galloyl)-rhamnopyranoside promotes the differentiation of hematopoietic stem cells or early progenitor cells into erythroid lineage in mice

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Quercetin 3-*O*-*β*-(2"-galloyl)-rhamnopyranoside (QGR) is a polyphenolic compound originally isolated from *Persicaria lapathifolia* S. F. Gray (Polygonacease). In the present study, we examined the erythropoietic potential of QGR in mouse bone marrow mononuclear cells (BMNCs). BMNCs including hematopoietic stem cells (HSCs), early progenitor cells (EPCs), stromal cells and other precursor cells were isolated from femurs and tibias of 8 to 12-week-old male C57BL/6 mice. QGR significantly increased the cell viability of BMNCs even in hypoxic condition, and mRNA expressions of stem cell factor, granulocyte macrophage-colony stimulating factor and erythropoietin from bone marrow stromal cells. In a colonogenic assay, formation of burst-forming unit-erythroid colony was enhanced by QGR treatment. Immunocytochemistry and flow cytometry against TER-119, a surface marker of erythroid precursor cells revealed that QGR enhanced the number of positive cells. These results suggest that QGR has erythropoietic effect by activating the differentiation of HSCs or EPCs in early stages of hematopoiesis.

Key words: Quercetin 3-*O*-*β*-(2''-galloyl)-rhamnopyranoside, bone marrow mononuclear cells, bone marrow stromal cells, burst-forming unit-erythroid, erythropoiesis.

INTRODUCTION

Hematopoiesis is a process of differentiation of hematopoietic stem cells (HSCs) into the mature blood cells to maintain homeostasis in the body. This process occurs in bone marrow, a microenvironment that regulates self-renewal, proliferation and differentiation of HSCs (Allen and Dexter, 1984; Deans and Moseley, 2000; Gardner, 1999; Gardner et al., 2003; Slovak et al., 2010). The developmental pathways of hematopoiesis contain diverse lineages including erythropoiesis, granulopoiesis, lymphopoiesis and thrombopoiesis. Once a lineage is chosen in reaction to physiological needs, the lineage potentials of progenitors become increasingly restricted. The commitment of pluripotent HSCs into self-renewing lineage specific progenitor cells including common myeloid progenitor, megakaryocyte/erythroid progenitor, burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) is the early stage for erythropoiesis (Beauchemin et al., 2004; Chung et al., 2005; Goodnough and Marcus, 1998; Kostaridou et al., 2004). Erythropoiesis is regulated by several cytokines such as stem cell factor (SCF), thrombopoietin (Tpo), erythropoietin (Epo), granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-1, -3, -5, -6, -7, -11 and -13, which are secreted from bone marrow stromal cells (Brugger et al., 1993). These variable

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Table 1. The primers used in real-time RT-PCR.

Genes[a]	Sequences	NCBI ref. seq.
GAPDH	5′-TGCATCCTGCACCACCAACTGCTTAG-3′	NM_008084.2
SCF	5′-CATTACAAAACTGGTGGCAAATCTT-3′	NM_013598.2
GM-CSF	5′-GCCCCCCAACTCCGGAAACGGACTG-3′	NM_009969.4
Epo	5′-AGAAAATGTCACGATGGGTTGTGCA-3′	NM_007942.2

[a] Murine.

cytokines stimulate the survival, proliferation and differentiation of the HSCs (Banu et al., 1995; Guerriero et al., 1997; Kaushansky et al., 1995; Kaushansky et al., 1994). Signal transduction pathways such as Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway activated by these hematopoietic cytokines play essential roles for cytokine receptor-mediated intracellular signaling and hematopoietic cell development (Nosaka and Kitamura, 2000).

Quercetin 3-*O*-*β*-(2''-galloyl)-rhamnopyranoside (QGR) is a polyphenolic compound originally isolated from *Persicaria lapathifolia* S. F. Gray (Polygonacease). It inhibits superoxide production in unopsonized zymosan-stimulated human monocytes (Kim et al., 2000), nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells (Kim et al., 2005) and in LPS-induced Balb/c mice (Jo et al., 2008) and peroxynitrite production in zymosan A-stimulated RAW 264.7 (Kim et al., 2007). Also, *P. lapathifolia* S. F. Gray has traditionally been used for hemostasis. In the present study, we investigated the erythropoietic potential of QGR in mouse marrow cells.

MATERIALS AND METHODS

Reagents

For proliferation and differentiation of HSCs and early progenitor cells (EPCs), mouse recombinant IL-3 (rIL-3), recombinant SCF (rSCF) and recombinant Epo (rEpo) proteins were purchased from R&D Systems (Minneapolis, MN, USA). For immunocytochemistry and flow cytometry, biotinylated rat monoclonal anti-mouse TER-119 and phycoerythrin (PE)-conjugated monoclonal anti-TER-119 (R&D Systems) were used as first antibody. QGR (>98% pure) was isolated from aerial part of *P. lapathifolia* as described previously (Kim et al., 2000). QGR (586.45 kDa) was dissolved in dimethylsulfoxide to a final concentration of 100 mM as the stock solution and was diluted in culture medium before treatment.

Isolation of bone marrow mononuclear cells and stromal cells

Bone marrow mononuclear cells (BMNCs) were isolated from femurs and tibias of 8 to 12-week-old male C57BL/6 mice by a previously described protocol (Kroeger et al., 2009) and were separated from dead cells and red blood cells (RBCs) by Histopaque (1,077 g/ml) (Sigma-Aldrich Chemical, St. Louis, MO, USA) density gradient centrifugation. Thereafter, the cells were cultured in Iscove's modified Dulbecco's media (IMDM) with 15% heat inactivated fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_2 humidified air.

The separated blood was cultured as described previously (Oswald et al., 2004) to obtain bone marrow stromal cells in α-minimum essential medium (MEM) with 20% heat inactivated FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_2 humidified air.

Cell viability assay

The possible cytotoxicity of QGR was examined in BMNCs. The cells were plated with a density of 5×10^3 cells/well in 96 well plates in 200 µl of medium. The cells cultured for 24 h with variable concentrations of QGR in either normoxia or hypoxia conditions were evaluated using a cell counting-8 assay kit (CCK-8) according to the manufacturer's instructions (Dojindo Molecular Technologies, Rockville, MD, USA). Briefly, 20 µl of CCK-8 solution was added to each well. The plates were incubated for 2 h in an incubator at 37°C. The resulting color was assayed at 450 nm using an EL808 microplate absorbance reader (BIO-TEK, Winooski, VT, USA).

Real-time reverse transcription-polymerase chain reaction

To confirm whether QGR regulates the expression of hematopoietic cytokines in bone marrow stromal cells, real-time RT-PCR was performed. Bone marrow stromal cells (3×10^5 cells/plate) were incubated with either mouse rIL-3 (100 units/ml) plus rSCF (5 units/ml) plus rEpo (3 units/ml) or QGR (50, 100 or 200 µM/ml) in 60 mm-diameter cell culture plates. The cells were harvested after 4 h of incubation and washed with PBS and total RNA were extracted using RNA extraction kit (Applied Biosystems, Carlsbad, CA, USA). The concentration of total RNA was measured by spectrophotometer (Shimadzu Corporation, Tokyo, Japan) at 260 nm. First strand synthesis of cDNA was carried out with 1 µg of total RNA using cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR using TaqMan probe PCR master mix kit (Applied Biosystems) was performed according to the manufacturer's instruction using a model 7500 Real-Time PCR System (Applied Biosystems). The sequences of primers used are indicated in Table 1.

GAPDH mRNA was utilized as an internal standard (Assay on Demand # 4352932E, Applied Biosystems) to normalize target transcript expression. The relative ratios of SCF, GM-CSF, or Epo mRNA to GAPDH mRNA which can be used to quantify precisely the levels of each mRNA expression, were calculated with the standard curves.

Semisolid colonogenic assay

To investigate the effects of QGR on erythroid lineage development in mouse HSCs and EPCs, the colonogenic assay was performed according to the manufacturer's instruction in triplicate by plating 2 × 10^5 cells/plate using methylcellulose stock solution in IMDM (R&D Systems) with cocktail (rIL-3 100 units/ml plus rSCF 5 units/ml plus rEpo 3 units/ml) or/and QGR (0, 100 or 200 µM/ml) in 35 mm-diameter cell culture plates. These plates were put in 100 mm-diameter plates with 3 ml of autoclaved distilled water and incubated at 37°C in 5% CO_2 humidity air. 14 days later, BFU-E colonies were counted under an Axiovert 40 CFL inverted microscope (Carl Zeiss, Oberkochen, Germany).

Histochemistry and immunocytochemistry for erythroid cells

BMNCs were harvested 7 days after incubation with either rEpo (3 units/ml) or QGR (0, 100 or 200 µM/ml) washed with PBS, resuspended at a concentration of 2 × 10^5 cells/ml in PBS and subjected (100 µl/slide) to cytospin for 5 min at 1,000 rpm (Cytospin3; Shandon, ThermoElectron, Waltham, MA, USA). All slides were fixed using cold methanol. Wright-Giemsa stain was performed for morphologic analysis. Immunocytochemistry against TER-119 was carried out for cellular lineage with 100-fold dilution according to the manufacturer's instruction using avidin biotinylated enzyme complex kit (VECTASTAIN, Vector Laboratories and Burlingame, CA, USA).

Flow cytometric analysis for specific erythroid cells

To confirm the differentiation of mouse HSCs or EPCs into erythroid cells by QGR treatment flow cytometric analysis for mouse anti-TER-119 was performed. BMNCs were cultured with either rEpo (3 units/ml) or QGR (0, 100 or 200 µM/ml) in 6-well plates. Cells were harvested at day 7, washed with PBS, resuspended at concentration of 1 × 10^5 cells/ml in 100 µl of PBS containing 0.02% sodium azide and 2% FBS, incubated with mouse anti-CD16/CD32 (eBioScience, San Diego, CA, USA) 10 µl as FC receptor blocker for 3 min at room temperature and incubated with PE-conjugated anti-TER-119 for 20 min at 4°C. Flow cytometric analysis was performed using FACS Calibur (Becton Dickinson, Oxnard, CA, USA) and the percentage of positively stained cells was determined (Figure 6A).

Statistical analysis

Data are represented as mean ± S.D. of three independent replicates. Statistical comparisons were made using the Student-Newman-Keuls (SNK) *t*-test after one-way ANOVA using SAS 9.1 program.

RESULTS

QGR increases cell viability

As shown in Figure 1A, QGR was not cytotoxic and significantly increased cell viability at the concentration of 50 µM. Pretreatment of QGR increased the viable cells under hypoxic damage in a dose-dependent manner (Figure 1B). QGR treatment increased cell viability at the concentrations of 200 µM after hypoxic damage for 24 h (Figure 1C).

QGR enhances the expression of hematopoiesis-related cytokines

QGR increased the levels of mRNA expression of SCF, GM-CSF and Epo from bone marrow stromal cells in a dose-dependent manner (Figure 2). QGR significantly increased the mRNA expression of SCF at all concentrations especially 1.8-fold enhancement in 200 µM dose when compared to the positive control (p<0.01) (Figure 2A). GM-CSF and Epo were significantly increased at the concentrations of 100 and 200 µM QGR when compared to the positive control (Figures 2B and C).

QGR increases the formation and growth of BFU-E colony *in vitro*

As shown in Figure 3, the treatment of cocktail (rIL-3, rSCF and rEpo) and/or QGR to BMNCs significantly enhanced the number of BFU-E colonies in a dose-dependent manner compared with negative control (p<0.001). The level in treatment with 200 µM QGR was equal to the positive control (p = 0.606). In addition, the treatment of the cocktail plus 200 µM QGR significantly increased the formation and growth of BFU-E colonies 1.43-fold compared with the positive control (p<0.01).

QGR increases the number of erythroid precursor cells

As shown in Figure 4, enucleated erythroid cells increased in the positive control and QGR treatment groups. Immunocytochemical analysis for the TER-119 erythroid surface antigen showed that TER-119^+ cells increased in the QGR-treated group compared to the negative control (Figure 5). In flow cytometric analysis for TER-119, the ratio of TER-119^+ cells was significantly increased in the QGR treatment group dose-dependently when compared to the negative control. However, it did not exceed the level caused by Epo (positive control) even in QGR 200 µM treatment (Figure 6B).

DISCUSSION

Erythropoiesis is a process of commitment, proliferation and progressive differentiation of HSCs into mature RBCs. Although the triggering factor to commit the differentiation of HSCs into erythroid lineage is unknown, multipotent HSCs successively differentiate into common myeloid progenitor, megakaryocyte/erythroid progenitor and BFU-E. The BFU-Es are programmed to differentiate only to erythroid precursor cells such as proerythroblasts

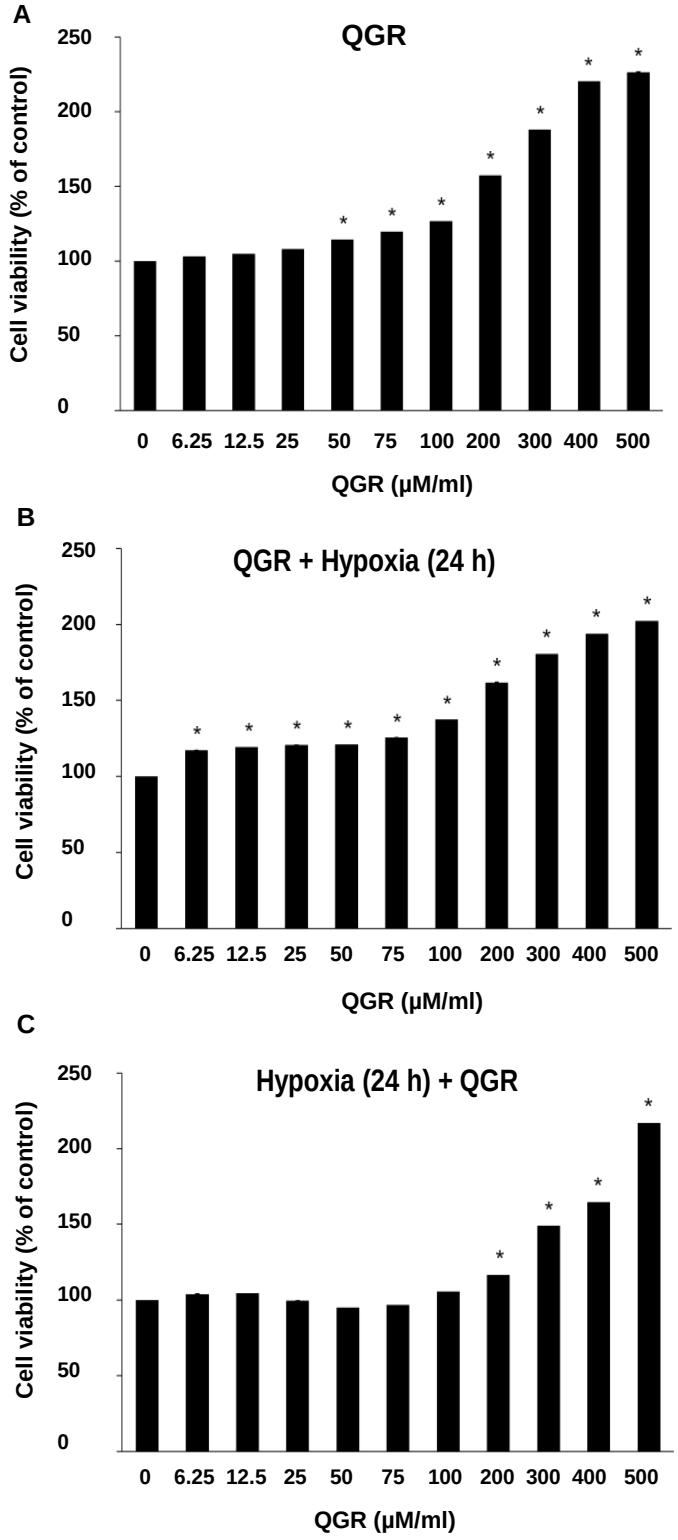

Figure 1. Effect of QGR on cell viability. Cells were treated with various concentration (μM/ml) of QGR for 24 h(A), exposed to hypoxic damage for 24 h after QGR treatment (B) or exposed to hypoxic damage for 24 h before QGR treatment (C). * Significantly different from negative control (p<0.01).

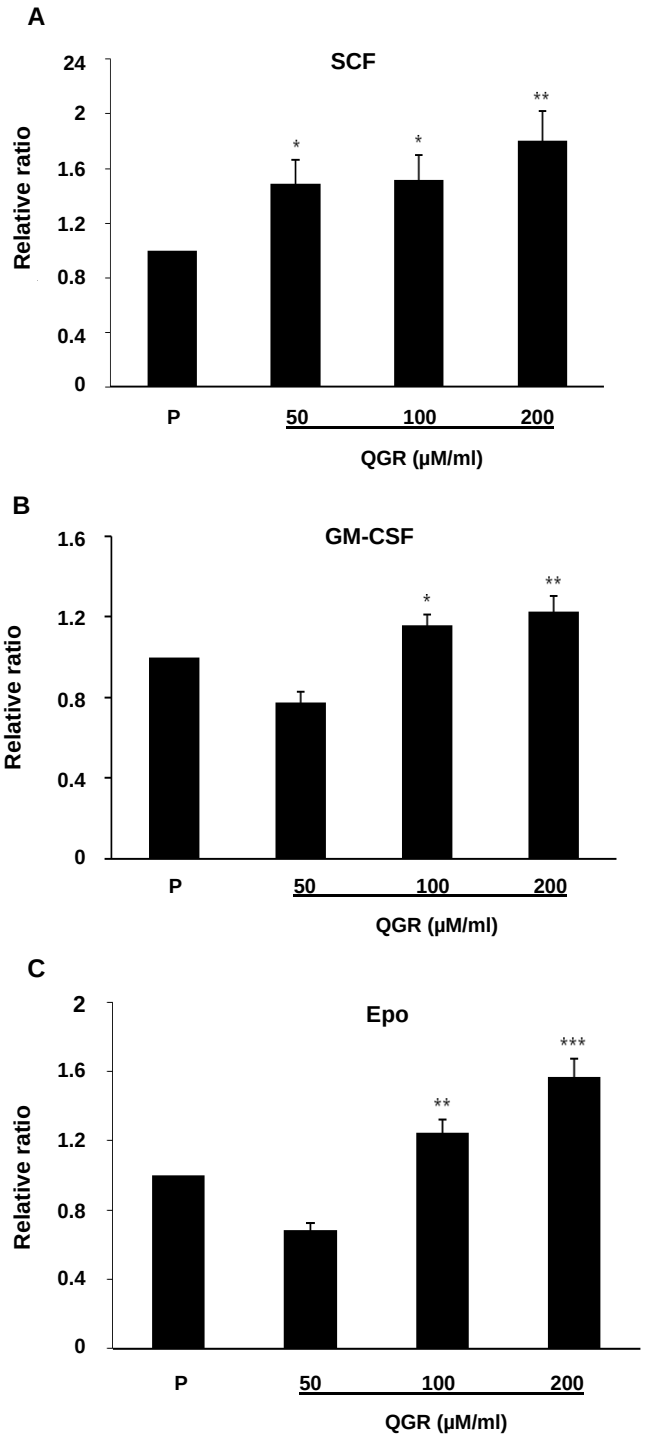

Figure 2. Effect of QGR on mRNA expression of hematopoietic cytokines in the bone marrow stromal cells. The cells were treated with either rIL-3 plus rSCF plus rEpo as positive control (P), or QGR (50, 100 or 200 μM/ml) for 4 h. The mRNA expression of SCF (A), GM-CSF (B) and Epo (C) was measured by real-time RT-PCR. The levels are indicated as relative ratio against positive control. *, **, *** Significantly different at p<0.05, p<0.01 and p<0.001, respectively.

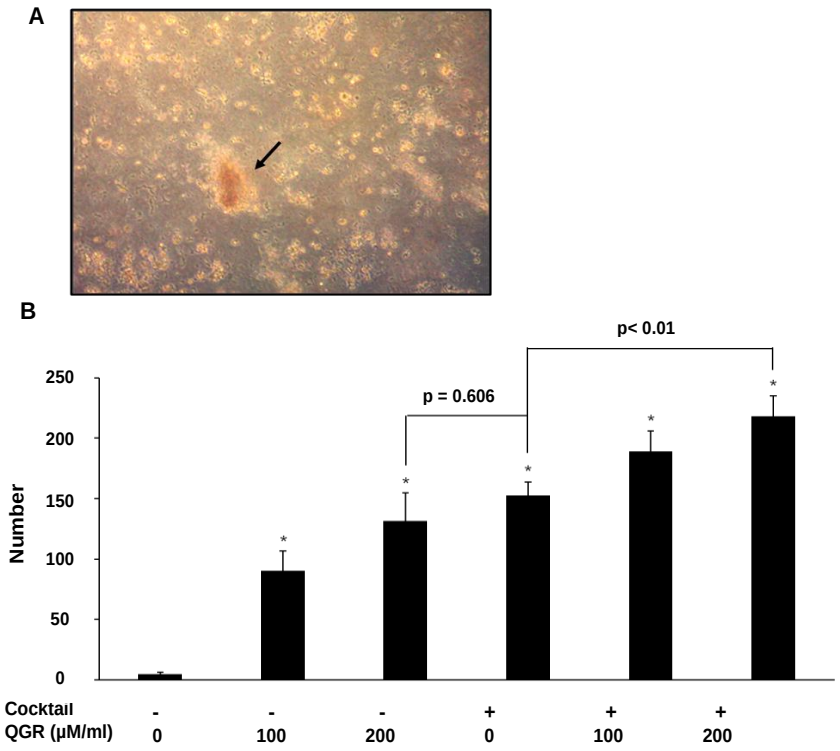

Figure 3. Effect of QGR on the formation and growth of BFU-E colony. BMNCs were treated with QGR (0, 100, 200 μM/ml) or cocktail of rIL-3, rSCF and rEpo as positive control or cocktail plus QGR. Morphology of BFU-E (A). It was assessed by the hematopoietic progenitor cell colony-forming assay on day 14. BFU-E colonies significantly increased in positive control and treatment group (B). * Significantly different from negative control (p<0.001).

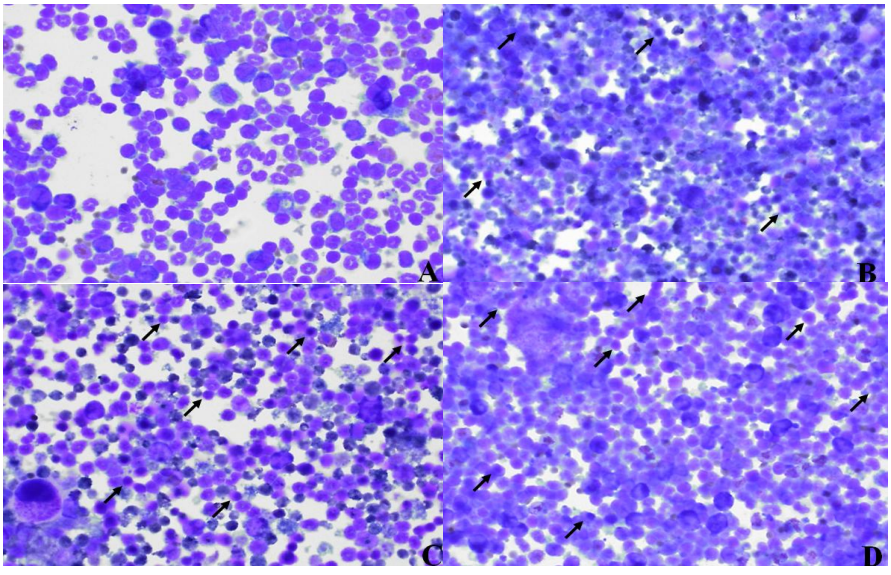

Figure 4. Cytology of erythroid precursor cells. BMNCs incubated with either QGR 0, 100, 200 μM/ml (A, B, C, respectively) or rEpo as positive control (D) for 7 days. Note the increased number of enucleated erythroid cells (arrows) in QGR treatment groups (Wright-Giemsa stain, × 400).

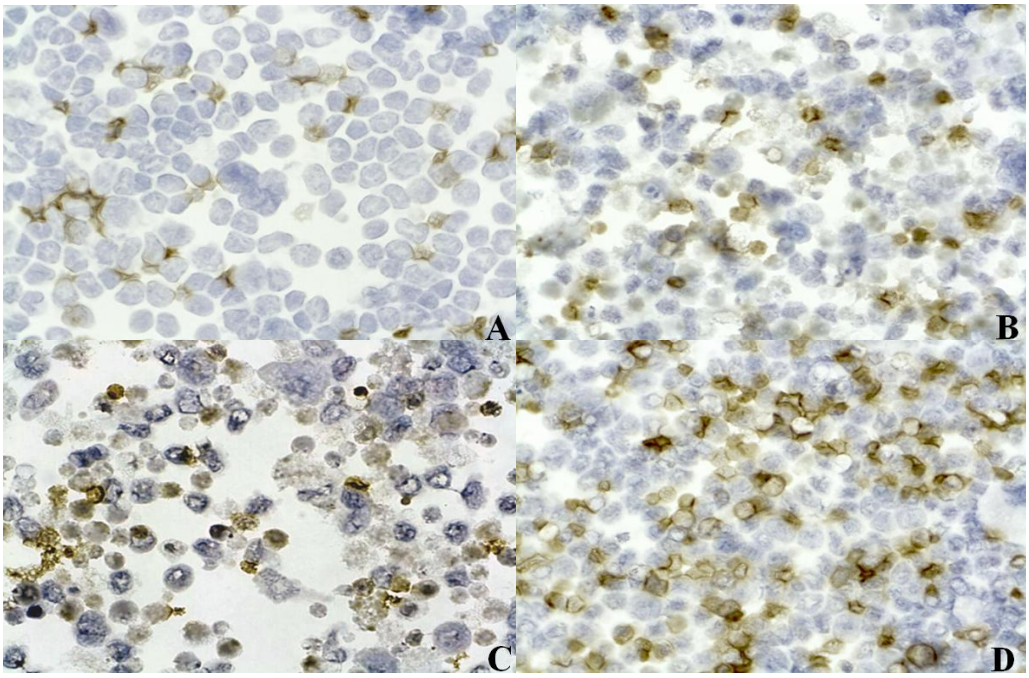

Figure 5. Immunostaining for erythroid surface antigen TER-119 in the BMNCs incubated with either QGR 0, 100, 200 μM/ml (A, B, C, respectively) or rEpo as positive control (D) for 7 days. Note the increased number of TER-119 positive cells in QGR treatment groups (ABC, × 400).

and basophilic erythroblasts. During this early stage of erythropoiesis, a large number of growth factors and transcription factors including IL-3, Epo, Tpo, SCF, GM-CSF, GATA-2 and SCL play important roles for the processes. Most of the cytokines are secreted from the bone marrow stromal cells and stimulate the survival, proliferation and differentiation of the HSCs (Banu et al., 1995; Brugger et al., 1993; Guerriero et al., 1997; Kaushansky et al., 1995; Kaushansky et al., 1994). Therefore, the relationship between HSCs and marrow stromal cells is important to self-renewal and quiescence. We have shown that QGR promotes or stimulates differentiation of HSCs or EPCs into the erythroid lineage. QGR significantly increased the expression of SCF, GM-CSF and Epo at the mRNA level. This indicates that QGR contributes to the stimulated secretion of the hematopoietic cytokines in the bone marrow stromal cells, which is an essential event to the differentiation of HSCs and/or EPCs.

Consistently, the treatment of QGR promoted the increase of the formation of BFU-E, a specific progenitor of erythroid lineage. Although the BMNCs used in colonogenic assay contain various marrow cells such as HSCs, EPCs, stromal cells and other precursor cells, the capacity to form BFU-E is a unique function of HSCs and their progeny EPCs (Saeland et al., 1992). Therefore, this result indicates that QGR stimulates the differentiation of HSCs or EPCs in erythropoiesis. Increased expression of erythroid cell surface marker TER-119 was demonstrated by immunocytochemistry and flow cytometry after QGR treatment. TER-119, a murine erythroid specific marker, is expressed in late stage of erythroid maturation, from proerythroblast to mature RBCs. However, EPCs including BFU-E or CFU-E do not express TER-119 (Kina et al., 2000). In our data, TER-119$^+$ erythroid precursor cells were significantly increased by QGR, and reached maximally to the level caused by Epo (positive control) when treated with 200 μM QGR. Considering that QGR has potential to increase BFU-E formation and Epo has potential to activate BFU-E to lead to TER-119$^+$ erythroid precursor cells, we can conclude that the enhancement of TER-119$^+$ cells by QGR was not because activation of BFU-E differentiation, but because of activation of HSC or EPC differentiation. Therefore, our data support the notion that QGR has potential to promote erythropoiesis in the early stage. Although the complex array of extrinsic and intrinsic factors control the differentiation of HSCs to erythroid cells, the detailed molecular controls are not fully understood.

Although JAK/STAT signaling pathway, one of major signaling pathways in erythropoiesis, is activated by hematopoietic cytokines including SCF,GM-CSF and Epo, the detailed influences of QGR to the activation of JAK/STAT pathway and several downstream signals such as phosphatidyl inositol 3-kinase/protein kinase B, STAT5-Bcl-XL and extracellular signal- regulated

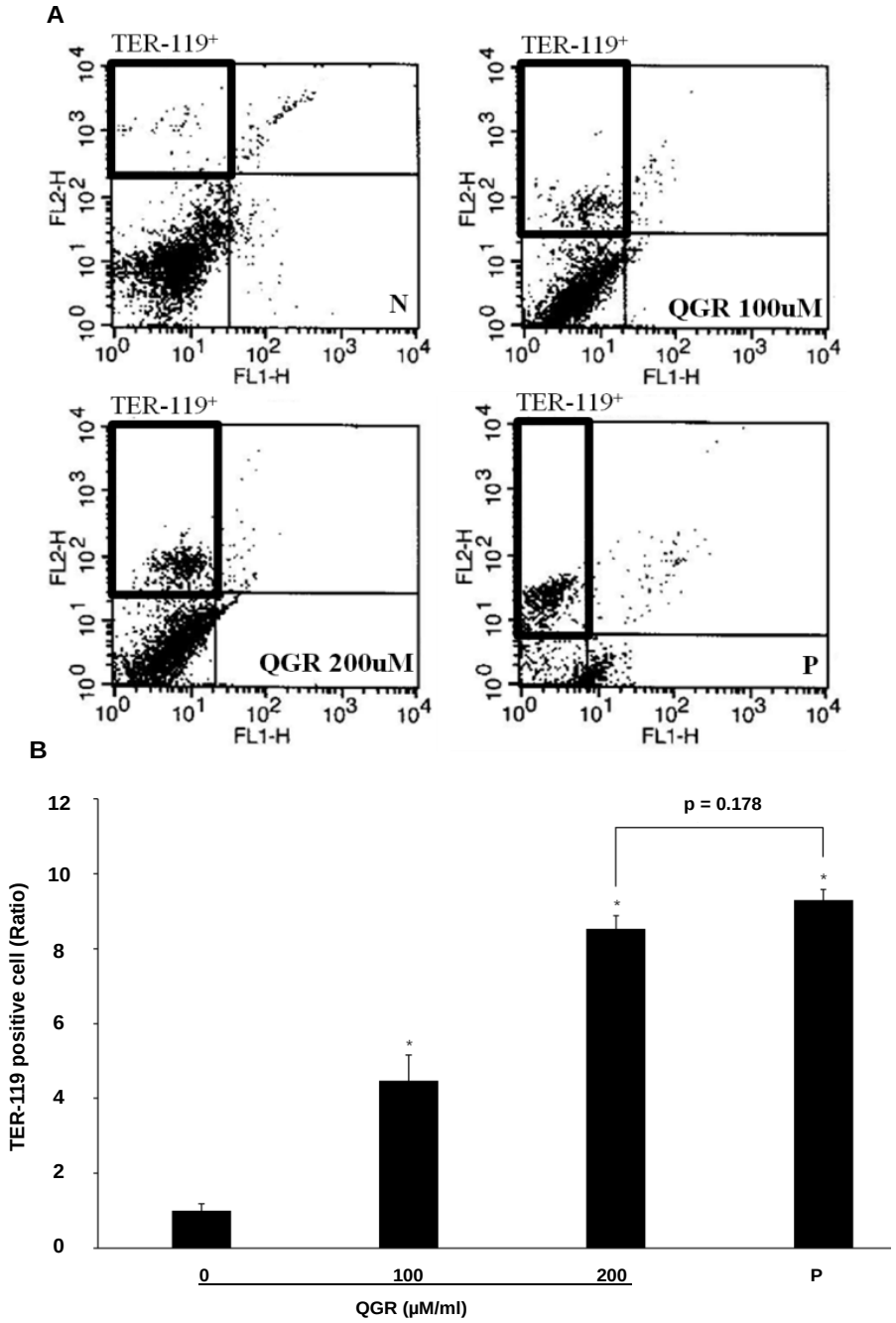

Figure 6. Flow cytometric analysis for erythroid precursor cells. BMNCs were incubated with either QGR 0, 100, 200 μM/ml or rEpo as positive control (P) for 7 days. The gate (bold rectangle) was set on cells stained positively with phycoerythrin-conjugated TER-119 antibodies (A). The ratios of TER-119 positive cells against the cells of negative control are indicated (B). * Significantly different from negative control (p<0.001).

kinase/mitogen-activated protein kinase were not revealed. We focused on the phenotypic expressions induced by QGR treatment in the present study. QGR, a natural compound isolated from a plant is different from the current commercial erythroid stimulating agents (ESAs) in mechanism to increase the number of RBCs. Although the effector molecules and exact signaling pathway of QGR in the erythropoiesis should be further

elucidated, this study clearly shows that QGR promotes the early stage of erythropoiesis. QGR activates secretion of the cytokines from stromal cells and, thus, the formation of BFU-E followed by the increase of erythroid precursor cells, whereas Epo or commercial ESAs play its key role at precursor cells to the right of BFU-E in erythropoietic lineage. This implies QGR mediates erythroid differentiation from pluripotent HSCs or EPCs and thus, can be a novel agent for erythropoiesis. This conclusion, of course, should be further confirmed by *in vivo* experiments utilizing an animal model of aplastic anemia.

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