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主 論 文

Effects of Histidine-Rich Glycoprotein on Erythrocyte Aggregation and Hemolysis: Implications for a Role under Septic Conditions

（赤血球凝集と溶血におけるヒスチジンリッチ糖タンパク質の作用：敗血症での役割の示唆）

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主 論 文

Effects of Histidine-Rich Glycoprotein on Erythrocyte Aggregation and Hemolysis: Implications for a Role under Septic Conditions

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[Introduction]

Eryptosis is the apoptotic-like suicidal process of erythrocytes, and is characterized by cell shrinkage and phosphatidylserine (PS) expression on the outer membrane. Active roles of erythrocyte in the pathological processes of sickle cell disease (SCD), diabetes mellitus, malaria, and beta-thalassemia have been suggested in recent studies. Kempe *et al.* reported that erythrocytes showed an apoptotic tendency in septic patients. However, it is still not clear whether this apoptotic tendency of erythrocytes affects the development of sepsis.

Histidine-rich glycoprotein (HRG) is a 75-kDa single-chain protein produced mainly in the liver. Various biological functions of HRG have been reported, including the clearance of immune complex/necrotic cells and regulation of cell adhesion, angiogenesis, coagulation and fibrinolysis. In the previous study, we demonstrated that HRG conferred protection against lethality to septic mice by suppressing immunothrombosis and relevant inflammation. However, it is still not clear whether HRG suppresses immunothrombosis through regulation of erythrocyte.

[Materials and Methods]

1. Erythrocyte aggregation, adhesion and PS/Ca^{2+} detection

Fresh blood samples were collected from healthy volunteers. After washing, erythrocytes were suspended with Hank's balanced salt solution (HBSS). Zn^{2+} (20 μM) was then added to the erythrocyte suspension to induce aggregation. PS expression on the outer membrane of erythrocyte was determined by using FITC-conjugated Annexin V. Fluo 4-AM was used to determine the erythrocyte intracellular Ca^{2+} changes after Zn^{2+} stimulation. EA.hy926 cells were used to examine the attachment of erythrocytes to vascular endothelial cells. Zn^{2+}-stimulated erythrocyte suspension was added to the cells and incubated for 30 min. After washing, Cy5-conjugated

anti-CD235a antibody was added to label the erythrocytes.

2. Animal experiment

All animal experiments were approved by the Institutional Animal Care and Use Committee of Okayama University, and performed in accordance with the guidelines of Okayama University on animal experiments. Cecal Ligation Puncture (CLP) model mice were conducted by two punctures on mice cecum with 18-gauge needle. HRG, human serum albumin (HSA) or PBS as a control was administered through a tail vein immediately after operation. The mice were sacrificed 24 h after operation. Hemoglobin levels in plasma were determined by Western Blot. Zn^{2+} levels in mice tissues were determined using a commercial kit.

3. Hemin-induced hemolysis and HRG-hemin binding affinity measurement

The level of hemin-induced hemolysis in human erythrocytes was determined by measuring the optical density (OD) at 578 nm. Binding between HRG and hemin was determined by calorimetry with a Microcal iTC200 calorimeter.

[Results]

Stimulation with 20 μM Zn^{2+} resulted in aggregation of washed human erythrocytes 1 h after incubation. HRG significantly prevented Zn^{2+}-induced erythrocyte aggregation and PS expression on the outer membrane. Intracellular Ca^{2+} levels were increased in Zn^{2+}-stimulated erythrocytes. Removing Ca^{2+} from extracellular environment or using Ca^{2+} channel blocker amiloride or nimodipine abolished erythrocyte aggregation induced by Zn^{2+}. HRG also decreased erythrocyte aggregation and PS expression of Zn^{2+}-pretreated erythrocytes. A Considerable number of erythrocytes were attached to the EA.hy926 cells after Zn^{2+}-stimulation. HRG treatment significantly inhibited the attachment of erythrocytes to the vascular endothelial cell monolayer. The level of plasma free hemoglobin was much higher in septic mice treated with PBS or HSA compared with normal mice, and the levels from HRG-treated mice were almost the same as the levels in the normal mice. Although a significant difference in PS expression was not observed among the CLP groups treated with PBS, HSA or HRG, incubation of erythrocytes for 4 h enabled the observation of beneficial effects of HRG on PS expression on erythrocytes. Zn^{2+} contents in the lung and kidney homogenate were increased significantly compared to those in intact mice. Hemin-induced hemolysis was also inhibited by HRG and

HRG-derived peptide. The calorimeter experiment showed that HRG binds hemin at ratio of 1:36, and the Kd value was estimated to be 9.17×10^{-6} M.

[Discussion]

Pathological immunothrombosis leads to thrombotic disorder, disseminated intravascular coagulation (DIC) and facilitated sepsis development. The cascade of immunothrombosis includes platelet activation and secretion, which may result in a local increase in Zn^{2+} concentration because of the presence of Zn^{2+} in platelet dense granules. Thus focusing on Zn^{2+}-stimulated erythrocytes might be an important new approach that could ultimately reveal the mechanism of immunothrombus formation in sepsis.

In the present study, we used 20 μM Zn^{2+} to induce PS expression and aggregation of erythrocytes. Aggregation of erythrocytes was dependent on the increase in intracellular Ca^{2+}, indicating that Ca^{2+} increase may be an important signaling in erythrocyte aggregation. HRG inhibited Zn^{2+}-induced PS expression and aggregation of erythrocytes and even reversed those processes. These inhibitory effects of HRG might be ascribed to its regulation on erythrocyte intracellular Ca^{2+} levels. However, further works are necessary to clarify the relationship between HRG effects and the regulation of Ca^{2+} channels on erythrocytes. HRG also inhibited Zn^{2+}-induced erythrocyte adhesion to endothelial cells, suggesting a specific regulatory role of HRG in immunothrombosis. CLP mice showed enhanced eryptosis and hemolysis, which were both inhibited by HRG supplementary treatment. Binding between HRG and hemin suggested that the beneficial effect of HRG *in vivo* might partially depend on its hemin-chelation effect. Taken together, our data suggest that HRG may protect erythrocytes from adhesion to vascular endothelial cells in combination with inhibition of erythrocyte PS expression and aggregation, leading to the down regulation of immunothrombosis under septic conditions and the protection of endothelial cells from damage.

[Conclusion]

In the present study, we have shown that erythrocytes play an important role in the immunothrombosis cascade through aggregation and adhesion. HRG showed protective effects in Zn^{2+}-induced aggregation and hemolysis in CLP model mice. The

effects of HRG in targeting erythrocytes might be an additional mechanism involved in the anti-septic effects of HRG.