

Establishment of *in Vitro* Binding Assay of High Mobility Group Box-1 and S100A12 to Receptor for Advanced Glycation Endproducts: Heparin's Effect on Binding

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Interaction between the receptor for advanced glycation end products (RAGE) and its ligands has been implicated in the pathogenesis of various inflammatory disorders. In this study, we establish an *in vitro* binding assay in which recombinant human high-mobility group box 1 (rhHMGB1) or recombinant human S100A12 (rhS100A12) immobilized on the microplate binds to recombinant soluble RAGE (rsRAGE). The rsRAGE binding to both rhHMGB1 and rhS100A12 was saturable and dependent on the immobilized ligands. The binding of rsRAGE to rhS100A12 depended on Ca²⁺ and Zn²⁺, whereas that to rhHMGB1 was not. Scatchard plot analysis showed that rsRAGE had higher affinity for rhHMGB1 than for rhS100A12. rsRAGE was demonstrated to bind to heparin, and rhS100A12, in the presence of Ca²⁺, was also found to bind to heparin. We examined the effects of heparin preparations with different molecular sizes—unfractionated native heparin (UFH), low molecular weight heparin (LMWH) 5000 Da, and LMWH 3000 Da—on the binding of rsRAGE to rhHMGB1 and rhS100A12. All 3 preparations concentration-dependently inhibited the binding of rsRAGE to rhHMGB1 to a greater extent than did rhS100A12. These results suggested that heparin's anti-inflammatory effects can be partly explained by its blocking of the interaction between HMGB1 or S100A12 and RAGE. On the other hand, heparin would be a promising effective remedy against RAGE-related inflammatory disorders.

Key words: RAGE, HMGB1, S100A12, heparin, inflammation

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell-surface molecules, which has been suggested to be involved in sustaining and amplifying inflammatory responses [1, 2]. RAGE can recognize a wide range of endogenous ligands, such as advanced

glycation end products (AGEs), amyloid- β peptide (A β), high mobility group box 1 (HMGB1), and the S100/calgranulin family [3]. RAGE's structure includes an extracellular immunoglobulin-like region essential for its binding to ligands, a single transmembrane domain, and a short cytoplasmic tail responsible for RAGE-mediated signal transduction [4]. There are soluble forms of RAGE—endogenous secretory RAGE (esRAGE) and soluble RAGE (sRAGE)—that lack a transmembrane domain and act

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as decoy receptors [5]. The binding of ligands to RAGE triggers intracellular signaling such as nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) activation in vascular endothelial cells and macrophages, leading to the development of inflammation-based diseases, such as diabetic complications, sepsis, Alzheimer's and rheumatic arthritis [6-9]. As a result, interfering with the binding of ligands to RAGE has been thought to be a means with which to block the inflammatory responses sustained by RAGE-dependent pathways.

Among the RAGE ligands, AGEs are the groups of nonenzymatically glycosylated proteins that accumulate in vascular tissue in a wide variety of disorders, especially in diabetes [10]. HMGB1 is a ubiquitous and abundant nuclear protein, which can be passively released from necrotic cells and actively secreted by macrophages. After HMGB1 is released into the extracellular environment, it functions as a pro-inflammatory mediator [11, 12]. S100/calgranulin is a multigenic family of EF-hand Ca^{2+} -binding proteins [13]. S100A12 is one member of the S100 protein family, which is found mainly in neutrophil granulocytes and monocytes [14]. S100A12 has a role in cell homeostasis as an intracellular molecule, but contributes to the pathogenesis of inflammatory lesions via interaction with RAGE after release to the extracellular compartment [15].

Heparin is a highly sulfated glycosaminoglycan, which has been traditionally used clinically as an anticoagulant [16]. It is biosynthesized and stored in the granules of mast cells [17]. More recent studies have highlighted the role of heparin as an anti-inflammatory substance that has been used in the treatment of some inflammatory settings [18-20]. However, the molecular mechanisms underlying the anti-inflammatory activities of heparin remain to be determined. Antagonism of AGE's effect by LMWH *in vivo* has been reported [21]. A similar effect of heparin on AGE-RAGE signaling also has been demonstrated by the inhibition of RAGE-dependent NF- κ B activation in glioma cells and expression of vascular cell adhesion molecule-1 (VCAM-1) and vascular endothelial growth factor (VEGF) in endothelial cells. HMGB1 has also been described as a heparin-binding protein [22-24]. Therefore, heparin may be an important regulator of RAGE-mediated responses through multiple ligands.

To investigate the complex binding properties of

RAGE to many ligands, we attempted to establish *in vitro* binding of sRAGE to HMGB1 and S100A12 using a microplate. Both *in vitro* binding assays were saturated in terms of sRAGE and ligands. We clearly showed Ca^{2+} , Zn^{2+} -dependent binding of S100A12 to sRAGE. We also observed the binding inhibition activity of each of the 3 heparin preparations, suggesting a mechanism for heparin's anti-inflammatory activity. This may provide insights into potential uses of heparin in some RAGE-related pathologies.

Materials and Methods

Materials. Recombinant plasmid pASK-IBA32-sRAGE was transformed into *E.coli* BL 21 (DE3) (Merck, San Diego, LA), and recombinant sRAGE (rsRAGE) proteins with a 6-histidine tag were expressed. rsRAGE proteins were partially purified by using a Ni-NTA column and further purified by heparin-sepharose affinity chromatography. Recombinant plasmids pGEX-6P-1-HMGB1 and pGEX-6P-1-S100A12 were transformed into *E.coli* BL 21 (DE3). Recombinant human HMGB1 (rhHMGB1) and recombinant human S100A12 (rhS100A12) proteins were expressed as GST-HMGB1 or GST-S100A12 fusion proteins, respectively. GST tag was cleaved by protease in Glutathione SepharoseTM 4B columns. Unfractionated heparin (UFH) (mol wt: 12000~15000 Da), low molecular weight heparin (LMWH) (mol wt: 5000 Da or 3000 Da), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2, 2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was a product of Tokyo Kasei. Kogyo. (Tokyo, Japan). Ni-NTA HRP Conjugate was from QIAGEN (Hilden, Germany). A 96-well ELISA plate (SUMILON[®], MS-8696F) was purchased from Sumitomo Bakelite (Tokyo, Japan).

Binding assay of rsRAGE to rhHMGB1 or rhS100A12. A flat-bottom 96-well plate was coated overnight with various concentrations of rhHMGB1 or rhS100A12 at 4°C (50 μ l/well). Control wells received only PBS (50 μ l/well). The plates were washed with PBS (200 μ l/well) 3 times and blocked with 10% (w/v) BSA in PBS (100 μ l/well) for 2 h at room temperature. After unbound rhHMGB1 or rhS100A12 was washed off, different concentrations of rsRAGE diluted with 10% BSA in PBS were

added into wells in triplicate (50 μ l/well), and incubation continued overnight at 4°C. Unbound rsRAGE was removed by washing 3 times with PBS at room temperature. Ni-NTA HRP Conjugate 1/500 diluted by 0.2% BSA in PBS (100 μ l/well) was added to the wells and incubated for 2h at 4°C. After washing with PBS, substrate solution, 0.1% ABTS dissolved in 20mM phosphate-citrate buffer (pH5.0) was added to each well (50 μ l/well) and incubated for 30min at room temperature. Finally, a stop solution of 1% SDS was added (100 μ l/well). The absorbance was measured at 415nm in a microplate reader (Model 680) from Bio-Rad Laboratories (Hercules, CA, USA).

To determine the effects of divalent cations on the binding, experiments for rsRAGE binding to rhHMGB1 were performed with PBS buffer containing no divalent cations; 1mM CaCl₂; 1mM CaCl₂ and 10 μ M ZnCl₂ throughout, and the experiments for rsRAGE binding to rhS100A12 were performed with PBS buffer containing no divalent cations; 1mM CaCl₂; 1mM CaCl₂ and 0.5mM MgCl₂; 1mM CaCl₂ and 10 μ M ZnCl₂; 1mM CaCl₂ and 0.5mM MgCl₂ and 10 μ M ZnCl₂ throughout. rsRAGE and rhHMGB1 were incubated for 24h, 48h, and 72h at 4°C, respectively, to determine the effect of incubation time on the binding of rsRAGE to rhHMGB1.

rsRAGE was purified by heparin-sepharose affinity chromatography. rsRAGE, partially purified by using a Ni-NTA column, was further purified by heparin-sepharose affinity chromatography. Heparin-sepharose CL-6B (GE Healthcare, Milwaukee, WI, USA) was poured into the column and equilibrated with 20mM phosphate buffer (pH7.4) containing 150mM NaCl. rsRAGE was mixed with heparin-sepharose for 3h at 4°C. The supernatant was removed after centrifugation for 15min at 1,500rpm. rsRAGE was eluted with buffer containing 600mM NaCl after washing with buffer containing 150mM NaCl. Purified rsRAGE was analyzed by SDS-PAGE.

Binding of rhS100A12 to heparin-sepharose. 100 μ l of 50% heparin-sepharose slurry was washed with 200 μ l PBS by centrifugation at 1,500rpm for 5min twice. PBS containing 1mM EDTA, no divalent cations, or 1mM Ca²⁺ was used throughout the experiment. To the washed gel was added 200 μ l of rhS100A12. Samples were mixed in rotation at 4°C for 3h. The supernatant and the sep-

harose gel were incubated with SDS-PAGE sample buffer at 99°C for 5min in a shaker. Then, 15 μ l of each sample was electrophoresed by SDS-PAGE.

The effects of different preparations of heparin with diverse molecular weights on the binding of rsRAGE to rhHMGB1 or rhS100A12. rhHMGB1 or rhS100A12 was coated on the plate as described above. Then, 25 μ g/ml rsRAGE was incubated with various concentration of heparin preparations diluted by 10% BSA in PBS for 30min at 4°C, and the mixture was added to the plate (50 μ l/well). The following procedures were the same as those described above in the binding assay.

Results

rsRAGE binding to rhHMGB1. Different concentrations of rhHMGB1 were immobilized on 96-well plates, and then various concentrations of rsRAGE were added to the plate. Bound sRAGE was determined by the addition of Ni-NTA HRP followed by the peroxidase reaction. The total binding was dependent on both rhHMGB1 and rsRAGE (Fig. 1A). The binding was saturable with respect to the rsRAGE concentration (Fig. 1B). The values of nonspecific binding in the absence of rhHMGB1 were very small compared with the specific binding. The inset shows a Scatchard plot of the specific binding obtained with rhHMGB1 at 6 μ g/ml for coating. The K_d value of sRAGE for rhHMGB1 determined from the Scatchard analysis was 0.71 μ M. rsRAGE bound to rhHMGB1 also in a time-dependent manner (Fig. 2). The presence of Ca²⁺ (1mM) on the combination did not influence the binding of rsRAGE to rhHMGB1 (Fig. 3).

rsRAGE binding to rhS100A12. Since S100A12 has been demonstrated to be a Ca²⁺- and Zn²⁺-binding EF-hand protein [28], we examined the effects of divalent cations on rsRAGE binding to rhS100A12. Ca²⁺ (1mM) alone significantly increased the binding of rsRAGE to rhS100A12, by 3-fold. Zn²⁺ (10 μ M) further enhanced binding, while Mg²⁺ (0.5mM) had no effect on it (Fig. 4). These results implied that Ca²⁺- and Zn²⁺-bound conformation of S100A12 is required for the optimal binding to rsRAGE. Fig. 5A shows the total and specific binding of rsRAGE to immobilized rhS100A12 (2 μ g/ml, 10 μ g/ml, and 50 μ g/ml for the coating plate) in the

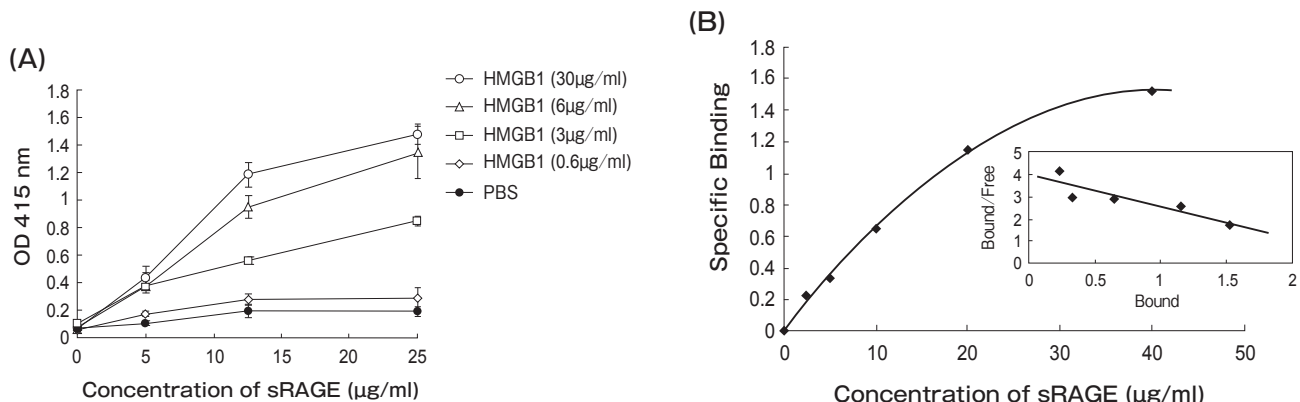


Fig. 1 Concentration-dependent binding of rsRAGE to rhHMGB1. (A) Different concentrations of rhHMGB1 were immobilized onto the wells of a 96-well plate. After blocking the plate with 10% BSA/PBS, the indicated concentrations of rsRAGE were added to the wells. The bound rsRAGE was quantified as described in Methods. The results were the means \pm SEM of 3 independent experiments. (B) Specific binding of rsRAGE to rhHMGB1 was calculated by subtracting nonspecific binding in the absence of rhHMGB1 from the total binding with 6 μ g/ml of rhHMGB1. The inset shows the Scatchard plot of the saturation curve. The K_d value of rsRAGE for rhHMGB1 binding was estimated to be 0.71 μ M.

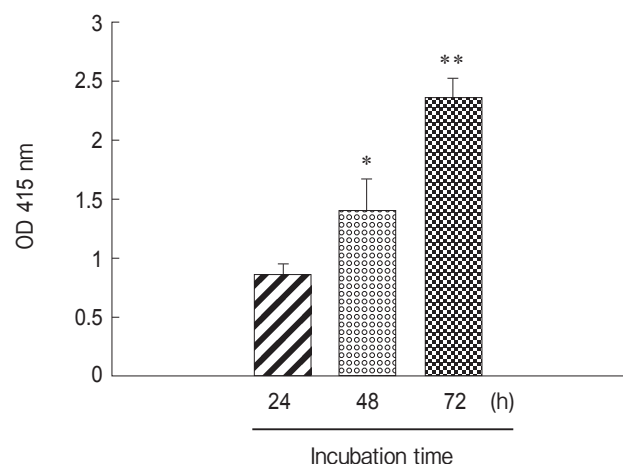


Fig. 2 Time-dependent binding of rsRAGE to rhHMGB1. First, 3 μ g/ml of rhHMGB1 was immobilized onto each well of a 96-well plate. After the plate was blocked with 10% BSA/PBS, 25 μ g/ml of rsRAGE was added to each well and the incubation continued for 24h, 48h, and 72h at 4°C. The results were the means \pm SEM of 3 independent experiments. * P <0.05, ** P <0.01 compared with the value at 24h.

presence of Ca^{2+} and Zn^{2+} . The K_d value of rsRAGE for rhS100A12 binding was determined to be 1.39 μ M by Scatchard analysis, as shown in the inset (Fig. 5B).

rsRAGE was purified by heparin-sepharose affinity chromatography. The fractions obtained from heparin-sepharose affinity chromatography were

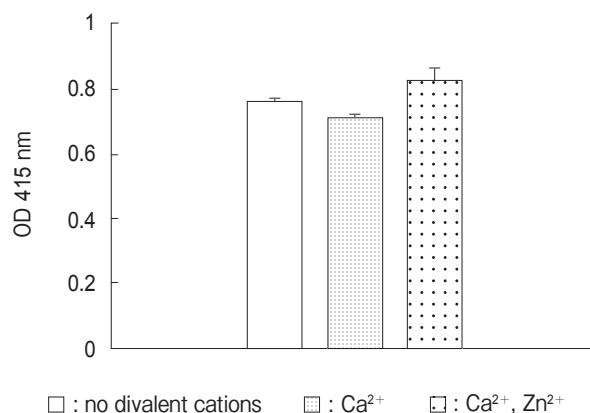


Fig. 3 The effects of Ca^{2+} and Zn^{2+} on rsRAGE binding to rhHMGB1. The plate was coated with 6 μ g/ml rhHMGB1, and 12.5 μ g/ml rsRAGE was added to each well. 1 mM Ca^{2+} and 10 μ M Zn^{2+} were added to the well indicated at the start of plate coating and were present throughout the period immediately before the final reaction. The results were the means \pm SEM of 3 independent experiments.

run on SDS-PAGE gels under denaturing conditions. Coomassie brilliant blue staining showed the presence of rsRAGE with a molecular size of 45000 Da (Fig. 6), which strongly suggested that rsRAGE can be regarded as a heparin-binding protein.

rhS100A12 binding to heparin-sepharose. After rhS100A12 was incubated with heparin-sepharose, the supernatant and the sepharose gel were analyzed by SDS-PAGE under denaturing conditions and

Coomassie brilliant blue staining. rhS100A12 bound to heparin-sepharose also in a Ca^{2+} -dependent manner. A band was obtained with a molecular size of 11000 Da in the presence of Ca^{2+} , while there was no clear band in the presence of EDTA or in the absence of Ca^{2+} (Fig. 7).

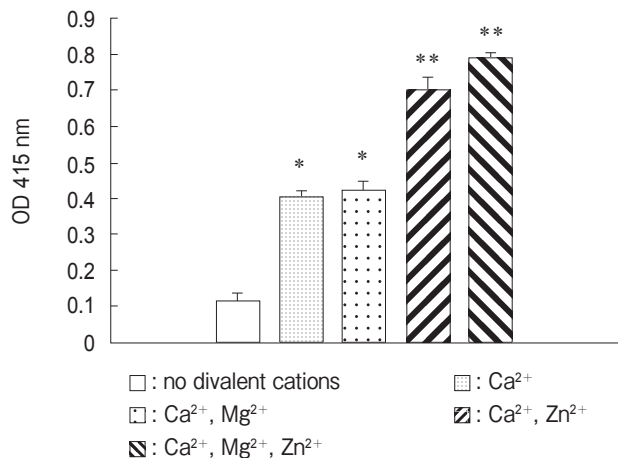
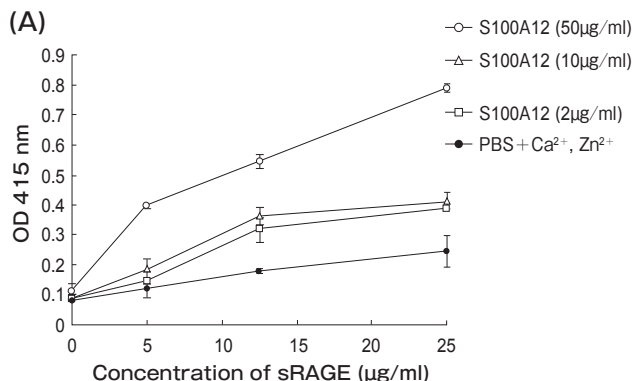


Fig. 4 The effects of divalent cations on rsRAGE binding to rhS100A12. The plate was coated with $50\mu\text{g/ml}$ rhS100A12, and $25\mu\text{g/ml}$ rsRAGE was added to each well. Ca^{2+} , Zn^{2+} , and Mg^{2+} were added to the wells indicated at the start of plate coating and were present throughout the period immediately before the final reaction. The results were the means \pm SEM of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the value with no divalent cations.



Heparin inhibits the binding of rsRAGE to rhHMGB1. The effects of each of the 3 preparations of heparin with different average molecular weights on rsRAGE binding to rhHMGB1 were examined. The concentrations of rhHMGB1 and rsRAGE were fixed at $3\mu\text{g/ml}$ and $25\mu\text{g/ml}$, respectively. All 3 preparations concentration-dependently inhibited the binding of rsRAGE to rhHMGB1 from 0.5 to $2\mu\text{g/ml}$ (Fig. 8). The rank order of inhibitory potency was UFH, LMWH 5000 Da, and LMWH 3000 Da.

Heparin inhibits the binding of rsRAGE to rhS100A12. The effects of the 3 preparations of heparin with different average molecular weights on the binding of rsRAGE to rhS100A12 were evaluated in the presence of 1mM Ca^{2+} and $10\mu\text{M}$ Zn^{2+} . The concentrations of S100A12 and rsRAGE were fixed at $50\mu\text{g/ml}$ and $25\mu\text{g/ml}$, respectively. Similar to the effects on the binding of rhHMGB1 to rsRAGE, all 3 preparations concentration-dependently inhibited the binding of rsRAGE to rhS100A12 (Fig. 9). However, the inhibitory potency against S100A12 binding was much lower than that against HMGB1 binding, and that was common to all 3 preparations. Even at $100\mu\text{g/ml}$ of heparin, the inhibition was about 50%, irrespective of the preparation.

Discussion

RAGE is a transmembrane protein and has been

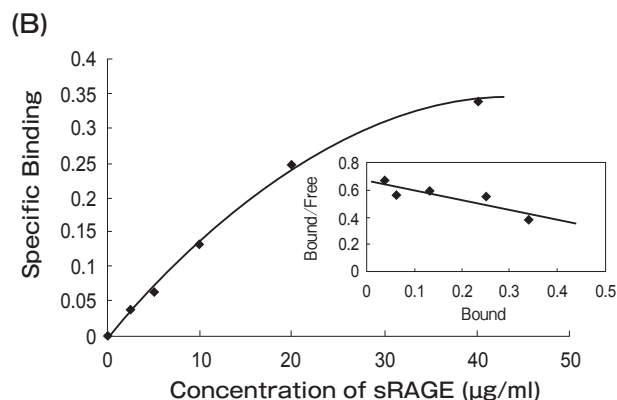


Fig. 5 Concentration-dependent binding of rsRAGE to rhS100A12. (A) Different concentrations of rhS100A12 were immobilized onto the wells of a 96-well plate in the presence of 1mM Ca^{2+} , $10\mu\text{M}$ Zn^{2+} , and 0.5mM Mg^{2+} . After the plate was blocked with 10% BSA/PBS, the indicated concentrations of rsRAGE were added to the wells. Ca^{2+} , Zn^{2+} , and Mg^{2+} were present throughout the period immediately before the final reaction. The results were the means \pm SEM of 3 independent experiments. (B) Specific binding of rsRAGE to rhS100A12 was calculated by subtracting nonspecific binding in the absence of rhHMGB1 from the total binding with $50\mu\text{g/ml}$ rhS100A12. The inset shows the Scatchard plot of the saturation curve. The K_d value of rsRAGE for rhS100A12 binding was estimated to be $1.39\mu\text{M}$.

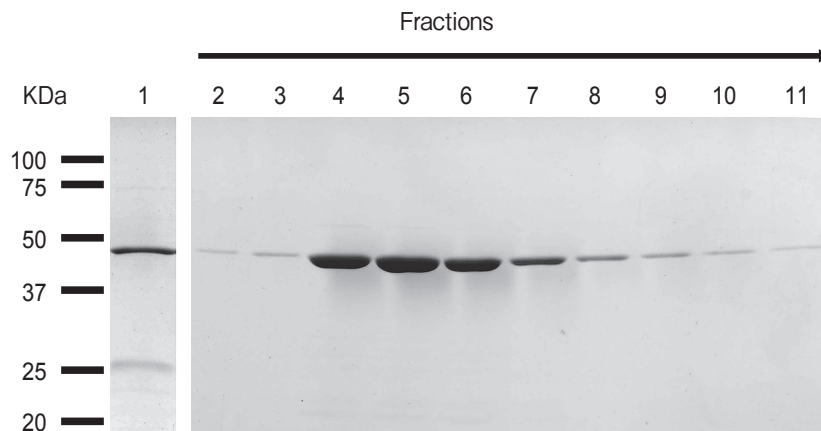


Fig. 6 Purification of rsRAGE using heparin-sepharose affinity chromatography. Partially purified rsRAGE preparations by using a Ni-NTA column (lane 1) were applied to heparin-sepharose. The bound proteins were eluted with 600 mM NaCl (lanes 4-11) after extensive washing (lanes 2 and 3). The eluted samples were electrophoresed on 12% separation gel, and SDS-PAGE gel was stained with Coomassie brilliant blue.

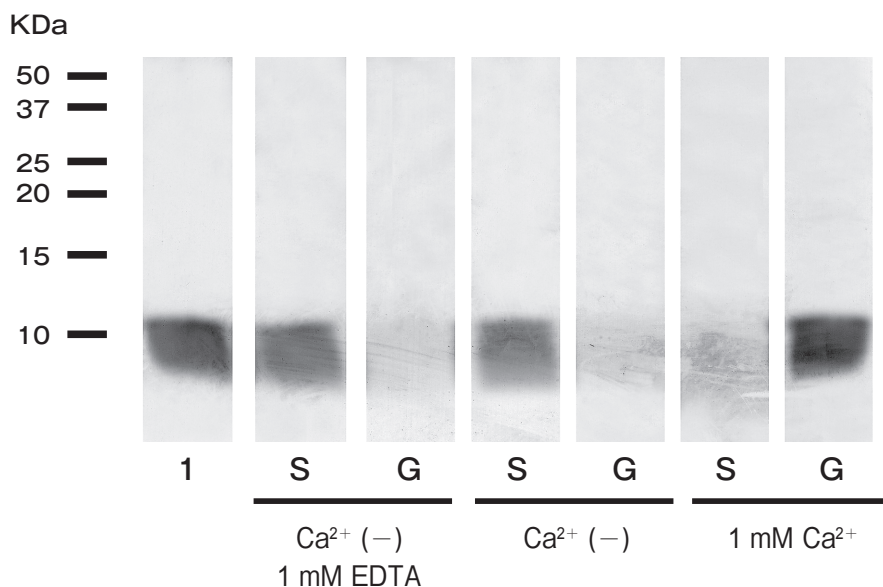


Fig. 7 rhS100A12 binding to heparin-sepharose. rhS100A12 was mixed with heparin-sepharose for 3 h at 4°C under different Ca^{2+} condition. After incubation, the supernatant (S) and the sepharose gel (G) were electrophoresed on 15% separation gel, and the SDS-PAGE gel was stained with Coomassie brilliant blue. Lane 1, loading S100A12.

reported to be a receptor for several ligands including HMGB1, S100A12, S100A8/9, AGEs, and $A\beta$. RAGE belongs to the immunoglobulin superfamily and has 3 immunoglobulin-like domain structures in the extracellular portion. We used recombinant human soluble RAGE with a 6-histidine tag at the C-terminus for the *in vitro* binding and established the binding

assay of HMGB1 or S100A12 to RAGE.

The binding of rhHMGB1 to rsRAGE was concentration-dependent and time-dependent (Fig. 1 and Fig. 2). The binding was saturable in terms of both rhHMGB1 and rsRAGE concentration for constant levels of the pair. rhS100A12 binding to rsRAGE was dependent on divalent cations (Fig. 4 and Fig. 5),

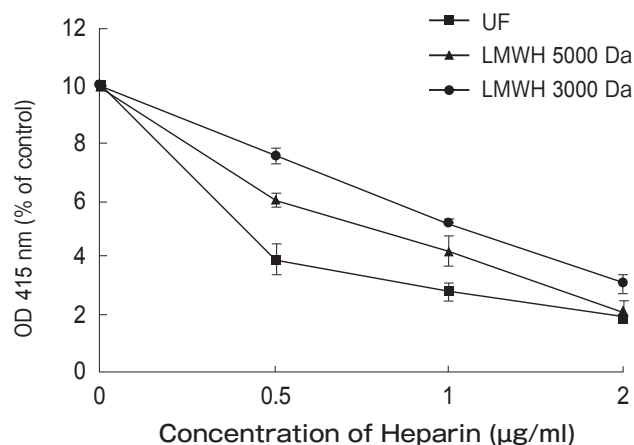


Fig. 8 Effects of heparin with different molecular sizes on rsRAGE binding to rhHMGB1. The different concentrations of UFH (■), LMWH 5000 Da (▲), and LMWH 3000 Da (●) were incubated with 25 µg/ml rsRAGE for 30 min at 4°C, and the mixture was added to the wells. The results were the mean ± SEM of 3 independent experiments.

whereas HMGB1 was not (Fig. 3). It is well known that S100A12 belongs to the EF-hand Ca^{2+} binding protein and forms a typical structure. X-ray crystallography analysis revealed both Ca^{2+} and Zn^{2+} binding sites on the structure of S100A12 [25]. The dependence of S100A12 binding to RAGE in the presence of Ca^{2+} and Zn^{2+} strongly suggested that the restrict structure of S100A12 mentioned above was required for its binding to RAGE. In contrast, rhHMGB1 binding to rsRAGE was not influenced by the presence of divalent cations. Thus, the effects of Ca^{2+} and Zn^{2+} were specific for S100A12. The binding site of HMGB1 or S100A12 on RAGE has been postulated from crystallographic studies. Hofmann *et al.* [26] suggested that S100A12 may bind to the V-domain of RAGE. On the other hand, there is controversy as to the binding site of HMGB1 on RAGE. Apparently, the affinity of HMGB1 ($K_d = 0.71 \mu\text{M}$) to RAGE was higher than that of S100A12 ($K_d = 1.39 \mu\text{M}$).

Since rsRAGE was able to be purified by heparin-sepharose affinity chromatography (Fig. 6), it was considered to bind to heparin, and rhS100A12 was also found to bind to heparin in the presence of Ca^{2+} (Fig. 7). By using the binding assay, we reported for the first time that heparin had inhibitory effects on the binding of rsRAGE to rhHMGB1 or rhS100A12. Dose-dependent inhibitory effects of UFH and LMWH on the binding of rsRAGE to rhHMGB1 (Fig. 8) or

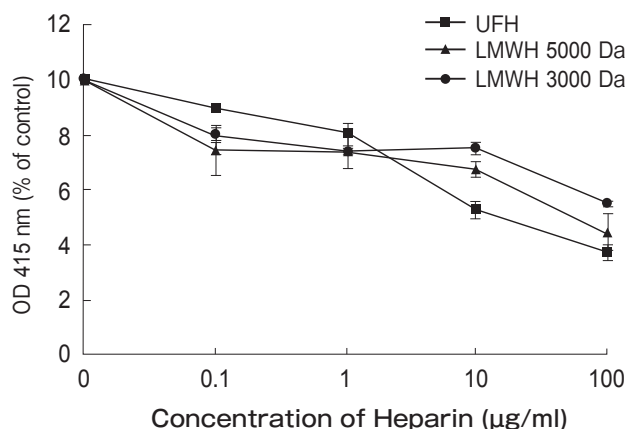


Fig. 9 Effects of heparin with different molecular sizes on rsRAGE binding to rhS100A12. The different concentration of UFH (■), LMWH 5000 Da (▲), and LMWH 3000 Da (●) were incubated with 25 µg/ml rsRAGE for 30 min at 4°C, and the mixture was added to the wells. The results were the mean ± SEM of 3 independent experiments.

rhS100A12 (Fig. 9) were demonstrated. UFH is a heterogeneous mixture of polysaccharides and has been clinically used as an anticoagulant. LMWH 5000 Da is obtained through chemical or enzymatic depolymerization of UFH and shows advantages in overcoming some of the limitations associated with the use of UFH. For instance, compared with UFH, LMWH 5000 Da more potently inhibits factor Xa, an essential component of the prothrombinase complex leading to the formation of thrombin [27–30]. The exact mechanisms by which heparin blocks the binding of rhHMGB1 or rhS100A12 to rsRAGE remain unclear under this situation. Considering that HMGB1 and RAGE are all heparin-binding proteins [21, 22–24], we hypothesize that one blocking mechanism could be heparin occupancy of the binding site on rsRAGE and/or rhHMGB1. However, further experiments are necessary for the analysis of molecular interaction between rsRAGE and its ligands.

It has been suggested that heparin functions as an anti-inflammation agent by affecting some components in the inflammatory cascade, such as complement activation, platelet activating factor production [31], and expression of P- and L- selectins [32]. In addition, heparin binds antithrombin III to form a complex capable of inhibiting thrombin proteolytic activity. This inhibition prevents clot formation and allows heparin to be utilized clinically as an anticoagulant

[33]. The concept that coagulation can play a role in the inflammatory response has been well illustrated [34]. In acute inflammatory diseases, like sepsis, inflammatory and coagulation systems coexist in delicate homeostasis [35], augment each other, and combine to influence disease progression [36, 37]. Accordingly, heparin's anti-inflammatory effect may be partly assigned to its own anticoagulant property. The present results imply that another anti-inflammatory mechanism of heparin might be the interference effect on the interaction between RAGE and its ligands.

RAGE has been considered a central player in the inflammatory response [38]. It is expressed at low levels in normal tissues and in the vasculature, but becomes upregulated at sites where its ligands accumulate [39]. The RAGE axis is involved in the pathogenesis of a wide range of inflammatory disorders via the integration of ligands. HMGB1 activated Mac-1 as well as Mac-1-mediated adhesive and migratory functions of neutrophils in a RAGE-dependent manner [40]. Interaction of S100A12 with cellular RAGE on vascular endothelial cells, mononuclear phagocytes, and lymphocytes triggers cellular activation, with the expression of VCAM-1, intercellular adhesion molecule-1 (ICAM-1), and tissue factor (TF) as well as production of pro-inflammatory mediators [15]. Therefore, the interaction between RAGE and its ligands should be a target for inflammation-based disease intervention. The *in vitro* RAGE binding established in the present study will provide a convenient assay system for screening candidate drugs to interfere with the binding between RAGE and HMGB1/S100A12.

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