らが著明に改善していた。さらに in vivo イメージング技術で循環血中にある好中球を共焦点レーザー顕微鏡で観察すると、敗血症マウスでは確かに静脈系血管床に多数の好中球が接着し、非常に多様な形態で動くことなく留まっていることがわかった。つまり肺組織切片で創造された病態が実際に動画上証明されたと言える。これらの実験結果を総合すると、HRG の治療効果については動物実験のレベルで Proof of Concept (POC) が確立されたと言える。

E. 結論

マウスの敗血症モデルを用いて、敗血症病態における血漿 HRG レベルの顕著な低下と、HRG の補充による致死率の改善作用を証明した。HRG 治療により、敗血症性 ARDS が抑制できることがわかった。HRG 治療は血管内皮細胞への異常な好中球の接着を抑制することが in vivo イメージングで示唆された。動物実験のレベルでは、HRG 治療の Proof of Concept が確立されたと考える。

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G. 知的財産権の出願・登録状況

1．特許取得

① 好中球活性化に起因する疾患の治療薬、治療方法及び検査方法
特願 2012-129232（2012.6.6 出願）
PCT/JP2013/64779（2013.5.28 出願）

2．実用新案登録

該当なし

3．その他

該当なし

資料 2

Manuscript

Histidine-Rich Glycoprotein Prevents Septic Lethality through Neutrophil Regulation

Hidenori Wake[1], Shuji Mori[6], Keyue Liu[1], Kiyoshi Teshigawara[1], Masakiyo Sakaguchi[2], Kosuke Kuroda[3], Hideo Takahashi[7], Aiji Ohtsuka[4], Tadashi Yoshino[5], Hiroshi Morimatsu[3], and Masahiro Nishibori[1]

[1]Department of Pharmacology, [2]Department of Cell Biology, [3]Department of Anesthesiology, [4]Department of Human Morphology, [5]Department of Pathology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; [6]Department of Pharmacology, Shujitsu University School of Pharmacy, Okayama, Japan; and [7]Department of Pharmacology, Kinki University School of Medicine, Osakasayama, Japan

Correspondence and requests for reprints should be addressed to Masahiro Nishibori, M.D., Ph.D., Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Shikata-cho 2-5-1, 700-8558 Okayama, Japan. Phone/Fax: +81-86-235-7140. E-mail: mbori@md.okayama-u.ac.jp

Author Contributions: H.W., S.M., H.T., and M.N. planned the study. H.W. and S.M. and M.S. performed the experiments using purified human neutrophils. H.W., K.L. and K.T. performed the sepsis mice experiments. A.O. and T.Y. analyzed neutrophil shape and histological features, respectively. K.K. and H.M determined the plasma HRG in septic patients. H.W., S.M. and M.N. wrote the manuscript.

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A short running head: HRG Prevents Septic Lethality

List ONE descriptor number: 17.5 (Pulmonary Circulation: Sepsis)

The total word count for the body of the manuscript: 3,411
The word count for the abstract: 250

At a Glance Commentary

Scientific Knowledge on the Subject: Sepsis is a critical disease condition with high mortality. Neutrophil adhesion on vascular wall may trigger platelet aggregation and microthrombus formation in septic acute respiratory distress syndrome.

What This Study Adds to the Field: A plasma protein HRG decreased markedly in septic mice with high lethality. Supplementary treatment with HRG improved the survival of septic mice. HRG kept circulating neutrophils quiescent morphologically and functionally. I.v. injection of HRG provides a novel therapy for sepsis based on pathology.

This article has an online supplement, which is acceptable from this issue's table of contents at www.atsjournals.org

Abstract

Rationale: Sepsis is a major cause of death worldwide. Although there have been many clinical trials for treatment of sepsis, no drugs are available at present.

Objectives: To clarify the involvement of histidine-rich glycoprotein (HRG) in septic pathogenesis and to develop a therapy for sepsis based on that.

Methods: Sepsis was induced in mice by cecal ligation and puncture (CLP). The mice were treated with human HRG after confirming the marked decrease in plasma HRG. We evaluated the beneficial effects of HRG administration on survival rate, lung inflammation, and the state of circulating neutrophils. Purified neutrophils from human blood were treated with HRG and analyzed with respect to neutrophil shape, adhesiveness to vascular wall, passage through microcapillaries, and production of reactive oxygen species.

Mesurements and Main Results: Supplementary treatment of septic mice with exogenous HRG for the decrease in plasma HRG improved survival, with strong inhibition of lung inflammation. Knockdown of HRG by siRNA exacerbated lethality. Purified human HRG reversibly induced morphological changes in human neutrophils *in vitro*; induction of spherical shape with reduced microvilli and adhesiveness to vascular endothelial cells. HRG maintained the passage of neutrophils through

microcapillaries and abolished production of reactive oxygen species.

Conclusions: We show that a plasma protein HRG is a crucial factor that keeps the circulating neutrophils quiescent and prevents unnecessary activation in blood-stream using cecal ligation puncture model in mice and human neutrophils. Thus, the supplementary therapy with HRG may provide a novel strategy for the treatment of septic patients through neutrophil regulation.

The word count for the abstract: 250

Key words: HRG, Sepsis, ARDS, Neutrophil

Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) associated with infection. The pathogenesis of sepsis includes the disturbance of blood-vascular homeostasis, which may cause multiple organ failure, circulatory shock, and disseminated intravascular coagulation, leading to high mortality (1-4). The proinflammatory cytokine response in the acute phase may be triggered by the constituents of invading pathogens and tissue damage-associated molecular patterns (5, 6), accompanied by the activation of vascular endothelial cells, a pivotal step for inducing the migration of leukocytes into inflammatory sites with pathogen invasion (7, 8). A recent study suggested that neutrophil adhesion on vascular endothelial cells may trigger platelet aggregation and microthrombus formation in septic acute respiratory distress syndrome (ARDS) (9-11). Thus, circulating neutrophils may play important roles in the pathogenesis of septic conditions in addition to infiltrating neutrophils. However, the uncontrolled activation of neutrophils has not been examined in detail due to methodological limitations (12). Neutrophils are easily activated by *in vitro* handling or even by withdrawing from blood vessels. Therefore, it might be rather difficult to know and speculate about the precise features of the circulating neutrophils by an *in vitro* analysis. Also, if any are present, a controlling

factor of neutrophils in plasma, one that might regulate a fundamental state of circulating neutrophils in both healthy and disease conditions, remains to be determined.

In the present study, we identified and characterized a plasma protein histidine-rich glycoprotein (HRG) (13-17) as a factor that maintains a neutrophil's spherical shape with minimal microvilli on the surface. These morphological features render neutrophils quiescent with respect to the cell adhesion to vascular endothelial cells, the passage of microvasculatures, and the spontaneous release of ROS. In CLP sepsis mice, plasma levels of HRG decreased significantly, and supplementary treatment with human HRG dramatically improved the survival rate of CLP mice associated with the inhibition of inflammatory responses in the lung and kidney, without interfering with the infiltration of neutrophils into the peritoneal cavity. The present study has shed light on our understanding of septic conditions by clarifying a crucial role of HRG in neutrophils.

Methods

Cecal ligation puncture model

All animal experiments were approved by the University's committee on animal experimentation and performed according to the guidelines of Okayama University on animal experiments. Male C57BL/6N mice (22-25 g, 7-8 wk) were obtained from Japan SLC (Shizuoka, Japan). Sepsis was induced in mice by cecal ligation and puncture (CLP). Animals were anesthetized and a ligature was placed below the ileocecal valve. The cecum was punctured once (mild sepsis) or twice (severe sepsis) with an 18-gauge needle and then returned to the peritoneal cavity. Animals were treated with vehicle (PBS) or with 4 or 20 mg/kg of human HRG or protein control (HSA; 20 mg/kg) immediately and at 24 h and 48 h after CLP induction. Sham mice underwent the same surgical procedures without ligation and puncture.

Neutrophil shape and adhesion assay

Purified human neutrophils pre-stained with Hoechst33342 (Nuclei) and Calcein-AM (Cytosol) were aliquoted in a volume of 100 μl ($5x10^4$ cells /well) to polystyrene wells or confluent wells of EA.hy926 cells (ATCC CRL-2922), a hybridoma of HUVEC and the human epithelial cell line A549. The incubation started with one of the reagents

(BSA, HSA, HRG, fMLP: each at a final concentration 1 μM) and continued for indicated periods at 37 °C. The cell shape and fluorescence intensity were analyzed by using an In Cell Analyzer 2000 (GE Healthcare, Waukesha, WI) and In Cell Analyzer Workstation software (GE Healthcare). Neutrophil adhesion was evaluated by measuring the fluorescence intensity of the wells by a Flexstation3 (Molecular Devices, Sunnyvale, CA) before and after the wells were gently washed twice to remove any nonadherent cells. When HRG's effects on intracellular calcium were examined, BAPTA or Fluo-4-AM was preloaded for 20 min.

Statistical analysis

The statistical analysis across multiple treatment groups was determined with ANOVA, followed by Dunnett test. The statistical difference between paired groups were determined using Student's t-test. All data are presented as the means ± SEM. P values < 0.05 were considered statistically significant. The Kaplan-Meier method was used for the survival experiments, and the differences were analyzed using the log-rank test.

Results

Effects of HRG on Lethality of CLP Septic Mice

In cecal ligation and puncture (CLP), a mouse model of sepsis, plasma HRG levels decreased significantly, by 77%, compared with the sham control levels 24 hours after CLP (Figures 1A and 1B). The survival rate of mice treated with phosphate-buffered saline (PBS) for 3 days after CLP was 0% at day 7. The administration of purified human HRG (4 and 20 mg/kg, i.v.) for 3 days significantly improved lethality in CLP mice, whereas the same dose of human serum albumin (HSA, 20 mg/kg, i.v.) had no effect on the survival rate, indicating the clear survival effects of i.v. injection of HRG (Figure 1C). In addition to survival rate, locomotor activity of mice treated with HRG was improved clearly (*see* Video E1). The colony-forming units of the blood from three groups of mice treated with PBS, HSA, and HRG showed similar values (data not shown). Also, the total number of infiltrating cells in the peritoneal cavity did not differ among the groups (data not shown). These results suggested that the beneficial effects of HRG were not ascribable to the increase in bacteriocidal activity (18) in the peritoneal cavity.

Pretreatment of mice with siRNA for mouse HRG reduced plasma levels of HRG by 90% at 5 days after i.v. injection as compared with those in nonrelevant siRNA-treated

mice (Figures 1D and 1E). The survival rate of HRG-knockdown mice after mild CLP (one cecum penetration by needle) was compared with that in control mice to see whether the depletion of HRG from plasma could exacerbate septic inflammation and lethality. The results clearly indicated that depletion of HRG reduced the survival rate significantly (Figure 1F). Moreover, plasma HRG levels in septic patients decreased significantly, by 67%, compared with those in healthy volunteers (Figure 1G), indicating a similar dynamics of plasma HRG in septic patients to those in septic mice. Taken together, the results of our experiments on supplementary treatment with HRG and the acute depletion of HRG strongly suggested that HRG may be an important plasma factor controlling the lethality of mice in septic conditions.

In vivo imaging of circulating neutrophils labeled with anti-Gr-1 Ab showed that the spherical shape of neutrophils circulated with different velocities in both mesenteric arterioles and venules in sham-operated mice. Rolling, spherical neutrophils on the vascular endothelial cells were sometimes observed along the marginal flow of the blood-stream in venules in sham mice (Figure 2A; *see* Video E2). Transient oval or teardrop shape changes were observed in the arterioles of sham mice. In contrast, deformed neutrophils with a multiangular and rigid appearance were observed in the circulation in CLP mice. Extremely deformed neutrophils were attached to vascular

endothelial cells in venules and were arrested there without migration during the observation periods (Figure 2A; *see* Video E2). Thus, the extreme morphological changes of circulating neutrophils and the enhanced interaction with vascular endothelial cells demonstrated by *in vivo* imaging appear to represent pathological features in the septic mouse model. In contrast, HRG-treated mice showed the spherical neutrophils in the circulation and had markedly fewer neutrophils attached to endothelial cells in venules compared with control CLP mice (Figure 2A; *see* Video E2), strongly suggesting the less interaction of circulating neutrophils with vascular endothelial cells in these mice.

The microcapillary passage of peripheral blood from CLP mice was examined using a micro-channel array flow analyzer (MC-FAN) ex vivo (Figure 2B; *see* Video E3). In mice treated with PBS or HSA, deformed leukocytes were attached to the microcapillary entrance very often, whereas there were few such leukocytes in HRG-treated mice (Figure 2B; *see* Video E3). The time required for the passage of 100 μl blood through the microcapillary reflected the adhesion of leukocytes (Figure 2C). On the other hand, there were no significant differences in hematocrit levels among the treated groups (data not shown).

Hematoxylin-eosin staining of lung tissue revealed lung inflammation 24 hours after

CLP in mice treated with PBS and HSA; this inflammation included increased thickness of the interstitial space of alveoli, infiltration of neutrophils, and congestion/hemorrhage (Figure 3A). However, HRG treatment (20 mg/kg, i.v.) ameliorated pathological findings in the lung remarkably (Figure 3A). Measurement of alveolar wall thickness showed that HRG treatment inhibited lung edema significantly (Figure 3B). Also, the number of neutrophils in the lung detected by anti-Gr-1 increased significantly in CLP mice treated with PBS and HSA, and HRG treatment inhibited the number of neutrophils by 57% (Figures 3C and 3D). In consistent with these histological findings, the results of mRNA expression of TNF-α, IL-6, PAI-1, iNOS, and neutrophil elastase in the lung using real-time PCR at 24 hours clearly showed the significant upregulation of these mRNAs in CLP mice treated with PBS or HSA, whereas HRG treatment strongly suppressed the mRNAs of all of these, especially in the cases of IL-6 and PAI-1 (Figure 3E). Thus, it is likely that HRG suppressed the inflammatory responses in septic ARDS efficiently. The expression of RAGE mRNA in the lung was inversely regulated by CLP and HRG. HRG treatment inhibited glomerular leukocyte infiltration and renal tubular swelling in CLP mice observed at 24 hours (Figure 3A).

Effects of HRG on Morphology, Adhesion and Microcapillary Passage of Purified Human Neutrophils

To examine the effects of HRG on neutrophils in detail, we used human neutrophils purified from peripheral blood by density gradient centrifugation and labeled with calcein-AM and Hoechst33342. HRG (1 μM)-induced morphological changes were observed under a fluorescent microscope 60 min after incubation without a fixation procedure (Figure 4A). The main features induced by HRG were the following: spherical shape change, loss of microvilli on the cell surface, and shortening of the diameter. When compared with other media containing the same concentration of bovine serum albumin (BSA), HSA, or fMLP, the spherical shape-inducing effects of HRG were evident (Figures 4A and 4B). The adhesion of neutrophils to the plastic well was determined by counting the residual neutrophils after the wells were washed twice. The results clearly showed that the HRG-induced spherical shape were less adhesive to the plastic material (Figure 4C). Quantification of HRG's spherical shape-inducing effects revealed HRG's concentration-dependent effects with the maximal response at 0.8 μM or above and EC_{50} around 0.1 μM (Figure 4D). It took 15-30 min for HRG to induce a stable spherical shape from a freshly prepared cell suspension (*see* Figure E1A). Moreover, the roundness of the completely flattened

neutrophils was restored by the addition of HRG (1 μM) time-dependently, suggesting the reversibility of the shape-change response (*see* Figure E1B). The spherical structure-inducing effects of HRG were inhibited by the addition of rabbit polyclonal Ab against HRG but not by control IgG (Figures 4E and 4F), confirming the specificity of HRG's effects. In addition to the inhibition of spherical shape, anti-HRG Ab antagonized HRG's inhibitory effect on neutrophil adhesion (Figure 4G). The adhesion property of HRG-treated neutrophils on vascular endothelial cells (EA.hy926) was then examined (Figures 4H-4K). As shown in Figure 4K, HRG significantly inhibited the adhesion of neutrophils on the EA.hy926 surface.

HRG's effects on the vertical transfer of neutrophils through micropores (5 μm diameter) were examined in a Boyden chamber. The results indicated that increasing concentrations of HRG as well as fMLP (1 μM) stimulated the transfer of neutrophils to the lower chamber (*see* Figure E2A). However, the chemotaxis-inducing activity to the horizontal direction was detected solely in fMLP and not in HRG (*see* Figure E2B). Thus, the apparent transfer of neutrophils to the vertical direction may be ascribed to the decrease in diameter, the loss of microvilli, and gravity.

Scanning electron microscopic observation confirmed the loss of surface microvilli structures from neutrophils treated with HRG at least 15 min after the start of incubation

(Figures 5A and 5B). The microvilli on neutrophils were observed after incubation with buffer alone, BSA, HSA, and fMLP, as was the case with the washed neutrophils immediately after isolation (Figures 5A and 5B). The cytochemical staining of G- and F-actin in neutrophils demonstrated that F-actin was dominant in HRG-treated neutrophils and that the HRG-induced spherical shape was accompanied by the F-actin ring formation beneath the plasma membrane of neutrophils (Figure 5C). On the other hand, cytosolic G-actin was dominant in neutrophils treated with HBSS, BSA, and HSA (Figure 5C). Few neutrophils had an F-actin ring under these conditions.

The passage of purified neutrophils through microcapillary slits (7.0 μm width, 4.5 μm depth) was evaluated by a MC-FAN under different conditions. Figures 6A and 6B and Video E4 show that neutrophils treated with HRG (1 μM) can pass microcapillaries more easily than can other treatment groups, in which the trapping of neutrophils with irregular shapes sometimes occurred before (white arrowheads) or on (red arrowheads) the microcapillary slits. HRG-treated neutrophils flowed through the slits much more smoothly in a teardrop shape (*see* Video E4). In contrast, the addition of anti-HRG mAb to whole blood retarded the passage of whole blood significantly, probably due to the trapping of leukocytes on the slits (Figures 6C and 6D; *see* Video E5).

HRG-fluorescein was taken up into neutrophils. There were two types of staining pattern of HRG-fluorescein: granular and homogeneous (Figures 7A and 7B). The total number of HRG-fluorescein-positive granules was much higher than that of HSA-fluorescein-positive granules, implying a receptor-mediated or transport carrier-mediated uptake system for HRG in neutrophils (Figure 7C). The incubation of EA.hy926 with HRG-fluorescein showed a homogeneous staining pattern, implying the cell surface binding of HRG-fluorescein (*see* Figures E3A and E3B).

Pharmacological Analysis of HRG-inducing Signal Transduction in Neutrophils

Pretreatment with BAPTA for 30 min (50 μM) prevented HRG from inducing spherical shape formation (Figure 8A). Determination of [Ca2+]i after the prolonged incubation with HRG revealed that HRG induced time-dependent and very slow increases in [Ca2+]i levels in neutrophils (Figures 8B and 8C). Wortmannin and LY294002, inhibitors of PI3-kinase, concentration-dependently induced the spherical shape changes in neutrophils, similar to the effect of HRG (Figure 8D). SB239063, an inhibitor of p38, and SP600125, an inhibitor of JNK, partially mimicked HRG's effects on neutrophil shape, whereas FR180204, an inhibitor of ERK, exhibited the opposite effects (Figure 8E). Toxin B (100 ng/ml), a nonselective inhibitor of three small G

proteins, Cdc42, Rac, and Rho, partially inhibits HRG's spherical-shape inducing effects (Figure 8F).

The production of reactive oxygen species (ROS) outside the neutrophils was determined at 15 min after the start of incubation by the detection of iso-luminol chemiluminescence under different conditions (Figure 8G). The ROS production levels in HRG (1 μM)-treated neutrophils was less than 5% the levels in the HBSS-, BSA-, and HSA-treated groups (Figure 8G). The ROS production inside the neutrophils was determined by DCF fluorescence after incubation in the presence of HRG (1 μM) or other factors (Figure 8H). Consistent with the extracellular ROS, ROS production inside the cells was lower in HRG-treated neutrophils than in any of the other groups (Figure 8H). These results as a whole indicated that HRG-induced spherical shape changes were accompanied by functional alterations of the neutrophils, including changes in ROS production, adhesion to vascular endothelial cells, and passage through microcapillaries.