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The effects of plasma gelsolin on human erythroblast maturation for erythrocyte production

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ABSTRACT

Gelsolin is an actin binding protein present in blood plasma and in cytoplasm of cells including macrophages. Gelsolin has important functions in cell cycle regulation, apoptotic regulation, and morphogenesis. Even though bone marrow macrophages and serum factors are critical for regulating erythropoiesis, the role of gelsolin on human erythroblasts has not been studied. Here, we investigated the effects of human recombinant plasma gelsolin (pGSN) on human immature erythroblasts. CD34+ cells isolated from cord blood were differentiated into erythroid cells in serum-free medium. When pGSN was applied to the culture medium, it accelerated basophilic and polychromatic erythroblast maturation and increased the enucleation rate with highly expressed erythropoiesis-related mRNAs. Also, pGSN was effective in reducing dysplastic changes caused by vincristine, suggesting its role in cell cycle progression at G2/M checkpoints. Also, pGSN activated caspase-3 during maturation stages in which caspase-3 functions as a non-apoptotic maturational signal or a pro-apoptotic signal depending on maturation stages. Our results suggest that pGSN has a pivotal role in maturation of erythroblasts and this factor might be one of the way how bone marrow macrophages and previously unknown serum factors work to control erythropoiesis. pGSN might be used as additive for *in vitro* production of erythrocytes.
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1. Introduction

Erythroblasts proliferate, differentiate, and finally enucleate within specialized niches called erythroblastic islands in which erythroblasts cluster around macrophages (Chasis and Mohandas, 2008). Even though it is very clear that macrophages are critical for erythropoiesis, the factors through which they act are not fully understood. Gelsolin (GSN) is a multifunctional actin binding protein involved in cell motility, cell morphogenesis, and remodeling of the cytoskeleton, controlled by Ca^{2+} and phospholipid (Yin, 1987; Yin and Stossel, 1979). GSN was first found in the cytosol of macrophages (Yin and Stossel, 1979), and three isoforms have been identified till now: an intracellular type (cytoplasmic type, cGSN), an extracellular type (secreted or plasma type) that is 23 amino acids longer than the cytoplasmic isoform, and a third isoform (GSN-3) that is 11 amino acids longer than the cytoplasmic isoform. Intracellular GSN is present in the cytoplasm and mitochondria, while extracellular GSN is found in the blood plasma (Chaponnier et al., 1986; Wen et al., 1996; Yin et al., 1984; Zapun et al., 2000; Li et al., 2012). GSN is involved in cell cycle control and enhances the G2/M checkpoint function of many cancer cells (Sakai et al., 1999; Deng et al., 2015). It may also inhibit apoptosis by preventing the loss of mitochondrial membrane potential (Koya et al., 2000). However, depending on the cell types and experimental conditions, GSN showed pro-apoptosis or anti-apoptosis (Nishio and Matsumori, 2009). Moreover, GSN might have a different function between species (Leifeld et al., 2006).

Little is known about the role of GSN in human erythroblasts. Cantu et al. reported that in GSN knock- out mice, terminal maturation and enucleation of erythroblasts failed due to an altered balance between actin polymerization and depolymerization (Cantu et al., 2012). However, the observed defective erythropoiesis may have been due to gelsolin-defective abnormal macrophages or altered gelsolin levels in the bloodstream, not just due to the gelsolin-defective erythroblasts. Moreover, that study was performed in mice not in human cells. Hence it could not establish the effects of GSN itself on human erythroblasts.

Erythroblasts committed from hematopoietic stem cells (HSCs) undergo a multi-step process of differentiation from proerythroblasts to

Abbreviations: GSN, gelsolin; pGSN, human recombinant plasma gelsolin; cGSN, cytoplasmic gelsolin; HSCs, hematopoietic stem cells; GATA-1, GATA-binding factor 1; VLA-4, very late antigen-4; ICAM-4, intracellular adhesion molecule-4; DLC-1, deleted in liver cancer-1; RhoA, ras homolog gene family member A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Cdk, cyclin-dependent kinase.

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basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, enucleated reticulocytes and finally mature red blood cells (RBCs) (Gabet et al., 2011; Testa, 2004). Cell morphology, cellular characteristics, and apoptotic mechanisms all differ depending on the degree of maturation of erythroid cells (Testa, 2004). These processes might be related to gelsolin especially plasma isoforms that are secreted by adjacent macrophages and other erythroblasts.

In the present work, we examined the effects of human plasma gelsolin on the various developmental stages of human immature erythroblasts by adding human recombinant plasma gelsolin protein (pGSN) to erythroblast differentiation medium. Our results indicate that pGSN facilitates erythroblast maturation, acts as a G2/M checkpoint, and corrects the myelodysplasia induced by serum-free culture conditions or by a G2/M blocker.

2. Materials and methods

2.1. Isolation of human CD34+ cells

Umbilical cord blood was collected after obtaining a written consent from healthy pregnant women. The study was approved by the IRB of Hanyang University Guri Hospital in Korea. Mononuclear cells (MNCs) of human umbilical cord blood were separated on Ficoll-Paque gradients and CD34+ cells were isolated using an Easysep Human CD34 Positive Selection Kit (Stemcell Technologies, Vancouver, Canada). The erythroid cell purity was evaluated by the expression of the glycophorin A (GPA) by flow cytometry and was higher than 90% after culture day 8.

2.2. Cell culture

CD34+ cells were cultured in serum-free medium (Stemline II, Sigma, Aldrich, St Louis, MO) supplemented with 150 μg/ml holotransferrin (Sigma), 90 ng/ml ferric nitrate, 30.8 μM vitamin C, 160 μM 1-thioglycerol, 50 μg/ml insulin (Sigma), 4 μM L-glutamine, 2 μg/ml cholesterol, 0.05% pluronic F-68, 0.5 μl/ml lipid mixture. Growth factors supplemented in the media were erythropoietin (6 U/ml, R&D systems, Minneapolis, MN), stem cell factor (100 ng/ml, R&D systems), and interleukin-3 (10 ng/ml; Sigma) for 0–7 days of culture, erythropoietin (3 U/ml) and stem cell factor (50 ng/ml) for 8–13 days, and erythropoietin (2 U/ml) for 14–18 days. The medium was changed every 2 days.

2.3. pGSN treatment

As Koya et al. have reported that recombinant gelsolin inhibited mitochondrial membrane potential loss at 0.4 μM (Koya et al., 2000), and the median level of GSN levels in human plasma is about 200 μg/ml (2.3 μM) (Jagadish et al., 2012), erythroblasts were treated with pGSN at concentrations of 0, 0.4, 1.0, and 2.0 μM. Around at culture day 8 or 13, the cultured cells were evaluated for their maturation states by cell morphology after Wright-Giemsa staining. If >70% of cells were at basophilic (experiment 1) or polychromatic erythroblasts (experiment 2), pGSN was added to culture media for 24–48 h.

For the toxicity test, cells were cultured for 24 h in the presence of pGSN dissolving solution (50% glycerol in Tris buffer).

2.4. Morphological analysis

Cells were stained with Wright-Giemsa (Sigma) after sedimentation using a cytospin (Hanil Science Industrial, Korea). Cell size, maturation, and myelodysplasia were evaluated by a microscope (Nikon Eclipse E400) in a blinded manner by two experts.

2.5. Flow cytometry

Cells were harvested and washed with phosphate buffered saline (PBS) containing 1% fetal bovine serum (FBS). Cells were labeled with CD235a (GPA)-PE, CD235a-FITC (Life Technologies, CA, USA), CD71-PE/Cy7 (BioLegend, San Diego, CA, USA), CD71-PE (BioLegend), CD49-FITC (BD Pharmingen, San Diego, CA), CD44-FITC (BioLegend) antibodies at 4 °C, for 20 min. Cells were then fixed with 1.3% formaldehyde for 10 min and permeabilized with 0.2% TX-100 at room temperature (RT) for 5 min. Cell were washed 2 times with 1% FBS and labeled with CD233-PE (Miltenyi Biotec, Auburn, CA) for 20 min. To confirm purity, cells were double-labeled for anti-CD13-APC (BD Biosciences) and anti-CD11b-FITC (BD Biosciences). In order to measure the activation of caspase-3, we used a CaspGLOW Fluorescein Active Caspase-3 Staining Kit (BioVision, California, USA). Labeled cells were analyzed by flow cytometry (Accuri™ C6, BD Biosciences).

2.6. qRT-PCR

Total RNA was purified from cells with Trizol Reagent (Ambion, Austin, TX) and measured using a Nanodrop (BioSpecNano Spectrophotometer, Shimadzu, Japan). To synthesize cDNA, we used SuperScript III Reverse Transcriptase (Invitrogen). mRNA expression levels were measured by qRT-PCR in duplicate. GAPDH mRNA was used for normalization. The primer sequences (forward/reverse) used were:

GATA-1, 5′-CCAAGCTTCGTGGAACTCTC-3′/5′-CCTGCCCGTTTACTGACAAT-3′

VLA-4, 5′-AGGATGGTGTAAGCGATGGC-3′/5′-TGCTGAAGAATTGGCTGAAGTGGTGG-3′

ICAM-4, 5′-CCGGACTAAGCGGGCGCAAA-3′/5′-AGCCACGAACTCCGGGCTCA-3′

DLC-1, 5′-AGTGCGTGCAACAAGCGGGT-3′/5′-TCCGGGTAGCTCTCGCGGTT-3′

RhoA, 5′-CTCATAGTCTTCAGCAAGGACCAGTT-3′/5′-ATCATTCCGAAGATCCTTCTTATT-3′

GAPDH, 5′-GAAGGTGAAGGTCGGAGT-3′/5′-GACAAGCTTCCCGTTCTCAG-3′

2.7. Cell cycle analysis

Cells were harvested and washed with PBS containing 1% FBS. Cells were fixed with 70% ethanol at −20 °C for 4 h. Thereafter, cells were washed twice with PBS containing 1% FBS and stained with propidium iodide (PI) and analyzed by flow cytometry (FACS Canto II, BD Biosciences).

2.8. PKH26 assay

Cells were washed twice with serum-free media, and the supernatant was removed. Cells were labeled with PKH26 (PKH26 red fluorescent cell

Fig. 1. Effects of pGSN on maturation of immature erythroblasts. (A) Experimental design. Basophilic erythroblasts or polychromatic erythroblasts were differentiated from cord blood CD34+ cells and treated with pGSN. (B) As a vehicle control, basophilic erythroblasts were treated with the pGSN dissolving solution for 24 h (n = 3). (C) Erythroblasts were treated with pGSN for 24–48 h as basophilic erythroblasts and polychromatic erythroblasts, respectively. Cell morphology was evaluated by Wright-Giemsa staining. The number of mature erythroblasts increased with concentration of pGSN. Black arrows, orthochromatic erythroblasts; red arrows, enucleated red cells (scale bar, 50 μm). Representative images are shown (n = 4). (D) The mean percentage of mature erythroblasts increased with pGSN dose of 4 independent experiments. The proportion of RBCs was increased especially at the mature stage of polychromatic erythroblasts (>200 cells scored in 4 independent fields by two experts, n = 4). (E) Erythroid cell surface markers were measured by flow cytometry for the maturation stage using antibodies against GPA, CD71, CD44, integrin α4 and Band 3 at 24–48 h of pGSN treatment in the basophilic erythroblasts and polychromatic erythroblast stage, respectively (n = 3). As other cell lineage markers, CD11b and CD13 were assessed for basophilic erythroblasts (n = 3) and polychromatic erythroblasts (n = 5).

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