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Review

Novel aspects of sepsis pathophysiology: NETs, plasma glycoproteins, endotheliopathy and COVID-19

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ABSTRACT

In 2016, sepsis was newly defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Sepsis remains one of the crucial medical problems to be solved worldwide. Although the world health organization has made *sepsis* a global health priority, there remain no specific and effective therapy for sepsis so far. Indeed, over the previous decades almost all attempts to develop novel drugs have failed. This may be partly ascribable to the multifactorial complexity of the septic cascade and the resultant difficulties of identifying drug targets. In addition, there might still be missing links among dysregulated host responses in vital organs. In this review article, recent advances in understanding of the complex pathophysiology of sepsis are summarized, with a focus on neutrophil extracellular traps (NETs), the significant role of NETs in thrombosis/embolism, and the functional roles of plasma proteins, histidine-rich glycoprotein (HRG) and inter-alpha-inhibitor proteins (IAIPs). The specific plasma proteins that are markedly decreased in the acute phase of sepsis may play important roles in the regulation of blood cells, vascular endothelial cells and coagulation. The accumulating evidence may provide us with insights into a novel aspect of the pathophysiology of sepsis and septic ARDS, including that in COVID-19.

1. Introduction

Sepsis is an infection-associated pathological condition that frequently leads to life-threatening and interactive organ failures such as acute respiratory distress syndrome (ARDS), circulatory shock, renal failure and disseminated intravascular coagulation (DIC).[1–3] Sepsis is also accompanied by a decreased level of consciousness due to CNS disturbance.[3] Much basic and clinical research has been conducted to extensively characterize the pathophysiology of sepsis.[4–7] Although this great research effort has led to accumulated knowledge of the clinical management of sepsis and gradually improved the outcome of patients,[8,9] sepsis remains the leading cause of death worldwide, with both high mortality and morbidity. Therefore, the world health organization recently made sepsis a global health priority.[10]

Because sepsis accompanies the systemic inflammation due to infection, the efforts to develop anti-septic drugs have generally targeted inflammation-related and coagulation-related molecules.[2,11] Table 1 provides a list of representative clinical trials. Unfortunately, this series of clinical trials has failed to yield any clinical breakthroughs, despite the various treatments showing favorable effects in animal models. Therefore, at present there is no available drug specific for sepsis treatment.[25]

In 2016, sepsis was renamed Sepsis-3 and redefined as life-threatening multi-organ failure with infection.[3] Although the original causes of infection, kinds of pathogens, and routes of infection and the preceding compromising conditions of patients vary from case to case, there might be a common biological cascade of events that includes respiratory failure, circulatory shock, renal failure and/or DIC.

In the last several years, the worldwide SARS-Cov-2 pandemic has led to the death of increasing numbers of patients due to ARDS and embolism/thrombosis.[26,27] Neutrophil extracellular traps (NETs),[28,29] one form of neutrophil activation, may not only inhibit diffusion of bacteria in the blood stream but also facilitate the intravascular coagulation and the damage to vascular endothelial cells, leading to the formation of immunothrombi.[30–32] Such

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Table 1
Major clinical trials for the treatment of septic patients.

Drugs or apparatus	Number of patients	Primary endopoint	Outcome	Reference
Anti-Lipid A mAb	543	Survival at 28 days	No effect	12
rh IL-1R antagonist	893	Survival at 28 days	No effect	13
Anti-TNF-α mAb	971	Survival at 28 days	No effect	14
rh Soluble TNF-R-Fc	141	Survival at 28 days	No effect	15
Antithrombin	2314	Survival at 28 days	No effect	16
rh TNF-R55-IgG1	1342	Survival at 28 days	No effect	17
rh Activated protein C	1690	Survival at 28 days	Effective	18
TAK-242	274	Survival at 28 days	No effect	19
rh Activated protein C	1697	Survival at 28 & 90 days	No effect	20
Eritoran	1961	Survival at 28 days	No effect	21
PolymyxinB-immobilized Column	450	Survival at 28 days	No effect	22
Vitamin C, thiamine, and hydrocortisone	501	Ventilation- and vasopressor- free days	No effect	23
Vitamin C, thiamine, and hydrocortisone	205	SOFA score at 72 h	No effect	24

rh: Recombinant human.

processes should involve the trapping of red blood cells (RBCs) in the clot and may be associated with the release of hemoglobin and subsequently heme and Fe^{2+} from the clot.[33–35]

In this review, I will summarize the recent advances in understanding of the sepsis pathophysiology, with a particular focus on NETs, endotheliopathy, and RBCs. I will also provide novel insights regarding specific plasma glycoproteins that are rapidly decreased during sepsis and that both regulate the interaction between blood cells and vascular endothelial cells and control the coagulation process.

2. Sepsis pathophysiology

2.1. Animal models of sepsis

Cecal ligation and puncture (CLP) has been used as a convenient animal model of polymicrobial sepsis in mice or rats because the severity and lethality can be controlled by the number of punctures, the size of the puncture needle and the volume of intestinal contents ejected. This model principally resembles the perforation in the lower intestinal tract in humans. Many candidate drugs for the treatment of sepsis have been evaluated using this model. Table 1 summarizes the major clinical trials of candidate drugs. As is well known, all of these pharmaceuticals failed to achieve the targeted effect -namely, an improvement of 28 day survival in patients (Table 1)- although these drugs were effective against animal models. One of the reasons for the clinical failure of these candidate drugs appears to the diversity of original diseases and the variation in individual disease severity and underlying conditions.[3,25,36] Another cause may be the sepsis-induced dysregulation of plural organs that are essential for life.[1,2,25] Multiorgan failure-related events during sepsis include respiratory failure, circulatory shock, renal failure, DIC and CNS disturbance.[11,37,38] These failures are interrelated and form a negative spiral response leading to crucial disorder of multiple organs, and finally to death.[38,39] The fundamental features of the common processes of sepsis have been repeatedly described in reviews.[1,2,40] Among the dysregulated responses, the vascular endotheliopathy or endothelitis,[38,41,42] a dysfunctional state of endothelial cells due to the inflammation associated with thrombosis/embolism, may make major contributions to the development of septic ARDS, circulatory shock and DIC.

2.2. Endotheliopathy or endothelitis in sepsis

The dysregulation of vascular endothelial cells may play a critical role in the development of organ failure,[38,41,43] and especially the failure of microcirculation in the lungs because healthy lung capillaries have a smaller diameter than the capillaries in other tissues. Activation of vascular endothelial cells in the lungs under a septic condition promotes the surface expression of adhesion molecules such as E-selectin, ICAM-1 and V-CAM-1. The contractile response of endothelial cells increases the permeability of capillaries, leading to the leakage of plasma proteins from blood to interstitial fluid and edema formation on the alveolar walls. Once the attachment of infiltrating neutrophils has been activated, the activation of endothelial cells may be enhanced.[44,45] Such excessive stimulation may lead to the injury and lesion of endothelial cells, including the loss of an anticoagulation system on their surface. In fact, recent studies suggested that the glycocalyx layer of lung capillaries was diminished during sepsis in an animal model[46–48] and a similar change is suspected in the lung tissue infected with SARS-Cov-2.[48] Since the glycocalyx probably regulates the interaction between blood cells and vascular endothelial cells, adjusts the trapping of many factors inside the glycosaminoglycan and finely modulates the coagulation cascade,[45,49] the diminution or loss of glycocalyx on the surface of vascular endothelial cells during sepsis would profoundly affect the very important roles of vascular endothelial cells.[45,48,50] Thus, the balance of regulation and activation of neutrophils and vascular endothelial cells by many factors, including cytokines, plasma factors and locally produced active substances, should be kept within a range that maintains the homeostasis of vascular endothelial cells.

2.3. Neutrophil extracellular traps (NETs) in sepsis

Neutrophil extracellular traps (NETs) are a host defense mechanism against bacteria first described by Brinkmann et al. (2004).[28] Under specific conditions, neutrophils can release chromatin DNA as a web-like structure called a NET without disrupting the plasma membrane, in association with an accompanying release of histones, neutrophil elastase and myeloperoxidase on web-like DNA (Fig. 1). NETs can trap and kill bacteria by using proteinases and myeloperoxidase, thereby preventing the diffusion of bacteria into the blood stream and surrounding tissues.[28,51,52] There is increasing evidence that this mode of neutrophil activation may be involved in different kinds of disease conditions.[53–55] In sepsis, uncontrolled generation of NETs that adhere to injured vascular endothelial cells facilitates platelet aggregation and coagulation on the attached sites.[56–58] The resultant formation of thrombus is called “immunothrombus” because it is initiated by the adhesion of NETs to vascular endothelial cells (Fig. 1). Recent research on sepsis suggests that the systemic occurrence of immunothrombosis is a state of DIC,[47,51,59] leading to the impairment of tissue microcirculation and oxygenation, and finally organ failure.[39,57] Therefore, the regulation of both NETs and immunothrombosis are thought to be important targets for

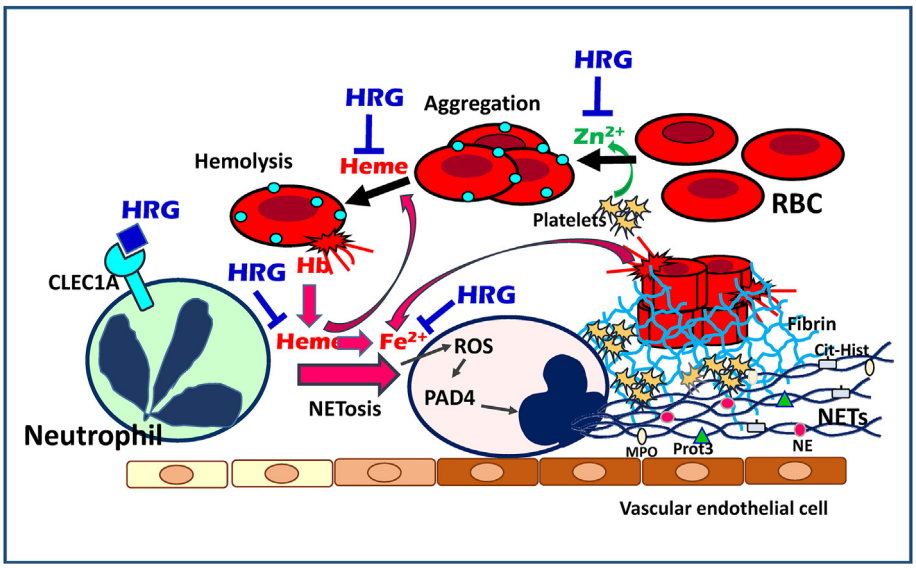

Fig. 1. Neutrophil extracellular traps (NETs), the involvement of NETs in immunothrombosis, and the effects of HRG. NETs play very important roles in the promotion of septic symptoms in collaboration with vascular endothelial cells and RBCs. HRG inhibits NETs by acting on multiple site including CLEC1A receptor and hemoglobin degradative products.

the treatment of DIC,[57,58] which leads to the impairment of tissue microcirculation and finally organ failure.[39]

Before we can regulate NETs, we first need to uncover their molecular mechanisms of NETs.[28,60,61] The major cascade of intracellular signaling that gives rise to NETs has been clarified as the following. Calcium mobilization from intracellular stores triggered by stimulation from various receptors leads to an increase in cytoplasmic free calcium levels. This in turn activates protein kinase C and NADPH oxidase to form reactive oxygen species (ROS). Intracellular ROS activates peptidylarginine deiminase 4 (PAD4)[62] and the subsequent conversion of arginine residue into citrulline, thereby facilitating decondensation of chromatin. Simultaneously, neutrophil elastase and myeloperoxidase move from azurophilic granules to the cytoplasm and then the nuclear compartment. Finally, the disruption of the nuclear membrane occurs and the neutrophils release DNA attaching citrullinated histones, proteinases and myeloperoxidase.[28,29]

To demonstrate the NETs in the tissue preparations from animals[59] and from autopsy samples,[32,63,64] one approach would be to detect the citrullinated histone on the extracellular DNA released from neutrophils. Usually, NETs are associated with aggregation of platelets and fibrin formation.[59] Immunothrombus has been reported to be present under many disease conditions, including ARDS in COVID-19.[31,32,63]

Autopsy studies have revealed that thrombosis and embolism are an exacerbating factors in COVID-19 and often increase the risk of mortality.[65–67] Clinical examinations have shown that the elevation of D-dimer, a degradation product of polymerized fibrin by plasmin, and an increase in the neutrophil/lymphocyte ratio appear to be markers that distinguish between severe and mild COVID-19.[68–70] An increase in red blood cell distribution width (RDW) is also a marker, which suggests that a disorder of RBC lineage or hemolysis may be present in severe COVID-19.[71–73] Consistent with the finding that RDW was higher in patients with severe than patients with mild COVID-19, haptoglobin, a hemoglobin-scavenging protein in plasma, was reported to be reduced in patients with severe COVID-19,[74] supporting the notion that hemolysis may be involved in the elevation of RDW. Further research will be needed along this line, because hemoglobin and its degradation products, heme and Fe^{2+}, have strong toxic effects on different types of cells and enhance the inflammatory responses.[33,34,75]

Heme, a degradative product of hemoglobin, is a ligand for pattern recognition receptor RAGE[76] and MD-2.[77] The latter binding triggers MD-2/TLR-4 signaling.[78] Recently, lower concentrations of heme were demonstrated to induce the endothelial adhesion of human neutrophils associated with ROS production,[79] whereas at higher concentrations heme induced NETs.[80] These results suggest that heme stimulates neutrophils differentially depending on its concentration and mechanisms. It seems probable that a high concentration of heme in the extracellular space is one of the endogenous stimulants that induce NETs. Recently, NETs have been suggested to be involved in the development of thrombosis in association with different underlying diseases. It is possible that NETs and hemolysis are closely related[81] (Fig. 1).

3. A plasma protein, histidine-rich glycoprotein (HRG)

HRG is a multifunctional plasma protein of about 75 kDa. HRG was first isolated as one of four serum proteins adsorbed tightly to carboxymethyl-cellulose with high content of histidine residues[82]. The normal concentration of HRG in plasma is around 1–1.5 μM in humans.[83,84] The half-life of plasma HRG in human was estimated to be 3 days using ^{125}I-labeled HRG,[85] whereas that in mice was reported to be much shorter (15 h).[59] This suggests a species difference in the turnover of HRG in plasma.

HRG is composed of six domains: cystatin-like domain 1, cystatin-like domain 2, proline-rich domain 1, histidine-rich domain, proline-rich domain 2, and C-terminal domain.[83,86] One of the remarkable features of HRG is that HRG binds to a diverse range of factors, including fibrinogen,[87] plasminogen,[88] thrombospondin,[89] FXIIa,[90] C1q,[91] zinc,[92] heme,[93] Fe^{2+},[94] IgG,[91] heparin[95] heparan sulfate,[96] heparanase,[97] glutathione peroxidase,[94] DNA(RNA),[98] polyphosphate[99] and lipopolysaccharide[100] (LPS; an outer membrane constituent of the gram negative bacteria) (Table 2). Through the binding to fibrinogen, FXIIa and plasminogen, HRG appears to regulate both coagulation and fibrinolysis.[83,101,102] The interaction of HRG with thrombospondin may have dual effects on angiogenesis, depending on the coexisting factors in the micro-miliue.[83,103,104] HRG also antagonized the

Table 2
Binding factors of histidine-rich glycoprotein (HRG).

Factors	Classification	Functional implication	Reference
Fibrinogen	Coagulation factor	Regulation of coagulation	87
Plasminogen	Fibrinolysis factor	Regulation of fibrinolysis	88
Thrombospondin	Matricellular protein	Anti-angiogenesis	89
FXIIa	Coagulation factor	Regulation of coagulation	90
C1q	Complement component	Regulation of complement activation	91
Zn^{2+}	Metal ion	Modulation of HRG actions	92
Heme	Iron-bearing porphyrin	Scavenging of heme	93
Fe^{2+}	Metal ion	Chelation of Fe^{2+}	94
IgG	Immunoglobulin	Regulation of immune response	91
Heparin	Sulfated polysaccharide	Antagonism for antithrombin	95
Heparan sulfate	Sulfated polysaccharide	Retention on glycosaminoglycan	96
Haparanase	Enzyme	Modulation of heparanase action	97
Glutathione peroxidase	Redox enzyme	Enhancement of enzyme activity	94
DNA (RNA)	Nucleic acid	Inhibition of thrombosis	98
Inorganic polyphosphate	High-polymeric phosphate	Inhibition of thrombosis	99
LPS	Endotoxin	Inhibition of endotoxemia	100

angiogenesis induced by HMGB1 in the presence of heparin.[105] Moreover, HRG plays a protective role in host defense against bacteria[106,107] and HIV in vivo.[108] Finally, HRG was reported to be involved in tumor growth regulation.[109–111] Thus, HRG plays important functional roles in the regulation of coagulation/fibrinolysis, host defense, anti-angiogenesis, and tumor growth through the binding to these interacting molecules.[83,111,112]

3.1. HRG effects on neutrophils

Recent studies have shown that HRG has marked effects on human neutrophils.[59] In an in vitro study of purified human neutrophils from peripheral blood, physiological concentrations of HRG induced a round morphology with reduced microvilli on the cell surface.[59,113] Although the spontaneous release of extracellular ROS from the round neutrophils was strongly suppressed compared with that from non-round neutrophils in the absence of HRG,[59] the phagocytotic activity against bacteria was enhanced in the round neutrophils.[114] Moreover, the round neutrophils passed through the artificial microchannels more easily than non-round cells and exhibited a lower adhesion on the vascular endothelial cells.[59] Furthermore, the treatment of human whole blood with anti-HRG antibody was found to slow the passage of leukocytes through microchannels.[59] Taken together, these functional regulations of human neutrophils by physiological concentrations of HRG imply that HRG maintains the basal and quiescent state of circulating neutrophils with low levels of spontaneous ROS production.[114] Conversely, once they encounter bacteria, these round neutrophils exhibit a higher mobility and phagocytic activity, suggesting that HRG maintains neutrophils in a state ready for activation.[114] These observations are consistent with the observation of antibacterial effects of HRG in vivo.[106,107]

3.2. HRG effects on RBCs

The effects of HRG on red blood cells (RBC) have also been reported. Platelets and mast cells contain zinc ion in their granules, which is released from these cells during secretory response. A low concentration of zinc (20 μM) induced phosphatidylserine-dependent aggregation of RBCs. This aggregation was completely inhibited by the presence of a physiological concentration of HRG (1 μM).[115] Zinc ion elicited an increase in intracellular free calcium, which was inhibited by HRG, and the increase in calcium seemed to be a trigger of a cellular cascade leading to the expression of phosphatidylserine on RBCs.[115] In the same study, aggregated RBCs tended to attach to the surface of a monolayer of vascular endothelial cells in culture.[115] Once coagulation starts, RBCs should contribute to the clot formation by deforming themselves into a cuboid shape.[116,117] During the disseminated intravascular coagulation (DIC) under a septic condition, some degree of hemolysis may occur in the clots, leading to the release of hemoglobin and heme.[115,118,119] Since heme itself elicits hemolysis, there might be a vicious cycle between DIC and hemolysis. Very interestingly, in addition to zinc-induced RBC aggregation, HRG strongly inhibited heme-induced hemolysis through its high capacity binding activity to heme[115] (Fig. 1), implying that HRG might scavenge zinc and heme from the extracellular space. These effects of HRG should lead to the control or prevention of the vicious cycle. Hemoglobin as well as heme once released by hemolysis should in turn stimulate the vascular endothelial cells and even be harmful depending on the concentrations.[78,120,121] Thus, the aggregation of RBC may be one of the factors contributing to endothelial injury and subsequent formation of thrombi within the vasculature. It is worth pointing out that LPS may facilitate hemolysis via direct membrane interactions.[122]

Although relatively little attension has been paid to the shape change and the aggregatory response of RBCs, the release of hemoglobin and its degradative products into the extracellular space during hemolysis must be very important, because the hemolytic products exert harmful effects on different kinds of cells besides vascular endothelial cells through their peroxidase activity and ROS production.[33,34,75] Collectively, these effects of HRG suggest that HRG maintains the homeostasis and quiescence of RBCs in addition to neutrophils.

3.3. HRG effects on vascular endothelial cells: an emerging functional role of high mobility group box-1 (HMGB1) in systemic inflammation

Both LPS and TNF-α have been shown to stimulate endothelial cells in terms of expression of adhesion molecules, loosing of intercellular cadherin, cytoskeletal rearrangement and cytokine production, in association with an increase in endothelial permeability.[123,124] These effects of LPS and TNF-α were strongly inhibited by HRG, probably through a reduction of NF-κB activation and suppression of p38 and JNK kinases, as judged by an analysis of intracellular signaling.[124] Thus, HRG has strong protective effects on vascular endothelial cells in vitro.

Since the pathophysiologic processes of sepsis are extremely complex, there are many factors involved in the development of the septic cascade: pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and a diverse

range of mediators including coagulation factors, complements, cytokines, and many bioactive substances.[1,2,25]

In 1999, high mobility group box-1 (HMGB1) was rediscovered as a late mediator of endotoxemia in mice.[125] Originally, HMGB1 was called chromatin DNA-binding nuclear protein, and was known to play roles within nuclei, such as for the regulation of transcription activity, stabilization of chromatin structure and DNA repair.[126] Research into the extracellular roles of HMGB1 opened a new aspect of cell-derived alarmins, later called damage-associated molecular patterns (DAMPs).[127] HMGB1 is now characterized as a representative DAMP.[128,129] Initially, HMGB1 was reported to be released from necrotic cells: however, it was soon demonstrated that under different stimuli and stressors HMGB1 can be translocated from nuclei to the cytosolic compartment, and ultimately released extracellularly.[130] Once released into the extracellular space, HMGB1 appears to be an enhancer of inflammatory responses through the direct binding to RAGE and toll-like receptor-4/2.[127] In addition, HMGB1 enhances the inflammatory responses by forming complexes with IL-1β and CXCL12, since these complexes increase the affinities of cytokines for their cognate receptors.[131,132]

There have been numerous reports concerning the involvement of HMGB1 in a diverse range of inflammatory responses in many disease conditions, including stroke,[133–135] traumatic brain injury,[136,137] spinal cord injury,[138] epilepsy,[139] lung inflammation,[141] arthritis[142] and reperfusion injury of many organs.[143,144] HMGB1 is a ubiquitous protein, and therefore, any kind of cell could be a source of extracellular HMGB1 under different conditions. Among the candidate cells, the release of HMGB1 from macrophages has been well demonstrated.[145] Some chemical modifications on HMGB1, such as acetylation[146] or phosphorylation,[147] may be involved in the initiating the dissociation of HMGB1 from chromatin DNA and triggering its translocation. The secretory pathway of HMGB1 has not been clarified yet.[148]

Gao et al.[123,124] showed that TNF-α and LPS induced the translocation and release of HMGB1 from vascular endothelial cells. This release response was strongly inhibited by physiological concentrations of HRG[123,124] (Fig. 2). The inhibition of HMGB1 release was accompanied by a simultaneous reduction in the sectretion of cytokines such as IL-6, IL-8, IL-1α and IFN-γ (Fig. 2). The effects of HRG on vascular endothelial cells appears to be mediated at least in part by a cell surface receptor, C-type lectin family protein A1 (CLEC1A).[124] The results of profiling of HRG activity on different types of cells are summarized in Fig. 3.

HRG maintained the E-cadherin-dependent intercellular attachment of vascular endothelial cells and the integrity of cellular contact, as discussed above.[123,124] Thus, HRG appears to control the cellular shape, intercellular adhesion, HMGB1 release and activation state of vascular endothelial cells and to maintain their quiescence under a healthy condition. The rapid decrease in plasma levels of HRG observed in septic mice[59] and patients[84,149] suggests that the quiescence-controlling effects of HRG on vascular endothelial cells will be weakened or lost under a septic condition, contributing to the strong interaction between blood cells and endothelial cells. The surface plasmon resonance showed that HRG directly binds to HMGB1 (Nishibori, unpublished observation). These results, as a whole, suggest that plasma protein HRG plays a very important role in the inhibition of mobilization of a representative DAMP, HMGB1, and in the regulation of its activity in the extracellular space. Since HMGB1 has been proposed as a good drug target for different kinds of diseases,[128] much attention should be paid to its relationship with HRG.[39]

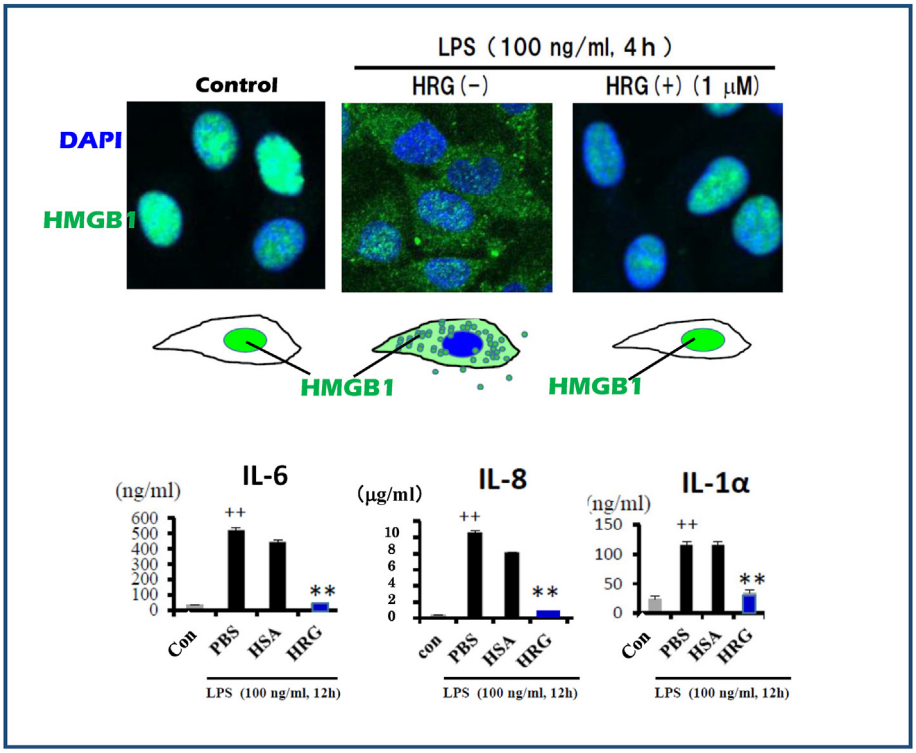

Fig. 2. Effects of HRG on LPS-induced translocation of HMGB1 and cytokine production in vascular endothelial cells (EA.hy926) in culture. The cells were incubated with LPS (100 ng/ml) in the presence or absence of HRG (1 μM) for 4 or 12 h. The translocation of HMGB1 was visualized immunohistochemically (upper panels). The cytokine levels were determined in the supernatants of cultures (lower panels) (modified from the data[123]).

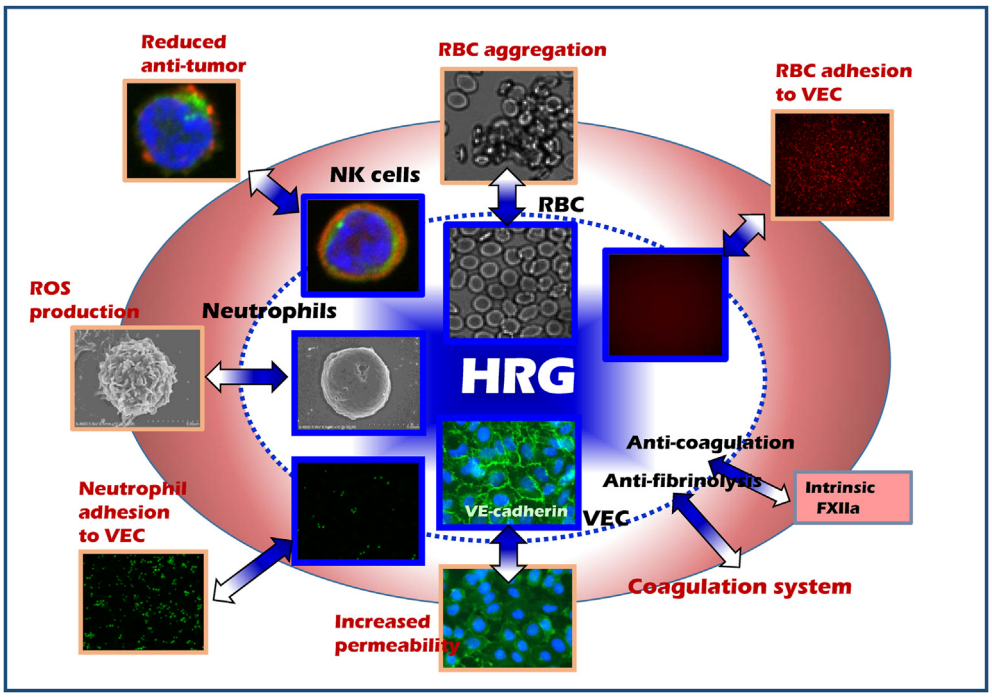

Fig. 3. Profiling of the effects of HRG on blood cells, vascular endothelial cells and coagulation. HRG plays the multifunctional roles in neutrophils, NK cells, RBCs and vascular endothelial cells. HRG also regulates the coagulation/fibrinolysis system. These effects of HRG contribute to the homeostasis of the interface between the blood and vasculatures.

3.4. HRG effects on a mouse sepsis model

Wake et al.[59] observed a marked decrease in plasma levels of HRG in a CLP septic mouse model with high mortality[59] (Fig. 4). This rapid decrease in plasma HRG levels was due to a reduction in hepatic HRG production, degradation by proteases such as thrombin, and consumption of HRG deposited on intravascular thrombi.[59] The septic mice developed lung inflammation associated with the cytokine production and infiltration of neutrophils, which sometimes exhibited the NET formation and contributed to immunothrombosis. In addition, hematological analysis showed that the mice developed a DIC state with reduced platelet counts and prolonged PT/APTT. All these manifestations were strongly inhibited by the supplementary treatment with purified HRG from fresh frozen human plasma, in association with a remarkable improvement of mortality[59] (Fig. 4). In contrast, knockdown of HRG expression in the liver by siRNA and subsequent marked reduction of plasma levels of HRG significantly exacerbated the mortality in the mild CLP mice[59] (Fig. 4). These observations were consistent with the above-mentioned homeostatic and regulatory effects of HRG on blood cells and vascular endothelial cells in vitro. HRG binds to Fe^{2+}, leading to a diminution of hydroxyl radical production by Fenton reaction.[94] HRG also exhibits antioxidant activity against peroxyl radical by oxidation of HRG itself as a substrate. Moreover, HRG enhanced the activity of glutathione peroxidase, a well-known antioxidant enzyme.[94] All these effects of HRG observed in cell-free systems may contribute to the antiseptic effects of HRG, because the oxidative stress, including that from ROS, causes tissue damage and injuries at inflammatory sites. Taken together, these effects of HRG on both cells and cell-free systems suggest that HRG might be a very important factor for preventing the progression of septic state into lethality.

4. Other important plasma protein IAIPs in sepsis

Inter-α-inhibitor proteins (IAIPs) consist of one or two heavy peptide chains connected by glycosylaminoglycan to a light chain, called bikunin[150]. Heavy chains and a light chain are encoded by different genes and there are six kinds of splicing variants for heavy chains. Thus, IAIPs are the family composed of a short form with bikunin and one of heavy chains, and a long form with bikunin and the heterogenous combination of heavy chains[150]. IAIPs show Kunitz-type proteinase inhibitor activity against a broad spectrum of proteinases and share the additional activities. One of these activities is an inhibitory effect on histone H3-induced platelet aggregation.[151] Another is a spherical shape-inducing effect on neutrophils, as observed in HRG.[152] The latter effect appears similar to the spherical-shape induction by HRG, but the distribution pattern of fibrous actin in neutrophils suggests that the regulation of the cytoskeletal arrangement may differ between IAIPs and HRG.[152] IAIPs also maintain the basal state of neutrophils, keeping the spontaneous ROS production at a level as low as that in HRG. The plasma levels of IAIPs are decreased significantly in neonatal[153,154] and mature[155] septic mice, and the administration of purified IAIPs from human plasma has been shown to improve the lethality in septic animals.[155] We can conclude that the kinetic pattern as negative acute phase protein during systemic inflammation, the in vivo antiseptic effects and the effects on neutrophils were common to both HRG and IAIPs.[39,156] In septic patients suffering from dengue, a virus infection, there was a good correlation between the extent of decrease in plasma IAIPs and severity of disease.[157] Thus, it was suggested that plasma protein IAIPs exert anti-septic effects along with HRG.[39] Similar to HRG, IAIPs were demonstrated to bind directly to HMGB1.[158] Investigation of the functional modulation of HMGB1 activity by IAIPs should be pursued.

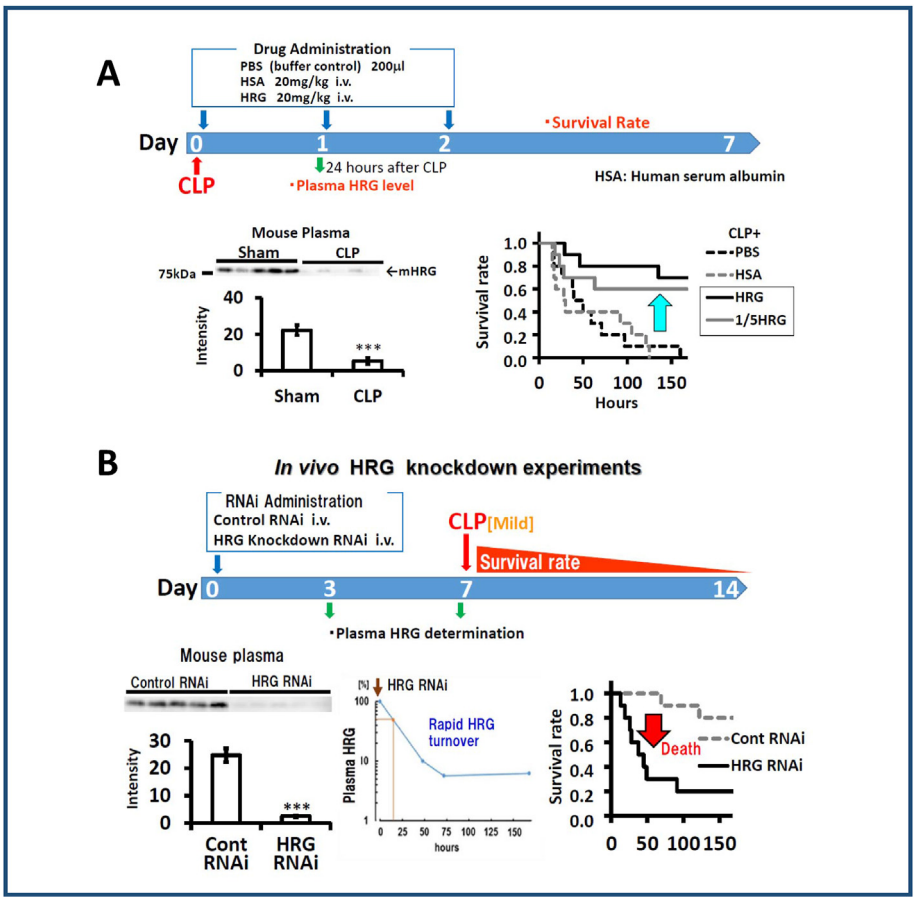

Fig. 4. The supplementary treatment with HRG reduced the lethality of CLP-induced septic mice. (A) CLP was performed in mice, and plasma levels of HRG were determined 24 h after CLP by Western blotting. Kaplan-Meier survival curve of mice treated with PBS, HSA (20 mg/kg), or human-purified HRG (4 or 20 mg/kg) was shown. The supplementary treatment with HRG remarkably ameliorated the lethality of CLP mice. (B) Effects of knockdown of liver HRG by siRNA on the plasma levels of HRG and on the lethality of mild-CLP mice. The plasma levels were determined at the time points indicated. The knockdown of liver HRG by siRNA and subsequent reduction of plasma HRG levels significantly exacerbated the lethality of mild-CLP mice (modified from the data[59]).

5. Novel aspects of septic pathophysiology

ARDS is one of the major manifestations in severe sepsis and often the direct cause of death in septic patients.[2,3] In ARDS, the respiratory function is severely impaired by lung inflammatory responses, which include the activation of alveolar epithelial cells and residual macrophages, the permeability increase of lung capillaries, and the adhesion and infiltration of leukocytes into lung parenchyma.[37,43] In addition, lung vascular endothelial cells should be activated strongly or injured expressing adhesion molecules on their surface. Such vascular endothelial cells may undergo a dramatic change in their surface properties from an anti-coagulation state to a pro-coagulation state, losing thrombomodulin, diminution of glycocalyx and expression of tissue factor.[41,43] The increase in capillary permeability may also induce plasma protein leakage and interstitial edema formation. All these cellular and plasma components should contribute to the inflammatory responses in the lung that lead to the impairment of gas exchange in the lung.

Among these contributing factors, recent studies have suggested that NETs may play an important role not only in endothelial injury by ROS but also in the formation of intravascular immunothrombosis.[30,31,38] Thus, it is suggested that NETs play a role in the expansion of the inflammatory response between capillary and alveolar epithelial cells that goes beyond mere bacterial trapping.[32,38]

The pathological processes in the progression of sepsis can be drawn as mutually-related negative spirals incorporating respiratory failure, circulatory shock, renal failure, CNS disturbance, DIC and immune paralysis. In the clinical studies, Kuroda et al.[84,149] observed that the stratified plasma levels of HRG in septic patients upon admission to the ICU can predict the prognosis and outcome of patients (Fig. 5). The efficacy of the determination of plasma HRG as a biomarker was much greater compared with that of the current plasma markers, presepsin and procalcitonin.[84,149] This means that we may be able to identify among the total septic patients in the ICU those critically ill patients who will steadily worsen and that we may be able to make this determination at an earlier time point. Collectively, these results lead us to hypothesize that the decrease in plasma HRG occurs somewhat upstream of the sepsis cascade.[39,149,159] The causal relationship between the decrease in plasma HRG levels and the development of septic symptoms may be one of the reasons why supplementary treatment with HRG had beneficial effects in septic animals.[59] This was also true for IAIPs.[155,160] The flow diagram in Fig. 6 shows a hypothetical cascade of sepsis incorporating functional roles of HRG and IAIPs in the maintenance of homeostasis of blood cells, vascular endothelial cells and coagulation/fibrinolysis.

6. COVID-19

SARS-Cov-2 infection gave rise to a world wide pandemic starting from the end of 2019. It is well known that there are considerable variations in manifestation among the infected

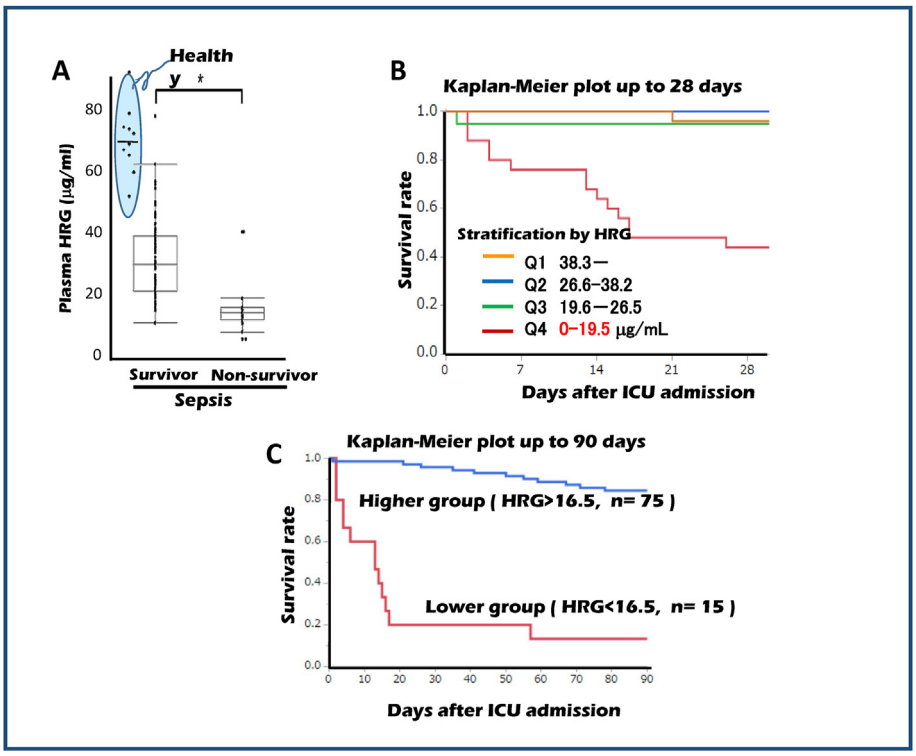

Fig. 5. Data from clinical study on the plasma levels of HRG in septic patients (modified from refs.[83,148]). The plasma levels of HRG in septic patients were determined on the first day of admission to an ICU. The analyses by Kaplan-Meier plot were performed based on the stratification of plasma HRG levels.

population-ranging from no symptoms to severe ARDS with high mortality due to complications such as embolism/thrombosis.[161] Clinical examinations of the COVID-19 patients have revealed that the increases in D-dimer, RDW and the ratio of neutrophils/lymphocytes are more typical findings in severely ill patients when compared with those in patients with mild symptoms.[68,71,72] The

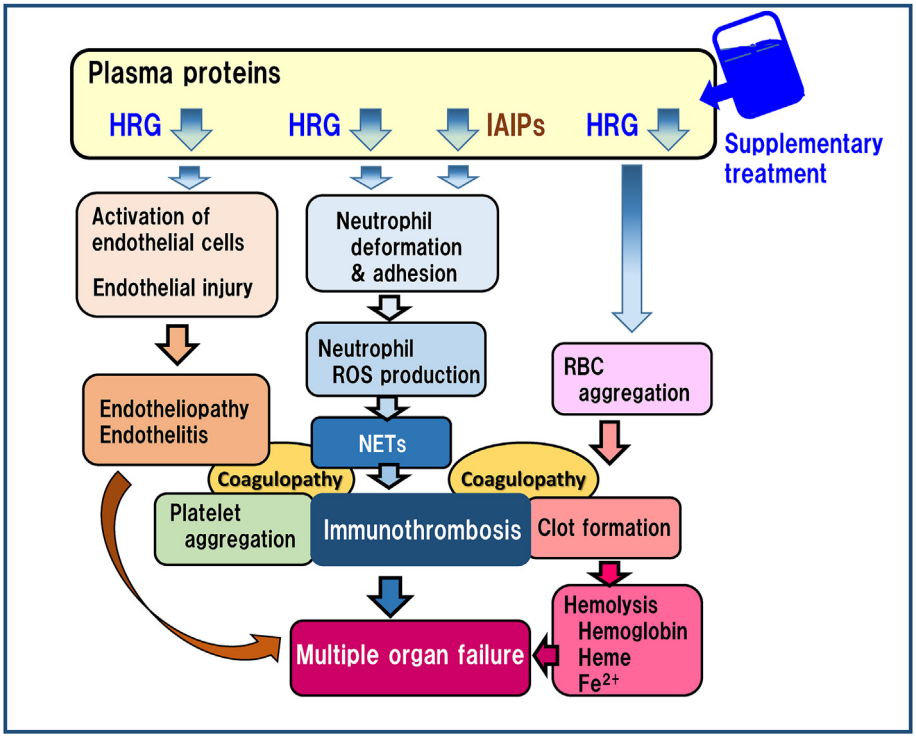

Fig. 6. Flow diagram of a hypothetical cascade of sepsis starting from the decrease in plasma glycoproteins, HRG and IAIPs. There are four major cellular elements depicted: vascular endothelial cells, neutrophils, platelets and RBCs. Coagulopathy participates in immunothrombosis and facilitates multiple organ failure.

increase in D-dimer implies coagulopathy. Consistent with this finding, autopsy studies have demonstrated that different types of embolism and thrombosis are observed among patients deceased from COVID-19, including deep vein thrombosis, lung embolism, multiple microvascular thrombosis, and thrombi in cerebral and cardiac arteries.[65,66,69] In these cases, embolism/thrombosis may have been the cause of death. On the other hand, it was reported that other parameters suggesting typical DIC were lacking.[68–70] Taken together, these results show that the elements of endotheliopathy and endothelitis, but not simple coagulopathy, may contribute to the thrombosis/embolism in severe COVID-19.[69,70]

Recent proteomic analyses on plasma proteins using mass-spectrometry revealed that several changes were prominent in COVID-19 patients compared with normal individuals and between severe and mild COVID-19 patients.[162,163] Very interestingly, these include a dramatic and significant decrease in the levels of HRG and some of IAIPs at an early time point in those patients who go on to develop a severe course of illness.[162,164] It has thus been proposed that the HRG and IAIPs levels may be of prognostic value as early biomarkers of COVID-19.[162,163]

Based on the activities of HRG and IAIPs and their characteristic kinetics in plasma, Nishibori & Stonestreet (2021)[39] suggested that these plasma proteins represent not only excellent biomarkers of COVID-19 but also possible therapeutic targets. In regard to HRG, Gao et al. (2020)[124] identified a receptor for HRG, CLEC1A, by using co-transfection of genes for a transmembrane tethered HRG ligand and candidate receptor into HEK293T cells and by immunoprecipitation of a complex. Therefore, it might be possible that a drug with agonist activity for CLEC1A could be an alternative therapy for sepsis in addition to supplementary HRG.

HRG appears to maintain vascular endothelial cells in a basal state, preventing their unnecessary interaction with blood cells. This may lead to the maintenance of the integrity of endothelial cells by restricting the capillary permeability and the homeostatic regulation of endothelial cells with anti-inflammatory and anti-coagulation activities on their surfaces. Since one of the deleterious and lethal factors in severe COVID-19 seems to be thrombosis/embolism due to endotheliopathy/endothelitis, the above mentioned effects of HRG on vascular endothelial cells could play a homeostatic role and protect against damage induced by SARS-Cov-2. Therefore, it is worth mentioning again that a decrease in plasma HRG from the early time point of COVID-19 might be an excellent marker to estimate the prognosis of patients and distinguish between mild and severe cases.[162] Further studies are necessary along this line.

In a model of highly pathogenic influenza A (H3N2) infection model to human lung microvascular endothelial cells in vitro, Namba et al. (2021)[165] observed that the infection-dependent translocation and release of HMGB1 in non-infected cells. The direct trigger to induce HMGB1 release from non-infected cells was demonstrated to be TNF-α that was secreted from infected cells. If this is also true for endothelitis after the infection with SARS-Cov-2, and the released HMGB1 facilitates the NETosis predisposing to the immunothrombosis on the surface of vascular endothelial cells, then anti-HMGB1 therapy may be useful for inhibiting the progress of inflammatory responses in capillary vessels as shown in influenza A infection.[141,166]

7. Perspectives

We focus our attention to the understanding of pathogenesis of sepsis, including SARS-Cov-2 virus-induced ARDS. The vigorous studies revealed that endotheliopathy/endothelitis should be very important for pathophysiology of both ARDS and accompanying thrombosis/embolism. In addition to the well known factors, such as inflammatory cytokines, DAMPs/PAMPs, coagulation factors and complements, the decrease in specific plasma glycoproteins, HRG and IAIPs, may play crucial roles in impairment of homeostatic interaction between blood cells and vasculatures with regard to the prevention of NETs and hemolysis. Further works are needed along this line.

Declaration of competing interest

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