Regular Article

Nucleolin Is a Receptor for Maleylated-Bovine Serum Albumin on Macrophages

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Scavenger receptors have a broad range of functions that include pathogen clearance, and identification of the scavenger receptor family has been of great benefit to the field of physiology. The shuttling-protein nucleolin has recently been shown to possess scavenger receptor-like activity. We therefore investigated whether or not nucleolin is a receptor for maleylated-bovine serum albumin (maleylated-BSA), which is a common ligand for scavenger receptors. Binding and phagocytosis of native control-BSA by thioglycollate-elicited mouse peritoneal macrophages was weak, but that of maleylated-BSA was strong. Surface plasmon-resonance analysis revealed that nucleolin strongly associated with maleylated-BSA but not control-BSA or maleic anhydride. Further, co-treatment of macrophages with anti-nucleolin antibody, but not control-immunoglobulin G, inhibited binding of maleylated-BSA. In addition, antineoplastic guanine rich oligonucleotide (AGRO), a nucleolin-specific oligonucleotide aptamer, inhibited binding of maleylated-BSA. Further, binding of maleylated-BSA to nucleolin-transfected HEK293 cells was higher than that by control HEK cells. These results indicate that nucleolin is a receptor that enables macrophages to recognize maleylated-BSA.

Key words macrophage; maleylated-bovine serum albumin; nucleolin; scavenger receptor

Macrophages rapidly recognize and remove physiologically or chemically modified proteins, lipids, lipoproteins, and cells to maintain tissue homeostasis. Failure to remove these components results in their accumulation, injury to surrounding tissues, disease, and abnormal autoimmune responses that potentially trigger a number of functional disorders. Therefore, macrophages are critical for maintaining homeostasis and healthy tissues by clearing various discarded elements and reducing inflammation.^{1–4)}

Scavenger receptors (SRs) consist of classes A to I and are expressed on the surfaces of macrophages, dendritic cells, and endothelial cells and initiate the removal of a large repertoire of discarded elements. SRs were identified by their ability to recognize and remove modified or damaged proteins (e.g. β -amyloid), lipoproteins (e.g. oxidized low density lipoprotein (LDL)), lipid (e.g. oxidized phosphatidylserine), and damaged cells (e.g. apoptotic cells).³⁾ The affinity of SRs for endogenous elements is involved in the pathogenesis of multiple diseases. For example, SRs participate in the binding and internalization of amyloid β 42, oxidized LDL, and in the transport of fatty acids. SRs have also been implicated in diseases as diverse as Alzheimer's disease, atherosclerosis and type 2 diabetes mellitus.³⁾

In addition to modified endogenous molecules, SRs recognize conserved pathogen-associated molecular patterns (PAMPs) expressed on exogenous microbial surfaces, which results in the clearance of various microbial species and structures such as bacteria and bacterial lipopolysaccharides, lipoteichoic acid, CpG DNA and viruses.³⁾ SRs therefore have a broad range of functions including not only the binding of modified self-molecules [danger-associated molecular patterns (DAMPs)], but also that of several exogenous PAMPs.^{3,5)} These findings suggest that SRs have a wide range of functions and that the identification of members of the scavenger

receptor family might help characterize tissue homeostasis and diseases.

Nucleolin is present in the nucleus, cytoplasm, and surface of macrophages.⁶⁾ Our previous studies have shown that macrophages utilize nucleolin to recognize and phagocytose discarded elements, including amyloid β 42,⁷⁾ glycated proteins,⁴⁾ apoptotic⁸⁾ and oxidized cells,⁹⁾ to maintain tissue homeostasis. In addition, nucleolin is also a receptor for microbes and their substituents, including lipopolysaccharide,¹⁰⁾ enterohemorrhagic *Escherichia* (*E.*) *coli* O157:H7,¹¹⁾ human parainfluenza virus type 3,¹²⁾ human immunodeficiency virus¹³⁾ and coxsackie B virus.¹⁴⁾ Taken together, these observations suggest that macrophages expressing nucleolin on their surface might have a general scavenger-like ability.

SRs generally have affinity for polyanionic molecules.¹⁵⁾ Maleylated protein derivatives, such as maleylated-bovine serum albumin (maleylated-BSA), have excess negative charge and are ligands for SRs on macrophages.^{16,17)} Maleylated-BSA induces various physiological effects in macrophages, including tumor necrosis factor (TNF)- α , interleukin-1 β , and nitric oxide production.^{17,18)} However, the molecular mechanisms underlying these effects of maleylated-BSA on macrophage function are unclear. In addition, whether or not macrophage cell surface nucleolin recognizes maleylated-BSA is unknown. Here, we investigated whether or not nucleolin is a receptor for maleylated-BSA.

MATERIALS AND METHODS

Materials BSA and PKH 26 red fluorescent cell-linker kits were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Alexa Fluor® 488 C5 maleimide was obtained from Life Technologies (Carlsbad, CA, U.S.A.). rNUC284, a truncated recombinant human nucleolin containing residues

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284–710 and corresponding to the C-terminal and two-thirds of the molecule, was produced in *E. coli* and purified as previously described.⁸⁾ Phycoerythrin-labeled anti-CD11b antibody was obtained from BD Pharmingen (Tokyo, Japan). Anti-nucleolin antibody was obtained from Bethyl Laboratories, Inc. (Montgomery, AL, U.S.A.). Control-rabbit immunoglobulin G (IgG) was obtained from Santa Cruz Biotechnology (Dela-ware, CA, U.S.A.).

Preparation of Maleylated-BSA Maleylated-BSA was prepared as described by Butler and Hartley.¹⁶⁾ Briefly, maleic anhydride powder was added to BSA (5 mg/mL) in 0.2 M Na₂B₄O₇ and pH maintained at 8.5 by Na₂CO₃. Addition of maleic anhydride was terminated once there was no decrease in pH. After incubation, the sample was dialysed 6 times with 100 times the volume of Ca²⁺, Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS[–]) at 4°C to remove low-molecular-weight reactants and maleic anhydride, and protein concentration was measured *via* the Lowry method.¹⁹⁾ Trinitrobenzene sulfonic acid titration²⁰⁾ confirmed that 95.2% of free amino groups in maleylated-BSA preparation was modified, compared to native-BSA.

Fluorescent Labeling of Control-BSA and Maleylated-BSA Using Alexa Fluor® 488 C5 Maleimide Control-BSA or maleylated-BSA was incubated with 20 times the volume of Alexa Fluor® 488 C5 maleimide overnight at 4°C in DPBS(–) under nitrogen replacement and then dialysed 6 times with 100 times the volume of DPBS(–). Protein concentrations were measured *via* the Lowry method,¹⁹⁾ and fluorescence intensities were measured using a VARIOSKAN microspectrometer (excitation: 492 nm, emission: 519 nm). The strengths of Alexa-control-BSA, Alexa-maleylated-BSA fluorescence per unit dosage were each adjusted by adding unlabeled proteins.

Binding of Control-BSA and Maleylated-BSA to Mouse Macrophages The protocol was approved by the committee on the Ethics of Animal Experiments of Tokyo University of Pharmacy and Life sciences (Permit Number: P14–15). All surgery was performed under diethyl ether anesthesia, and all efforts were made to minimize suffering.

Thioglycollate-elicited mouse macrophages were prepared from male ddy mice (Japan SLC, Inc., Shizuoka, Japan), as previously described.^{4,8,21)} Macrophages were suspended at 5×10⁵ cells/mL in RPMI-1640 medium buffered with 20 mM 4-[2-hydroxyethyl]-piperazineethanesulfonic acid (RPMI 1640-HEPES, pH 7.2) and then incubated with 0–20 µg/mL Alexa Fluor® 488-labeled control-BSA or maleylated-BSA at 37°C for 2 h. After 2 h, the cells were incubated with phycoerythrin-labeled anti-CD11b antibody for 30 min on ice, then washed and immediately analyzed using a flow cytometer (FACS Calibur, Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) with CellQuest software (Becton-Dickinson) and gating for forward scatter (FSC) and side scatter (SSC) regions of intact cells. Since fluorescent labeled anti-CD11b was used for selection of macrophages at flow cytometer analysis, anti-CD11b positive cells were regarded as macrophages.^{4,8,21)}

Confocal Images of Phagocytosed Maleylated-BSA Macrophages were seeded on a coverslip as previously described⁸⁾ and incubated with 0–10 µg/mL Alexa Fluor® 488-labeled control-BSA or maleylated-BSA, and membranes were stained with the fluorescent cell-linker compound PKH 26 red. BSAs taken up by macrophages were identified using

confocal laser-scanning fluorescence microscopy (FV1000D; Olympus, Tokyo, Japan).

Surface Plasmon-Resonance (Biacore) Binding of nucleolin to control-BSA or maleylated-BSA was analyzed using a Biacore 2000X (GE Healthcare, Tokyo, Japan). This biosensor directly measures the binding of a recombinant protein to its ligand in real time and in a quantitative and highly reproducible manner.²²⁾ Briefly, DPBS(–) was used for sample dilution and analysis. rNUC284 was produced in *E. coli* and purified as previously described⁸⁾ and immobilized on a research-grade CM5 dextran sensor-chip.⁴⁾ To evaluate binding, control-BSA or maleylated-BSA were diluted in DPBS(–), analyzed at different concentrations, and passed over the sensor chip at a flow rate of 20 µL/min. An activated and blocked flow-cell without immobilized ligands was used to evaluate nonspecific binding. Results were calculated using BIAevaluation 4.0 software (Biacore).

SDS-PAGE Analysis BSAs (17 µg) were analyzed *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli, using a 10% polyacrylamide gel under reducing conditions.²³⁾

Binding of Maleylated-BSA to Nucleolin-Transfected HEK Cells Human nucleolin cDNA was transfected into the monolayer of HEK 293 cells (HEK cells) (Health Science Research Bank, Osaka, Japan) as previously described.⁸⁾ Sixteen hours after transfection, adherent HEK cells were dislodged by incubation with Puck's ethylenediaminetetraacetic acid (EDTA) solution (5 mM *N*-(2-hydroxyethyl)perazine-*N'*-2-ethanesulfonic acid (HEPES), 0.1 M NaCl, 5 mM KCl, 4 mM NaHCO₃, 1 mM EDTA, and 5.6 mM glucose) for 10 min and gentle pipetting. HEK cells were then incubated with 10 µg/mL Alexa Fluor® 488-labeled maleylated-BSA at 37°C for 2 h and immediately analyzed using a flow cytometer with CelQuest software with gating for FSC and SSC regions of intact HEK cells.

Statistical Analysis Data are presented as the mean±standard deviation (S.D.) of at least triplicate determinants and were analyzed using Student's *t*-tests.

RESULTS

Maleylated-BSA Was Bound and Phagocytosed by Macrophages The purpose of present study was to elucidate whether or not nucleolin is a receptor for maleylated-BSA. First, we compared the proportion of macrophages that bound and phagocytosed maleylated-BSA and native-BSA. Although mouse macrophages did not bind control-BSA (Fig. 1A, *open circle*), they did bind maleylated-BSA in a dose-dependent manner (*closed circle*). The binding of Alexa Fluor® 488 labeled maleylated-BSA was suppressed by unlabeled maleylated-BSA dose-dependently (Fig. 1B), suggesting that Alexa Fluor® 488 labeled maleylated-BSA was specifically binding to macrophages. In addition, although macrophages did not exhibit a high level of phagocytosis for control-BSA (Figs. 2B, D), they did for maleylated-BSA (Figs. 2C, D), indicating that they regard maleylated modified-protein as a target for removal.

Binding of Nucleolin with Maleylated-BSA We next investigated whether or not nucleolin is a receptor for maleylated-BSA. We first observed the interactions between nucleolin and maleylated-BSA using surface plasmon-resonance (SPR).

Fig. 1. Binding of Maleylated-BSA to Macrophages

(A) Binding of 0–20 µg/mL Alexa Fluor® 488-labeled control-BSA (open circle) and maleylated-BSA (closed circle) to macrophages as measured by the mean fluorescence intensity (flow cytometer analysis). (B) Co-incubation with 0–100 µg/mL unlabeled maleylated-BSA suppressed binding of 10 µg/mL Alexa Fluor® 488-labeled maleylated-BSA. Each point represents the mean ± S.D. of triplicate determinations. * $p < 0.05$; *** $p < 0.001$.

Fig. 2. Phagocytosis of Maleylated-BSA by Macrophages

Phagocytosis of Alexa Fluor® 488-labeled control-BSA, 10 µg/mL (B) and maleylated-BSA, 10 µg/mL (C) by macrophages was observed by confocal laser microscopic analysis. BSA-untreated macrophage (A) is shown as a control image. Green, BSAs. Red, macrophage cell-surface. (D) Relative fluorescence intensities of intracellular green-fluorescence. Columns represent mean ± S.D. *** $p < 0.001$.

rNUC284, a truncated recombinant human nucleolin containing residues 284–710 and corresponding to the C-terminal and two-thirds of the molecule, was produced in *E. coli* and purified as previously described.⁸⁾ We used native-BSA as an affinity control, as we have previously shown that native-BSA was not bound to rNUC284.⁴⁾ Control-BSA, maleic anhydride, and maleylated-BSA were applied to a CM5 dextran sensorchip, which was immobilized with rNUC284. The sensorgram demonstrated that maleylated-BSA had a high concentration-dependent affinity and did not easily dissociate upon washing (Fig. 3C), while control-BSA (Fig. 3A) and maleic anhydride (Fig. 3B) had no affinity for rNUC284. SDS-PAGE analysis showed that the molecular weight of control-BSA and maleylated-BSA were calculated as 61.3 kDa (77 mm of electrophoretic mobility) and 88.4 kDa (54 mm of electrophoretic mobility) (Fig. 3D). Therefore, the respective k_a , k_d , and KD values between rNUC284 and each BSA were calculated as follows: maleylated-BSA: 7.62×10^2 , 2.08×10^{-2} and 3.69×10^{-4} , control-BSA: $k_a = 2.86$ (k_d and KD value were not shown, since control-BSA was hardly shown the affinity for rNUC284). These results indicate that maleylated-BSA bound to nucleolin

and that control-BSA and maleic anhydride did not.

Involvement of Macrophage-Surface Nucleolin in Binding of Maleylated-BSA We next investigated whether or not cell surface-expressed nucleolin is a receptor for maleylated-BSA. As shown in Fig. 4A, binding of maleylated-BSA by macrophages was inhibited by anti-nucleolin antibody but not by control-rabbit IgG (Fig. 4A). Further, when nucleolin was blocked using the nucleolin-specific oligonucleotide aptamer antineoplastic guanine rich oligonucleotide (AGRO),²⁴⁾ macrophage binding of maleylated-BSA was inhibited, whereas adding cytosine rich oligonucleotide (CRO) as a control had no such effect (Fig. 4B). In addition, we expressed recombinant nucleolin on the surface of non-macrophage HEK cells by transiently transfecting nucleolin on HEK cells as previously described.⁸⁾ Binding of maleylated-BSA to these HEK cells was slightly but significantly increased compared to non-transfected HEK cells (p value = 0.0366) (Fig. 4C). These results indicate that nucleolin is a receptor for maleylated-BSA.

Fig. 3. Binding of Nucleolin with Maleic Anhydride, Control-BSA, and Maleylated-BSA

Surface plasmon-resonance analyses of maleic anhydride, control-BSA, and maleylated-BSA binding to nucleolin. Control-BSA (A), maleic anhydride (B), and maleylated-BSA (C) were washed over rNUC284-immobilized CM5 dextran-sensor chips. Maleic anhydride, control-BSA, and maleylated-BSA were applied at 1–20 µg/mL. An activated and blocked flow cell without immobilized ligands was used to evaluate nonspecific binding. (D) SDS-PAGE analysis of control-BSA and maleylated-BSA. Post-electrophoresis, bands were visualized by Coomassie Brilliant Blue staining (left panel). Analytical curve of molecular weight and electrophoretic mobility (right panel).

Fig. 4. Involvement of Macrophage-Surface Nucleolin in Binding of Maleylated-BSA

(A) Co-incubation with 10 µg/mL anti-nucleolin antibody inhibited binding of 10 µg/mL maleylated-BSA. (B) Co-incubation with 10 µM nucleolin-specific aptamer AGRO inhibited binding of 10 µg/mL maleylated-BSA. (C) Binding of 10 µg/mL maleylated-BSA to nucleolin-transfected HEK cells. Bars represent mean ± S.D. of triplicate determinations. * $p < 0.05$; ** $p < 0.01$.

DISCUSSION

Nucleolin has recently been characterized as a phagocyte receptor that initiates the removal of a variety of discarded

elements.^{4,6–9,25)} Here, we showed that nucleolin expressed on the surface of macrophages is also a receptor for maleylated-BSA. These observations support the idea that phagocytes with cell-surface expression of nucleolin have a general scav-

enger-like ability.

In the present study, maleylated-BSA serves as the ligand for nucleolin, while the biological impacts of the ligand on nucleolin on macrophage have remained obscure. Alford *et al.*, reported that maleylated-BSA elicits transcription and secretion of proinflammatory cytokines, including, nitric oxide, TNF- α , and nuclear factor-kappa B (NF- κ B), suggesting that maleylated-BSA induces inflammation.¹⁸⁾ Although the precise mechanism of the inflammation induced by maleylated-BSA is still unknown, nucleolin might function as a receptor. Indeed, cell surface-expressed nucleolin is known to be involved in the inflammatory response induced by lipopolysaccharide.¹⁰⁾ In addition, TNF- α -inducing protein (Tip α), a carcinogenic factor released by *Helicobacter pylori*, induces TNF- α and chemokine gene NF- κ B activation through cell surface-expressed nucleolin.²⁶⁾ As down-regulation of cell surface nucleolin inhibited inflammatory response,²⁶⁾ an anti-inflammatory strategy that targets nucleolin might be a promising strategy for preventing inflammation.

Maleylation of BSA results in an affinity for nucleolin. Scavenger receptors generally have an affinity for polyanionic molecules (*e.g.* acetylated-LDL, oxidized-LDL, polysaccharides (polyvinyl sulfate, dextran sulfate and fucoidan) and polynucleotides (poly[G] and poly[C]).¹⁵⁾ The polyanionic nature of maleylated-BSA might therefore play a major role in nucleolin binding as maleylated proteins have an excess negative charge.¹⁶⁾ However, negative charge alone cannot explain the affinity between nucleolin and maleylated-BSA, as maleic anhydride does not have an affinity for nucleolin (Fig. 3B). In addition, the binding of rNUC284 and maleylated-BSA did not dissociate with NaCl treatments by SPR analysis (data not shown). In contrast, treatment with the protein-denaturing agent guanidine did dissociate binding, which indicates that the bond between nucleolin and maleylated-BSA is not only an ionic bond but also superstructural modifications due to maleylation are required.

Our previous studies revealed that nucleolin also bound to an aggregated sialylpolylactosaminyl chain of CD43, a negatively charged membrane protein on T-lymphocytes.^{8,27)} These studies showed that the three-dimensional structure of the CD43 cap is critical for binding to nucleolin. Therefore, negative charges might have an important role in binding between maleylated-BSA and nucleolin. In addition, the superstructure of the ligand composed by clustering or aggregation might also be important for binding to nucleolin.

As SRs play an important role in the pathogenesis of various diseases, including atherosclerosis, soluble SRs were investigated. Jalkanen *et al.* reported that soluble SRs inhibit receptor-mediated functions in atherosclerosis.²⁸⁾ Those authors generated a secreted macrophage scavenger receptor (sMSR) that consisted of the bovine growth hormone signal sequence and the human MSR-AI extracellular domains. sMSRs reduce the degradation of atherogenic modified LDL and monocyte/macrophage adhesion on endothelial cells. In addition, adeno-associated virus (AAV)-mediated sMSR gene transfer reduced atherosclerotic lesion area in the aorta. Even though eradication of established disease was not possible, atherosclerotic lesion formation could be modified using AAV-mediated gene transfer of the sMSR.²⁸⁾ As nucleolin is also detected in serum,²⁹⁾ nucleolin operation *in vivo* might also be a useful means of preventing and treating SRs-involved dis-

eases, including atherosclerosis.

Here, we demonstrated that, in addition to other discarded elements,^{4,6–9,25)} maleylated-BSA is also a ligand for nucleolin. This observation supports the notion that nucleolin on the surface of phagocytes has a general scavenger-like ability. Additional comprehensive evaluation of nucleolin, including the molecular composition of its ligands and the functional consequences of their composition, will further clarify the physiological role of SRs.

Conflict of Interest The authors declare no conflict of interest.

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