The E-Selectin Ligand Basigin/CD147 Is Responsible for Neutrophil Recruitment in Renal Ischemia/Reperfusion

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ABSTRACT
E-selectin and its ligands are essential for extravasation of leukocytes in inflammation. Here, we report that basigin (Bsg)/CD147 is a ligand for E-selectin that promotes renal inflammation in ischemia/reperfusion. Compared with wild-type mice, Bsg-deficient (Bsg−/−) mice demonstrated striking suppression of neutrophil infiltration in the kidney after renal ischemia/reperfusion. Although E-selectin expression increased similarly between the two genotypes, Bsg−/− mice exhibited less renal damage, suggesting that Bsg on neutrophils contribute to renal injury in this model. Neutrophils expressed Bsg with N-linked polylactosamine chains and Bsg−/− neutrophils showed reduced binding to E-selectin. Bsg isolated from HL-60 cells bound to E-selectin, and tunicamycin treatment to abolish N-linked glycans from Bsg abrogated this binding. Furthermore, Bsg−/− neutrophils exhibited reduced E-selectin-dependent adherence to human umbilical vein endothelial cells in vitro. Injection of labeled neutrophils into mice showed that Bsg−/− neutrophils were less readily recruited to the kidney after renal ischemia/reperfusion than Bsg+/+ neutrophils, regardless of the recipient’s genotype. Taken together, these results indicate that Bsg is a physiologic ligand for E-selectin that plays a critical role in the renal damage induced by ischemia/reperfusion.


The selectins and their ligands are essential for leukocyte tethering/rolling on endothelial cells and the initiation of inflammatory response. The selectins are C-type lectins and consist of three members, i.e., P-, L-, and E-selectin.1,2 P-selectin is expressed upon inflammatory stimulation in platelets and endothelial cells. L-selectin is constitutively expressed on the tip of leukocyte microvilli and implicated in lymphocyte homing to lymph nodes.3 E-selectin is specifically induced in the endothelium upon inflammatory stimulation. Thus, E- and P-selectin closely collaborate with one another and play a major role in leukocyte recruitment to inflammatory sites.4–6 Among the several glycoproteins reported to bind to E-selectin, three have been identified as representative physiologic E-selectin ligands on neutrophils. There are P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1, and CD44, and all three play distinct roles during tethering and slow rolling of neutrophils on the endothelium.7 A minimal recognition motif for all selectins is sialylated and fucosylated glycan determinants, such as sialyl Lewis X, that decorate the terminal extensions of carbohydrates of these molecules.8,9 However, because of the poor immunogenicity of highly gly-
cosylated epitopes, it has proven difficult to identify selectin ligands.

Basigin (Bsg)/CD147 (Bsg is the name of the mouse gene) is a membrane glycoprotein that belongs to the Ig superfamily. Bsg was discovered in embryonal carcinoma cells as a receptor for *Lotus tetragonolobus* agglutinin and was determined to have the structure Galβ1→4(Fucα1→3)GlcNAc, which is known as the Lewis X structure. But it has been unclear whether Bsg has sialyl Lewis X structure and whether Bsg serves as a selectin ligand. Bsg is expressed in many cell types, e.g., blood cells, epithelial cells, endothelial cells, and germ cells. We previously generated Bsg-deficient (Bsg−/−) mice and found several abnormalities that included male and female sterility, progressive retinal degeneration, increased cell proliferation upon mixed lymphocyte culture, decreased memory function, and abnormal sensory function. In addition to these functions deduced through the study of Bsg−/− mice, two additional Bsg functions have recently been highlighted. First, Bsg activates matrix metalloproteases (MMPs), thereby promoting cancer invasion. Second, Bsg functions like a chaperone for monocarboxylate transporters (MCTs). In the present study, we found an additional and unexpected role of Bsg; namely, its glycosylation was crucial for inflammation.

Acute kidney injury (AKI) is a common complication that occurs in approximately 5% of hospitalized patients and in approximately 30% of patients in intensive care units. As the mortality of AKI is still unacceptably high, between 40% and 60%, this disease is being intensively studied. Renal ischemia/reperfusion injury is characteristic of acute renal inflammation involving marked infiltration of inflammatory cells, such as neutrophils, and is the most widely used model for human AKI. We used this model to investigate the role of Bsg in inflammation in the present study. Bsg−/− mice exhibited less renal damage after ischemia/reperfusion. To our surprise, this phenotype was attributable to Bsg on neutrophils, rather than Bsg on other cells in the inflammation area. We found that highly glycosylated Bsg on neutrophils bound to E-selectin on endothelial cells and led to neutrophil infiltration to the inflammatory lesion. Our results may shed light on the mechanisms underlying the pathogenesis of AKI.

**RESULTS**

**Bsg Deficiency Preserves Renal Function and Decreases Renal Injury after Ischemia/Reperfusion**

We subjected Bsg+/+ and Bsg−/− mice to renal ischemia/reperfusion injury. As shown in Figure 1, the renal damage was less pronounced in Bsg−/− mice than in Bsg+/+ mice. Thus, 2 d after ischemia/reperfusion, Bsg−/− mice showed less tubulointerstitial injury by all three criteria examined, i.e., tubular cast formation, dilation, and degeneration (Figure 1A and B). The increase of serum urea nitrogen levels was also significantly suppressed in Bsg−/− mice (Figure 1C). The serum urea nitrogen reached the maximum level on day 1 after ischemia in Bsg−/− mice versus day 2 in Bsg+/+ mice. Thus, postischemic renal injury was quickly terminated in Bsg−/− mice.

**Bsg Deficiency Reduces Neutrophil Infiltration into the Tubulointerstitium**

Renal ischemia/reperfusion injury is characterized by a massive influx of neutrophils early after reperfusion, which plays a crucial role in the pathogenesis of postischemic renal failure through the release of cytotoxic proteases and oxygen-derived free radicals.

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**Figure 1.** Renal injury is less severe in Bsg−/− mice after renal ischemia/reperfusion. (A) A representative image of tubular lesions 2 d after ischemia/reperfusion injury. Less renal damage was observed in Bsg−/− mice than in Bsg+/+ mice. Arrow, tubular cast; arrowhead, degeneration of the tubule; asterisk, dilation of the tubule. Scale bar, 50 μm. Periodic acid-Schiff staining. (B) Semiquantitative analysis of tubulointerstitial damage 2 d after ischemia/reperfusion injury. The degree of tubular cast formation, tubular dilation, and tubular degeneration were comparatively rated as described in the Concise Methods section. High values indicate more severe damage. White columns, Bsg+/+ mice; black columns, Bsg−/− mice. cast, cast formation; dil., dilation; degen., degeneration. Data are means (columns) and SEM (bars). *P < 0.05; **P < 0.01; n = 6. (C) Blood urea nitrogen (BUN) levels in Bsg−/− and Bsg−/− mice after ischemia/reperfusion. Renal function was better preserved in Bsg−/− than Bsg+/+ mice. White columns, Bsg+/+ mice; black columns, Bsg−/− mice. BUN is shown as mg/dl. Data are means (columns) and SEM (bars). *P < 0.05; n = 6.
radicals. We next compared the amount of neutrophil influx and renal function. Bsg deficiency greatly reduced the influx of neutrophils to the kidney 1 d after ischemia/reperfusion (Figure 2A). The number of infiltrated neutrophils reached the maximum level 1 d after ischemia/reperfusion (Figure 2A). This was in contrast to the macrophage influx, which peaked 4 d after ischemia/reperfusion (Figure 2B). As the difference in renal function between Bsg+/+ and Bsg−/− mice became apparent 2 d after ischemia/reperfusion (Figure 1C), it is conceivable that neutrophil influx, rather than macrophage influx, was crucial for the role of Bsg in renal damage in our model.

It is widely accepted that chemokines are generated by ischemic tubular epithelial cells in the early phase of renal ischemic injury. In addition, the tubular epithelial cells express Bsg. Accordingly, we next examined whether the difference in influx of neutrophils was mediated through differences in chemokine levels. Macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC) are representative CXC chemokines that are known to be induced after ischemia/reperfusion injury and to attract neutrophils. MIP-2 and KC levels were elevated in Bsg+/+ and Bsg−/− mice at days 1 and 2 after ischemia/reperfusion, but there was no difference in the degree of elevation between the two genotypes (Figure 2C).

Neutrophil recruitment in the postischemia/reperfusion kidney requires adhesion molecules. E-selectin and intercellular adhesion molecule-1 (ICAM-1) on peritubular capillary cells play particularly crucial roles in this model. In the present study, western blot analysis showed upregulation of E-selectin in the kidney 12 h after ischemia/reperfusion in both Bsg+/+ and Bsg−/− mice, but the expression was not significantly different between the two genotypes (Figure 2D). The expression of ICAM-1 in the kidney at 24 h postischemia was also comparable between the two genotypes (Figure 2E). E-
selectin and ICAM-1 expression was localized along the length of the peritubular capillaries (Supplementary Figure S1).

**Bsg Expression on the Surface of Neutrophils**

Figure 3A shows the schematic molecular structures of Bsg and two major E-selectin ligands, PSGL-1 and CD44. Bsg is a type 1 integral membrane protein with a predicted molecular mass of 28 kD, but its glycosylated form on various cells is between 35 and 66 kD, with the actual molecular mass being dependent on the cell type. Thus, highly glycosylated Bsg harbors long sugar chains. This is in contrast to PSGL-1 (approximately 120 kD) and CD44 (approximately 85 kD), which have relatively short glycans. Determinants for E-selectin-binding exist on O-glycans of PSGL-1 and N-glycans of CD44.25,26

The molecular mass of Bsg from peritoneal-elicited neutrophils was reduced by N-glycosidase F digestion as well as endo-β-galactosidase digestion, indicating that Bsg harbored N-linked polylactosamine chains (Figure 3B). This was consistent with a previous report.27 We then analyzed the subcellular distribution of Bsg in neutrophils. At the electron microscope level, neutrophils display a complex surface architecture with prominent microvillus-like membrane protrusions. These microvilli represent principal sites of initial contact with the vascular endothelium. As shown in Figure 3C, a and b, Bsg was widely distributed on both the planar cell surface and the microvilli of peritoneal-elicited neutrophils. A negative control experiment was performed with an isotype-matched antibody, but no signals were observed (Figure 3Cc). The microvillous presentation argues for the participation of Bsg in the early interaction between neutrophils and endothelial cells during extravasation. Bsg may also be expressed on the surface of unstimulated neutrophils as well, since Bsg is readily detected by FACS analysis on leukocytes obtained from peripheral blood.28

**Bsg Binds to E-Selectin**

Consistent with Figure 3C, Bsg expression on the cell surface of peritoneal-elicited neutrophils was confirmed by FACS analysis, but the expression was lost in neutrophils from Bsg+/− mice (Figure 4A). Peritoneal-elicited neutrophils from wild-type mice bound to soluble mouse E-selectin (E-selectin/Fc) and P-selectin (P-selectin/Fc) (Figure 4B). Notably, Bsg+/− neutrophils showed less binding to E-selectin (Figure 4B). On the other hand, there was no difference in binding to P-selectin between Bsg+/+ and Bsg−/− neutrophils (Figure 4B).

Since this result suggested that Bsg might bind to E-selectin, we next addressed this question. We examined Bsg isolated from HL-60 cells, a human promyelocytic cell line, that were pretreated with or without tunicamycin. Protein extracts from HL-60 cells were incubated with immunomagnetic beads coated with mouse anti-human Bsg antibody. Immobilized Bsg beads were then incubated with soluble human E-selectin and P-selectin with or without EDTA. Soluble E-selectin bound to the Bsg beads, and this binding was abrogated under a Ca2+ chelate condition (Figure 4C, left upper panel, solid line). Because it has been reported that mouse E-selectin/Fc has a higher level of binding activity to human leukocytes than human E-selectin/Fc,29 we also used mouse E-selectin/Fc. Mouse E-selectin/Fc bound to the Bsg beads (Figure 4C, left upper panel, dotted line). On the other hand, human P-selectin/Fc did not bind to the Bsg beads (Figure 4C). Tunicamycin treatment abolished N-linked glycans from Bsg (Figure 4C, right) and consequently di-
Figure 4. Neutrophil Bsg binds to E-selectin. (A) Bsg expression on mouse neutrophils. Peritoneal-elicited neutrophils were stained with anti-mouse Bsg antibodies (open histogram) or isotype-matched control antibodies (gray-filled histogram). Analysis gates were set on the granulocytic population using forward/side scatter distributions. The upper panel shows the data for Bsg+/+ mice, and the lower one the data for Bsg−/− mice. (B) E-selectin and P-selectin ligands on Bsg+/+ and Bsg−/− mouse neutrophils. Peritoneal-elicited neutrophils were stained with E-selectin/Fc or P-selectin/Fc. Gray-filled histograms represent EDTA treatment. The geometric mean fluorescence of E-selectin/Fc or P-selectin/Fc. Peritoneal-elicited neutrophils were stained with anti-CD11a (LFA-1) or anti-CD11b (Mac-1) antibodies (open histogram). Gray-filled histograms represent the isotype-matched control.

Expression of Other Adhesion-Related Molecules in Bsg-Deficient Neutrophils

The level of expression of the major E-selectin ligands PSGL-1 and CD44 on neutrophils was similar between Bsg+/+ and Bsg−/− neutrophils (Figure 4E). In the renal ischemia/reperfusion injury model, integrins on the neutrophils are another factor to be considered.31 However, we found that the expressions of lymphocyte function antigen 1 (LFA-1: CD11a/CD18) and macrophage-1 antigen (Mac-1: CD11b/CD18) on neutrophils from Bsg+/+ mice were comparable to those on neutrophils from Bsg−/− mice (Figure 4F).

In addition to PSGL-1 and CD44, CD43 is known to function as an E-selectin ligand.32 However, there was no difference of CD43 expression between Bsg+/+ and Bsg−/− neutrophils (Supplementary Figure S2A). Furthermore, the beads used for Figure 4C did not contain PSGL-1, CD44, or CD43 (Supplementary Figure S2B).
mentary Figure S2B). Together with the data shown in Figure 4D, these data exclude the possibility that the Bsg used for the bead experiments was co-isolated with associated molecules that exert an E-selectin-binding activity.

**Impaired Bsg<sup>−/−</sup> Neutrophil Adhesion to Cytokine-Activated Endothelial Cells**

We next investigated the biologic significance of Bsg on neutrophils using an in vitro adhesion assay. It is known that human umbilical vein endothelial cells (HUVECs) express E- and P-selectin upon stimulation with TNF-α. We found that, compared with Bsg<sup>+/+</sup> neutrophils, Bsg<sup>−/−</sup> neutrophils were less adherent to HUVECs at 4 h after TNF-α stimulation (Figure 5, A and B). This result was primarily due to the interaction between E-selectin and its ligands, since an E-selectin-blocking antibody significantly suppressed the adhesion, and the difference in adherence to HUVECs between Bsg<sup>+/+</sup> and Bsg<sup>−/−</sup> neutrophils was abolished (Figure 5A). The adhesion was further suppressed when Ca<sup>2+</sup> was chelated with EDTA, suggesting that, in addition to E-selectin, other components of the Ca<sup>2+</sup>-dependent adhesion machinery, e.g., other selectins, also played a role in this adhesion (Figure 5A). Indeed, P-selectin blocking antibody suppressed the neutrophil adhesion to HUVECs, but the difference in adhesion between the two genotypes remained intact (Figure 5B). It is noteworthy that the adhesion between HUVECs and Bsg<sup>+</sup> neutrophils was suppressed by approximately 50% by the E-selectin-blocking antibody, whereas the adhesion between HUVECs and Bsg<sup>−/−</sup> neutrophils was only suppressed by about 10% by this antibody (Figure 5A). Therefore, the results strongly suggested that Bsg on neutrophils played an indispensable role in adhesion to HUVECs through E-selectin.

**Few Neutrophils from Bsg<sup>−/−</sup> Mice Infiltrated into the Postischemic Kidney**

The suppressed infiltration of neutrophils in Bsg<sup>−/−</sup> mice after renal ischemia/reperfusion could be interpreted in two ways. First, Bsg on infiltrating neutrophils may have played a role. Second, Bsg on other cells in the inflammatory tissues may have been important. To examine these possibilities, fluorescence-labeled neutrophils were adoptively transferred into mice 5 min after renal ischemia/reperfusion surgery. After 6 h, the mice were sacrificed, and the postischemic kidneys were examined. The fluorescence-labeled neutrophils predominantly infiltrated around the vasa recta in the outer medulla, which was one of the main damaged areas after ischemia/reperfusion injury (Figure 6Aa). Much less infiltration was observed when Bsg<sup>−/−</sup> neutrophils were injected, compared with Bsg<sup>+/+</sup> neutrophils (Figure 6A, a and b, neutrophils are indicated by arrows). Labeled cells were barely detectable in the right kidney, which was not subjected to ischemia/reperfusion surgery (Figure 6Ac). Labeled cells were also not observed when saline was injected instead of labeled neutrophils (Figure 6Ad). The numbers of labeled cells infiltrating into the postischemic kidney are summarized in Figure 6B. Regardless of the genotypes of recipients, fewer adopted Bsg<sup>−/−</sup> neutrophils infiltrated than adopted Bsg<sup>+/+</sup> neutrophils. These data clearly indicated that Bsg on neutrophils, rather than Bsg on other cells at inflammatory sites, was responsible for the reduced infiltration of neutrophils into the postischemic kidney in Bsg<sup>−/−</sup> mice (Figure 2A). We confirmed that the infiltrating labeled cells (PKH26; Figure 6Cc, red) were indeed neutrophils by staining with anti-mouse neutrophil antibody (Figure 6Cb, green). Thus, the red and green spots were completely merged (Figure 6Ca). We also confirmed that infiltrating cells were located on the portions of the endothelium that were positive for E-selectin and thrombomodulin, a vascular endothelial marker (Figure 6C, d through f). Thus, E-selectin expression was found along the peritubular capillary blood vessels of the postischemic kidney, and the PKH26-labeled cells were in close proximity to E-selectin (Figure 6C, d through f). As the majority of the PKH26 staining in Figure 6C, c and f, represents autofluorescence, the PKH26-labeled cells are indicated by arrows in these figures.

**DISCUSSION**

In this study, we demonstrated that Bsg isolated from HL-60 bound to E-selectin, but not after N-linked polylactosamine
chains of Bsg were ablated by tunicamycin treatment. The massive neutrophil infiltration into the kidney after renal ischemia/reperfusion was partly attributable to Bsg on neutrophils, because (i) the level of neutrophil infiltration was lower in Bsg<sup>−/−</sup> mice and (ii) regardless of the recipients’ genotypes, exogenously injected Bsg<sup>−/−</sup> neutrophils were less readily recruited to the inflammatory kidney tissue. In vitro E-selectin-dependent adherence to HUVECs was also reduced in Bsg<sup>−/−</sup> neutrophils. Therefore, our data strongly suggest that Bsg is a physiologic ligand for E-selectin.

The selectins are required for leukocyte adhesion during inflammation. E-selectin but not P-selectin controls slow leukocyte rolling on inflamed venules, and this rolling may enhance an efficient transition to firm adhesion and extravasation. Although blocking of P-selectin ameliorates ischemia/reperfusion-induced AKI, platelet P-selectin, but not endothelial P-selectin, is the key component in P-selectin-mediated AKI. Therefore, we focused on endothelial E-selectin and its ligand in these renal ischemia/reperfusion experiments. Several glycoproteins have been found to bind to E-selectin in vitro, and the topographic distribution of the ligands on the surface of neutrophils is a major determinant of their ability to mediate initial contacts to the endothelium under flow. For example, PSGL-1 distributes on the very tip of neutrophil microvilli and contributes to the primary interaction between neutrophils and the endothelium. On the other hand, CD44 is exclusively distributed on the planar surface of the neutrophils and mediates steady slow rolling. In this context, it is of note that Bsg is equally distributed on the planar surface and microvilli of the neutrophils (Figure 3C). The localization of Bsg on microvilli is an indication of its possible role in the early steps of leukocyte endothelial contact formation.

Bsg is known to induce MMPs and thus, is referred to as an extracellular matrix metalloproteinase inducer (EMMPRIN). To confirm the effects of MMP activity in the postischemic kidneys on this model, we performed gelatin zymography. Both MMP-2 and MMP-9 activities in Bsg<sup>−/−</sup> mice were comparable to those in Bsg<sup>+/+</sup> mice at both 1 and 2 d postischemia/reperfusion (data not shown). Another molecule we considered was cyclophilin. It has been reported that extracellular cyclophilins can induce leukocyte chemotaxis, and Bsg is a signaling receptor for these proteins. Blocking the cyclophilin-Bsg interaction pharmacologically or by means of an antibody reduces the inflammation responses in a LPS-induced acute lung injury model and a bronchial asthma model in mice. Dear et al. found that cyclophilin is upregulated in the liver in a sepsis model, and inhibition of Bsg attenuates sepsis-induced AKI.

antibody (green in d through f). The arrows indicate fluorescence-positive cells. The capillary endothelium was also stained with anti-thrombomodulin (TM) antibody followed by rhodamine-conjugated secondary antibody (g through i). Scale bar, 50 μm.
However, the expression of cyclophilin A, the most representative cyclophilin, did not increase after ischemia in the kidney, and there was no difference in the expression of cyclophilin A between the two genotypes in the present study (data not shown). Thus, it is not likely that the function of Bsg is always exerted through cyclophilin in AKI, although further studies are needed to fully understand the involvement of cyclophilin in AKI. Furthermore, Bsg\(^{-/-}\) neutrophils expressed normal levels of PSGL-1, CD44, and integrins (LFA-1 and Mac-1). We also observed that the expression of E-selectin on renal microvessels and chemokines in the kidney was comparable between Bsg\(^{+/+}\) and Bsg\(^{-/-}\) mice. These data support the idea that the difference in renal damage between Bsg\(^{+/+}\) and Bsg\(^{-/-}\) mice was due to the difference in neutrophil infiltration mediated by Bsg on neutrophils.

Bsg on neutrophils has long sugar chains that are N-linked polylactosamines (Figure 3A). The biologic significance of these long sugar chains has long been obscure. In this context, it is noteworthy that O-linked glycans on PSGL-1 contribute to binding to its receptors, E- and P-selectins, while N-linked glycans on CD44 are responsible for the binding to E-selectin (Figure 3A). Based on the present results, Bsg has unique sugar chains, i.e., N-linked polylactosamines, that are responsible for its binding to E-selectin.

Renal ischemia/reperfusion leads to increased endothelial expression of a variety of adhesion molecules that promote endothelial-leukocyte interaction. Gene knockout, antibody, and pharmacologic inhibitor studies have suggested a role for E-selectin in ischemia/reperfusion injury. In particular, E-selectin-deficient mice show a 75% reduction in myeloperoxidase activity (an indicator of neutrophil infiltration) in the postischemic kidneys at 24 h compared with wild-type mice. Our Bsg\(^{-/-}\) mice showed a 50% reduction in neutrophil counts in the kidney compared with wild-type mice. Therefore, Bsg may not fully account for the function of E-selectin. Our in vitro binding assay between neutrophils and HUVECs also supports this idea. Therefore, other E-selectin ligands on neutrophils, such as PSGL-1 and CD44, may also be important for neutrophil recruitment and the subsequent renal damages induced by ischemia/reperfusion. Infiltrating neutrophils produce cytokines, growth factors, proteases, and reactive oxygen species, all of which can injure renal cells. Injured renal cells in turn produce factors that stimulate neutrophils. This chain reaction may contribute to the establishment of renal dysfunction. If neutrophil infiltration is moderately suppressed as in the case of Bsg\(^{-/-}\) mice, the suppression of renal dysfunction may be delayed. Therefore, the delayed effect of Bsg deficiency on BUN as compared with E-selectin knockout may not necessarily indicate that Bsg is not an early-acting E-selectin ligand. Rather, the localization of Bsg on the microvilli (Figure 3) may suggest that it participates in initial capture (tethering) on the endothelium, as in the case of PSGL-1.

Finally, our study has shed light on the mechanisms underlying AKI. There is no specific therapy for AKI except for supportive care, and AKI is associated with unacceptably high mortality that has been reported to range from 40% to 60%. The overwhelming majority of studies have exclusively looked at neutrophils as the most important prevalent leukocytes during AKI. Our study thus introduces a novel player in the pathogenesis of AKI. Bsg might be a good candidate target for intervention of AKI.

CONCISE METHODS

Bsg-Deficient Mice

Mice deficient in the Bsg gene were generated as described previously. All experiments were performed with Bsg\(^{+/+}\) and Bsg\(^{-/-}\) littermates. The mice used were 8- to 12-wk-old females weighing 20 to 25 g. The mice were housed under controlled environmental conditions and maintained with standard food and water.

The experiments described above were conducted according to The Animal Experimentation Guide of Nagoya University School of Medicine.

Renal Ischemia/Reperfusion Injury Model

We used a previously characterized model of renal ischemia/reperfusion injury in mice. Briefly, we anesthetized the mice by intraperitoneal administration of 40 mg/kg sodium pentobarbital. We placed the animals on a heating pad to maintain a constant body temperature of 37°C. Under general anesthesia, we removed the right kidney. This heminephrectomy procedure was omitted in the experiment of adoptive transfer of labeled neutrophils. After 7 d, we anesthetized the mice as described above and exposed the left kidney. We occluded the renal artery for 45 min with nontraumatic microvascular clamps. The animals received 30 ml/kg warm saline instilled into the peritoneal cavity after the procedure and were allowed to recover with free access to food and water. Sham-operated mice underwent the same procedure without clamping of the artery and were killed 1 d after surgery. Groups of mice (n = 6) were killed 1, 2, 4, and 7 d after surgery.

We determined serum urea by a standard diagnostic procedure using a kit from KAINOS Laboratories (Tokyo, Japan).

We measured the cytokine MIP-2 and KC in renal homogenates as described previously by specific ELISA according to the manufacturer’s instructions (MIP-2: R&D Systems; KC: Immuno-Biologic Laboratories Ltd., Gunma, Japan). We normalized the results for the total protein concentration.

Histology

We fixed renal tissues in 4% paraformaldehyde, embedded them in paraffin, and then cut them into 2-μm sections. We stained the sections with periodic acid-Schiff reagent. Using semiquantitative indices, we analyzed the sections to evaluate tubulointerstitial damage in each region by light microscopy, as described previously. Briefly, the extent of cast formation, tubular dilation, and tubular degeneration in the cortex, outer medulla, and inner medulla were scored according to the fol-
lowing criteria by two observers in a blind manner: 0, normal; 1, below 30% of the pertinent area; 2, 30% to 70% of the pertinent area; 3, over 70% of the pertinent area.

Parts of the kidney tissues were snap-frozen in liquid nitrogen. We cut 2-μm-thick sections with a cryostat and then fixed them in acetone. We stained the cryosections with rat anti-mouse neutrophil antibody (dilution, 1:200; clone 7/4; Serotec, Oxford, UK), rat anti-mouse macrophage antibody (dilution, 1:50; clone F4/80; Serotec), rat anti-mouse E-selectin antibody (dilution, 1:50; clone 96419; R&D Systems, Minneapolis, MN), or goat anti-mouse ICAM-1 antibody (dilution, 1:50; R&D Systems), followed by detection with FITC-conjugated rabbit anti-rat IgG (dilution, 1:100; Zymed Laboratories, San Francisco, CA) or FITC-conjugated rabbit anti-goat IgG (dilution, 1:100; Sigma-Aldrich, St. Louis, MO). We counted leukocytes positive for 7/4 and F4/80 in all renal regions (cortex, outer medulla, and inner medulla) under a microscope at ×200 magnification in a blind manner.

For immunoelectron microscopy, we washed peritoneal-elicited mouse neutrophils twice and then stained them with rat anti-mouse Bsg antibody (dilution, 1:25; clone OX114; Abcam Ltd., Cambridge, UK) or control rat IgG followed by HRP-conjugated goat F(ab′)2 fragment anti-rat IgG (Histofine; Nichirei Corporation, Tokyo, Japan). After fixation with 1% glutaraldehyde, we incubated the cells with 3,3′-diaminobenzidine (Dako, Carpinteria, CA) for 30 min, then washed them twice. The cells were postfixed in osmium tetroxide, dehydrated in alcohol, and embedded in epoxy resin (Quetol-812; Nissin EM Corporation, Tokyo, Japan). We examined ultrathin sections with a JEM-1400 electron microscope (JOEL Ltd., Tokyo, Japan).

Preparation of Mouse Peritoneal-Elicited Neutrophils
Mouse peritoneal neutrophils were elicited by intraperitoneal injection of 2 ml 3% thioglycollate medium (Wako, Osaka, Japan), which induced aseptic peritoneal inflammation. After 5 h, we collected peritoneal exudate fluid with 5 ml ice-cold PBS.47 We washed the isolated peritoneal cells three times. The purity of neutrophils was approximately 90% as confirmed by FACS analysis (anti-GR-1-positive cells) and May-Giemsa staining. The cell viability was more than 98% checked by trypan blue staining.

Cells
HUVECs (Cell Applications, San Diego, CA) were cultured using an EGM-2 BulletKit (Takara Bio, Shiga, Japan) at 37°C in 5% CO2 and used between the second and fifth passages. We obtained the human promyelocytic cell line HL-60 from the American Type Culture Collection (ATCC; accession no. CCL-240; Manassas, VA) and cultured them in RPMI 1640 (Sigma-Aldrich) containing 10% fetal bovine serum (Life Technologies BRL, Gaithersburg, MD) at 37°C in 5% CO2.

Deglycosylation
To remove N-glycans in the cell lysate, we treated the lysate of mouse peritoneal-elicited neutrophils with 10 U N-glycosidase F (Roche Diagnostics, Mannheim, Germany) at 37°C overnight in a buffer containing 50 mM sodium phosphate, pH 7.5, and 1% Nonidet-P 40. To remove polyglactosamine chains in the cell lysate, we treated the cell lysate with 5 μM endo-β-galactosidase (Seikagaku Corporation, Tokyo, Japan) at 37°C overnight in a buffer containing 10 mM sodium acetate, pH 6.0.

For the inhibition of N-glycosylation in cells, we cultured HL-60 cells in the presence of 15 μg/ml tunicamycin (Calbiochem, San Diego, CA) for 48 h.

Flow Cytometry E- and P-Selectin-Binding Assay
First, we prepared immunomagnetic beads. Lysates were prepared by incubation of HL-60 in lysis buffer (1% Triton-X 100 in PBS with EDTA-free Protease Inhibitor Cocktail; Nakalai Tesque, Kyoto, Japan) for 30 min on ice, and cell debris was removed by centrifugation at 17,000 × g for 10 min at 4°C. We incubated anti-mouse IgG-coated beads (M-280 Dynabeads; Dynal Biotech ASA, Oslo, Norway) with mouse anti-human Bsg antibody (clone MEM-M6/1; Abcam Ltd.) and control mouse IgG for 4 h under rotation. We then washed the beads twice with lysis buffer and incubated them overnight at 4°C under rotation with the prepared HL-60 cell lysate (2 × 106 cells/106 beads) or culture medium supernatant obtained after ultracentrifugation. We washed the beads three times with PBS or 1 M NaCl-PBS before the binding assay. To detach the protein on the prepared immunomagnetic beads, we boiled the beads with sample buffer for 5 min. We then subjected the supernatants to western blot analysis.

Mouse peritoneal-elicited neutrophils (5 × 106) were stained by incubation with antibody against Bsg (dilution, 1:50; clone OX114; Abcam Ltd.), PSGL-1 (dilution, 1:50; clone 4RA10; Becton Dickinson, Franklin Lakes, NJ), CD44 (dilution, 1:50; IM7; BioLegend, San Diego, CA), CD11a (dilution, 1:50; clone I21/7; SouthernBiotech, Birmingham, AL), CD11b (dilution, 1:50; clone M1/70; Cedarlane, ON, Canada), Gr-1 (dilution, 1:100; clone RB6–8C5; Cedarlane), and CD43 (dilution, 1:50; clone 1B11; Biolegend) or control antibodies. Subsequently, we washed these cells with PBS and then incubated them with FITC-conjugated anti-rat IgG (dilution, 1:100; Zymed Laboratories). Cells were washed three times before flow cytometry analysis. To assess the E- and P-selectin-binding property, we incubated mouse peritoneal-elicited neutrophils or prepared Bsg-coated beads with 10 μg/ml recombinant human E-selectin/Fc chimera, human P-selectin/Fc chimera, mouse E-selectin/Fc chimera, and mouse P-selectin/Fc chimera (R&D Systems) at 4°C with gentle shaking for 30 min in the presence or absence of 5 mM EDTA. We then incubated the cells with FITC-conjugated goat F(ab′)2 fragment anti-human IgG (Fcγ; Beckman Coulter, Fullerton, CA). In the analysis of mouse neutrophils, the gates were set on the granulocytic population using forward/side scatter distributions. All samples were analyzed using a FACSChallium flow cytometer and CellQuest software (Becton Dickinson).
Western Blot Analysis

We performed western blot analysis as described previously.48 Briefly, we separated the samples by 10% SDS-PAGE and then transferred the gels to a nitrocellulose membrane (Whatman, Florham Park, NJ). We blocked the membranes with 5% (wt/vol) dry fat-free milk in PBS with 0.1% Tween for 60 min at room temperature. We then incubated the membranes with rat anti-mouse E-selectin antibody (dilution, 1:1000; clone 966419; R&D Systems), goat anti-mouse ICAM-1 antibody (dilution, 1:1000; R&D Systems), goat anti-mouse Bsg antibody (dilution, 1:1000; R&D Systems), mouse anti-human Bsg antibody (dilution, 1:1000; clone HIM6; Biolegend), mouse anti-human PSGL-1 antibody (dilution, 1:1000; clone KPL-1; BD Biosciences), mouse anti-human CD44 antibody (dilution, 1:1000; clone 2C5; R&D Systems), mouse anti-human CD43 antibody (dilution, 1:1000; clone MEM-59; Biolegend), rabbit anti-mouse cyclophilin A antibody (dilution, 1:1000; Upstate, Lake Placid, NY), or mouse anti-β actin antibody (dilution, 1:1000; Sigma-Aldrich). Each primary antibody was incubated at 4°C overnight. After washing with PBS containing 0.1% Tween, we incubated these membranes with peroxidase-conjugated anti-rat IgG, anti-goat IgG, or anti-mouse IgG (dilution, 1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA), respectively, for 60 min at room temperature. We visualized the proteins with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, Amersham Biosciences, Piscataway, NJ).

Adhesion Assay of Mouse Neutrophils to HUVECs

We performed the adhesion assay on HUVECs as described previously.49 Briefly, we washed the mouse peritoneal-elicited neutrophils in HBSS without Ca2+ and Mg2+ and concentrated them to 107 cells/ml. We then incubated them to 107 cells/ml. We then incubated the suspensions with 5 ml of H9262 medium to adhere for 30 min with or without EDTA. This procedure was examined at 4°C, since this temperature is known to be optimal for the investigation of the selectin-mediated adhesion mechanism and does not activate integrins.31 We then inverted the plates for 30 min to eliminate unadherent cells and washed carefully. We then added an equal number of labeled cells (5 × 105) to the well to measure total fluorescence (Ft). The remaining fluorescence (Fb) and the fluorescence of a blank well (Ft) were measured.30 We measured all fluorescences on a Fluoroskan AscentCF (Labsystems, Helsinki, Finland). Calcein-AM-labeled cells were excited at 485 nm and evaluated at 530 nm. We calculated the percentage adhesion using the following formula:

\[
\text{% adhesion} = \frac{F_t - F_b}{F_t} \times 100.
\]

Adoptive Transfer of Labeled Neutrophils

We washed peritoneal-elicited neutrophils from Bsg+/+ and Bsg−/− mice with PBS and stained them with a PKH26 red fluorescence cell linker kit (Sigma-Aldrich) according to the manufacturer’s instructions. The viability of neutrophils was more than 98% after labeling with trypan blue exclusion. Five million neutrophils from Bsg+/+ and Bsg−/− mice were injected intravascularly into Bsg+/+ and Bsg−/− mice, respectively, at 5 min after renal ischemia/reperfusion surgery. After 6 h of reperfusion, we harvested the postischemic kidney. We prepared frozen sections of the kidney at 4-μm thickness. We counted the number of transferred neutrophils (PKH26-labeled cells) by examining all renal regions in 20 continuous sections of the short axis hilum of the kidney. Then we fixed other sections as described above and stained them with rat anti-mouse neutrophil antibody (clone 7/4; Serotec), followed by FITC-conjugated rabbit anti-rat IgG (Zymed Laboratories) to confirm that the infiltrated PKH26-labeled cells were neutrophils. We incubated other sections with chicken anti-mouse E-selectin antibody (dilution, 1:50; R&D Systems), followed by FITC-conjugated rabbit anti-chicken IgG (Zymed Laboratories) to check the positional relations of transferred neutrophils in the kidney. For double-immunofluorescence staining of E-selectin and the endothelium, we first stained the section with E-selectin as described above. It was then incubated with rabbit anti-rat thrombomodulin antibody32 (dilution, 1:1000) followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (dilution, 1:100; Zymed Laboratories).

Statistical Analysis

We expressed all values as means ± SEM. We performed statistical analysis with unpaired, two-tailed t test for single comparisons. Values of P < 0.05 were considered to indicate statistically significant differences.

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DISCLOSURES

None.

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