morphological features render neutrophils quiescent with respect to the cell adhesion to vascular endothelial cells, the passage of microvasculatures, and the spontaneous release of reactive oxygen species (ROS). In cecal ligation and puncture (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 immunothrombosis in pulmonary vasculatures, disseminated intravascular coagulation (DIC) state, hypercytokinemia and 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.

Results

Effects of HRG on Lethality of CLP Septic Mice. In CLP, a mouse model of sepsis, plasma HRG levels decreased significantly, by 77%, compared with the sham control levels 24 hours after CLP (Fig. 1A). 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 (Fig. 1B). In addition to survival rate, locomotor activity of mice treated with HRG was improved clearly (Supplementary Fig. 1A and Supplementary Video 1). The therapeutic time window of i.v. HRG appeared to be 6 hours of the CLP induction in which 30% survival rate was obtained (Supplementary Fig. 1B) The colony-forming units of the blood from three groups of mice treated with PBS, HSA, and HRG showed similar values (Supplementary Fig. 1C). Also, the total number of infiltrating cells in the peritoneal cavity did not differ among the groups (Supplementary Fig. 1D). These results suggested that the beneficial effects of HRG were not ascribable to the increase in bacteriocidal activity[33] 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 (Fig. 1C). 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 (Fig. 1D). HRG mRNA levels decreased time-dependently after CLP or LPS injection, leading to less than 15% of sham control at 24h (Fig. 1E). The resultant plasma levels of HRG in CLP mice were less than that in LPS-injected mice (Fig. 1F). Moreover, plasma HRG levels in septic patients decreased significantly, by 77%, compared with those in healthy volunteers (Fig. 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 (Fig. 1H and Supplementary Video 2). 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 (Fig. 1H and Supplementary Video 2). 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 (Fig. 1H, I and Supplementary Video 2), 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 (Fig. 1J and Supplementary Video 3). In mice treated with PBS or HSA, deformed leukocytes were attached to the microcapillary entrance very often indicated by red arrowheads, whereas there were few such leukocytes in HRG-treated mice (Fig. 1 J and Supplementary Video 3). The time

required for the passage of 100 μl blood through the microcapillary reflected the adhesion of leukocytes (Fig. 1K). On the other hand, there were no significant differences in hematocrit levels among the treated groups (Supplementary Fig. 1E).

Analysis of HRG Effects on Septic ARDS. 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 (Fig. 2A). However, HRG treatment (20 mg/kg, i.v.) ameliorated pathological findings in the lung remarkably (Fig. 2 A). Measurement of wet/dry weight ratio showed that HRG treatment inhibited lung edema significantly (Fig. 2B). 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% (Fig. 2C, D). Triple immunohistochemical staining of neutrophil, platelet and fibrin clearly revealed that most of the adherent neutrophils were accompanied by platelet aggregation and fibrin deposits, namely immunothrombus, in CLP mice treated with PBS or HSA (Fig. 2C, E) whereas the total number of overlapping spots in mice treated with HRG was much lower (Fig. 2C, F). HRG immunoreactivities were observed on neutrophils from sham

and CLP mice (on immunothrombus) (Supplementary Fig. 2A, B) Extracellular localization of DNA specific for NETosis (yellow arrowheads) was evident on immunothrombosis (Fig. 2G). The NETs in CLP mice treated with PBS or HSA were significantly higher than that in sham mice and the treatment with HRG inhibited the number of NETs (Fig. 2H). 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 (Fig. 2I). 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, increase in mesangeal matrix and renal tubular swelling in CLP mice observed at 24 hours (Fig. 2A).

Effects of HRG on coagulation and hypercytokinemia in CLP Septic Mice. The platelet counts in CLP mice treated with PBS or HSA were significantly lower than that in sham mice and the treatment with HRG restored the platelet counts (Fig. 3A).

Consistent with the consumption of platelets in CLP mice treated with PBS or HSA, APTT and PT values, disorder markers of intrinsic and extrinsic coagulation pathways, were prolonged in these mice compared with sham control value (Fig. 3B). HRG treatment significantly reduced APTT and PT values. HRG did not affect the changes in plasma levels of fibrinogen and ATIII levels in CLP mice (Fig. 3B).

Determination of blood cytokines 24 hours after CLP revealed the marked increase in IL-6, TNF-α and IL-10 levels in the CLP mice treated with PBS or HSA (Fig. 3C). HRG treatment almost completely inhibited the cytokine responses.

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 (Fig. 4A). The main features induced by HRG were the following: spherical shape change, loss of irregularity of shape, 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 (Fig. 4A, B). 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 (Fig. 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 (Fig. 4D). It took 15-30 min for HRG to induce a stable spherical shape from a freshly prepared cell suspension (Supplementary Fig. 3A). 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 (Supplementary Fig. 3B). The spherical structure-inducing effects of HRG were inhibited by the addition of rabbit polyclonal Ab against HRG but not by control IgG (Fig. 4E, F), 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 (Fig. 4G). The adhesion property of HRG-treated neutrophils on vascular endothelial cells (EA.hy926) was then examined (Fig. 4H-K). As shown in Figure 4K, HRG significantly inhibited the adhesion of neutrophils on the EA.hy926 surface. On the other hand, HRG did not change in the shape and adhesion of mononuclear leukocytes on vascular endothelial

cells (Supplementary Fig. 4A, B).

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 (Supplementary Fig. 5A). However, the chemotaxis-inducing activity to the horizontal direction was detected solely in fMLP and not in HRG (Supplementary Fig. 5B). 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 (Fig. 5A, B). 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 (Fig. 5A, B). 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 (Fig. 5C). On the other hand, cytosolic G-actin was dominant in neutrophils treated with HBSS, BSA, and HSA (Fig. 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. Figure 5D, E and Supplementary Video 4 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 (red arrowheads) or on (white arrowheads) the microcapillary slits. HRG-treated neutrophils flowed through the slits much more smoothly in a teardrop shape (Fig. 5D, E and Supplementary Video 4). 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 (Fig. 5F, G and Supplementary Video 5).

HRG-fluorescein was taken up into neutrophils. There were two types of staining pattern of HRG-fluorescein: granular and homogeneous (Supplementary Fig. 6A, B). 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 (Supplementary Fig. 6C). The incubation of EA.hy926 with HRG-fluorescein showed a homogeneous staining pattern, implying the cell surface binding of HRG-fluorescein (Supplementary Fig. 6D, E).

Pharmacological Analysis of HRG-inducing Signal Transduction in Neutrophils. Pretreatment with BAPTA for 30 min (50 μM) prevented HRG from inducing spherical shape formation (Fig. 6A). 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 (Fig. 6B, C). Wortmannin and LY294002, inhibitors of PI3-kinase, concentration-dependently induced the spherical shape changes in neutrophils, similar to the effect of HRG (Supplementary Fig. 7A). 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 (Supplementary Fig. 7B). Syk inhibitor, BAY61-3606 had no effects on neutrophil shape in the presence or absence of HRG (Supplementary Fig. 7C). 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 (Fig. 6D).

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 (Fig. 6E). The ROS production levels in HRG (1 μM)-treated neutrophils was less than 5% the levels in the HBSS-, BSA-, and HSA-treated groups (Fig. 6E). The ROS production inside the neutrophils was

determined by DCF fluorescence after incubation in the presence of HRG (1 μM) or other factors (Fig. 6F). Consistent with the extracellular ROS, ROS production inside the cells was lower in HRG-treated neutrophils than in any of the other groups (Fig. 6F). Although HRG did not affect the expression of CD11b, CD62L, CD162 on neutrophils in resting as well as agonist (C5a, IL-8 and fMLP)-stimulated condition, HRG inhibited the expression of activated form of CD11b, irrespective of the presence of agonists (Supplementary Fig. 8). 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.

Effects of HRG on LPS- or TNF-α- induced Expression of Adhesion Molecules and Cell Death in Vascular Endothelial Cells. Vascular endothelial cells must be activated or lesioned under septic condition. Therefore, we determined the effects of HRG on vascular endothelial cells (EA.hy926), especially focused on adhesion molecule expression and cell death. As shown in Fig. 6G-J, HRG (1μM) inhibited LPS (10ng/ml)- or TNF-α (10ng/ml)- induced expression of ICAM-1 and P-selectin. Also, HRG (1μM) strongly inhibited the endothelial cell death induced by both LPS and

TNF-α (Fig. 6K, L), suggesting a protective role of HRG against endothelial injury.

Discussion

In the present study, we clearly showed a novel and important role of HRG in controlling the shape, adhesiveness, passage and basal ROS-producing activity of neutrophils. No plasma protein has ever been reported to have such activity. Judging from the plasma HRG levels (around 1 μM) in healthy human[17, 34], the effects of HRG on neutrophil shape in the circulation under normal condition are speculated to be maximal. A marked decrease in plasma levels of HRG in septic conditions due to rapid reduction in HRG gene expression (Fig. 1C, D) may lead to increased adhesion of neutrophils to vascular endothelial cells and retardation of neutrophil passage in the microvasculature demonstrated by *in vivo* imaging and *in vitro* experiments; the changes are associated with the enhancement of ROS production. Thus, the neutrophil shape maintained by HRG should be suitable for their passage through the capillary vessels, sustaining rheological stability, and preventing unnecessary activaton of vascular endothelial cells. The spherical shape probably minimizes the surface attachment area of neutrophils to vascular endothelial cells and reduces physical contact between neutrophils and endothelial cells in the microvasculature, easing the passage of neutrophils through capillary vessels as observed by *in vivo* imaging of circulating neutrophils in CLP mice treated with HRG. In addition, it was revealed that HRG

slightly inhibited the expression of activated but not inactivated form of CD11b, irrespective of the presence of neutrophil activation agonists. This effect of HRG may also limit the unnecessary interaction between circulating neutrophils and vascular endothelial cells. In contrast, the deformed neutrophils in septic condition sometimes occupy the flow of microcirculation by forming a cell cluster, implying enhanced interaction between deformed neutrophils and vascular endothelial cells or deformed cells themselves. This attachment pattern seems quite different from that observed during the migration of neutrophils infiltrating inflamed sites[35-37].

Immunohistochemical analysis of septic ARDS in CLP mice clearly showed that the number of adhension sites of circulating neutrophils to pulmonary vascular walls increased remarkably. Above 70 % of the sites, we observed the overlapping platelets aggregation and fibrin deposits. This strongly indicated that a major portion of neutrophil attachment sites in the lung developed immunothrombosis[12]. The marked inhibitory effects of HRG on immunothrombosis in pulmonary vasculatures (Fig. 2C, E) were presumably due to suppression of initial neutrophil attachment by keeping morphological and functional quiescence of circulating neutrophils because the relative percentage of CD42d and fibrin positive sites on neutrophils were not different among CLP groups (Fig. 2F). In addition, almost complete inhibition of the increase in

plasma IL-6 and TNF-α by HRG administration could contribute to cancellation of activation of vascular endothelial cells that facilitates immunothrombosis. The finding that HRG antagonized the pro-DIC state together with the anti-immunothrombotic effects as well as strong inhibition of lung inflammation implies that HRG administration suppressed the major and fatal responses in sepsis; DIC, multiple organ failure due to microthrombus formation, and severe inflammatory injuries in plural organs. It was reported that neutrophil extracellular trap (NET) formation[13, 14] was involved in different types of immunothrombosis. In the present model, we confirmed NETosis occurring in the lung vasculatures leading to immunothrombosis (Fig. 2G, H).

Analysis using pharmacological tools suggested that cytoskeltal arrangement by HRG may be mediated by inhibition of PI3-kinase and p38 kinase but not ERK. These possible signal transduction pathways were consistent with the reported pathways for controlling neutrophil shape and ROS production[38-41]. Moreover, the HRG-induced shape change of neutrophils may be accompanied by the regulation of cytosolic calcium judging from the antagonizing effects of BAPTA and the time-dependent increase in intracellular calcium by HRG. Further studies are needed to clarify the molecular mechanisms underlying HRG's action on neutrophils.

C5a, IL-8, and fMLP induced the expression of CD11b and CD162 on the neutrophil

surface and shed off CD62L, as detected by FACS (Supplementary Fig. 8). HRG did not influence the basal and agonist-induced changes in adhesion molecules on the neutrophil surface except activated form of CD11b (Supplementary Fig. 8). Since HRG did not influence the expression of PSGL-1 and Mac-1 induced by IL-8, C5a and fMLP, HRG was speculated not to inhibit the agonists-induced facilitation of neutrophil migration to the destination. This speculation was supported by the present finding that there were no differences in the total number of infiltrating neutrophils in the peritoneal cavity between HRG-treated and control CLP mice. Therefore, it is possible that HRG does not interfere with neutrophil activation and migration of neutrophils which are responsible for the recruitment of neutrophils into inflamed sites while playing a role in maintaining the basal state of circulating neutrophils. In contrast, HRG concentration-dependently antagonized agonist-induced morphological changes and vice versa (Supplementary Fig. 9).

Excess activation and even lesion of vascular endothelial cells represent the disorder of septic condition[42]. The inhibition of expression of ICAM-1 and P-selectin on vascular endothelial cells induced by LPS or TNF-α *in vitro* by the addition of HRG (1μM) strongly suggested that physiological concentration of HRG in the circulation constantly suppresses the activation of vascular endothelial cells. Furthermore, HRG

appeared to play a protective role against endothelial cell death induced by LPS and TNF-α. Thus, it was concluded that HRG is a crucial regulatory factor of vascular endothelial cell function.

Taken together, the results in the present study strongly suggested that the decrease in plasma HRG constitutes the fundamental pathway for septic pathogenesis. The supplementary treatment of CLP mice with HRG may simultaneously improve complex and multiple aspects of the serious disorders found in septic conditions: the uncontrolled activation of circulating neutrophils, the activation and lesion of vascular endothelial cells, the immunothrombosis, cytokine overproduction, and the disorder of coagulation and fibrinolysis cascades (Supplementary Fig. 10). Supplementary therapy with HRG may provide a novel strategy for the treatment of septic patients[6, 43-46] although there might be a therapeutic time window for the treatment.

Methods

Reagents. Wortmannin, LY294002, SB239063, BAY61-3606, isoluminol, and horseradish peroxidase type IV were obtained from Sigma (St. Louis, MO). DAPI, SYTOX Blue, Hoechst33342, Calcein-AM, Fluo-4, CM-H_2DCFDA, Phalloidin-Alexa584, DNaseI-Alexa488, BAPTA-AM, and Invivofectamine were obtained from Life Technologies. FR180204, SP600125 and anti-phosphatidylserine Ab were obtained from Merck (Darmstadt, Germany). fMLP was obtained from Peptide Institute, Inc. (Minoh, Japan). Toxin B from *C. difficile* was obtained from List Biological Laboratories (Campbell, CA). C5a, IL-8, and anti-mouse CD42d Ab were obtained from R&D systems (Minneapolis, MN). Anti-mouse fibrinogen/fibrin Ab, DyLight488-labeled anti-human CD54 Ab, Alexa Fluor 647-labeled anti-rabbit IgG, Alexa Fluor 647-labeled anti-sheep IgG, Alexa Fluor 568-labeled anti-rabbit IgG and Alexa Fluor 568-labeled anti-sheep IgG were obtained from abcam (Cambridge, UK). Anti-mouse Gr-1-FITC and FITC-labeled anti-human activated CD11b Ab were obtained from eBioscience (San Diego, CA). Alexa Fluor 488-labeled anti-human CD62P Ab was obtained from BiossAntibodies (Woburn, MA). Anti-mouse Gr-1-Alexa Fluor 594 was obtained from BioLegend (San Diego, CA). FITC-labeled anti-human CD11b Ab and FITC-labeled anti-human CD62L Ab were obtained from

Beckman Coulter (Brea, CA). PE-labeled anti-human CD162 Ab was obtained from Novus Biologicals (Littleton, CO). Anti-human HRG monoclonal antibody (Rat) (#75-14) and anti-human HRG polyclonal antibody (Rabbit) were made in our laboratory.

Cell preparation. In accordance with the ethics approval and guidelines of Okayama University, written informed consent was obtained from healthy volunteers (n=5), and blood was drawn from the cubital vein. The blood was layered onto Polymorphprep or Lymphoprep (Axis-Shield, Oslo, Norway) prior to density-gradient centrifugation. The neutrophils or peripheral blood mononuclear cells (PBMCs) purified were suspended in HBSS buffer. The EA.hy926 cells (ATCC CRL-2922), a hybridoma of HUVEC and human epithelial cell line A549, were cultured in DMEM (ATCC) containing 10% FBS (Invitrogen). These cells were grown in a humidified atmosphere and passaged every 3-4 days.

Purification of HRG from human plasma. HRG was purified from human plasma (supplied by the Japanese Red Cross Society) as previously described[47]. In brief, human plasma was incubated with nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen,