Nucleolin Acts as a Scavenger Receptor for Acetylated Low-Density Lipoprotein on Macrophages

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Although macrophage phagocytoses modified low-density lipoprotein (LDL), excessive accumulation of modified LDL induces macrophage foam cell formation, which is a feature of atherosclerotic plaque. Thus, the identification of scavenger receptor for modified LDL will provide better understanding of an atherosclerotic event. We recently showed that nucleolin expressed on macrophages acts as a scavenger receptor for various endogenous discarded products. Here, we investigated whether or not nucleolin is involved in the uptake of acetylated LDL (AcLDL). In contrast to normal LDL, AcLDL directly bound to immobilized nucleolin. AcLDL exhibited a higher affinity for macrophages than normal LDL. This binding of AcLDL was inhibited by anti-nucleolin antibody and antineoplastic guanine-rich oligonucleotide (AGRO), a nucleolin-specific oligonucleotide aptamer. In addition, AcLDL exhibited a higher affinity for HEK cells transfected with nucleolin than those without. Further, intracellular accumulation of AcLDL was also inhibited by antinucleolin antibody. The results of this study suggest that nucleolin expressed on macrophages is a receptor for AcLDL.

Key words nucleolin; macrophage; acetylated low-density lipoprotein (AcLDL); scavenger receptor

Low-density lipoprotein (LDL) elevates cholesterol deposits in macrophage foam cells, which are involved in the development of atherosclerotic lesions.1) As the uptake of normal LDL via its receptor is controlled by feedback regulation, excess levels of LDL-derived cholesterol in macrophages might be attributed to modifications of LDL such as oxidation, acetylation, and aggregation.2) Scavenger receptors (SRs) and other unknown receptors account for up to 95% of the uptake of modified LDL.3,4) Acetylated-low-density lipoprotein (AcLDL), which is a chemically-modified form of LDL, mimics oxidized-low-density lipoprotein (OxLDL) found in atherosclerotic lesions.5) Normal LDL is considered to bind to LDL receptors, while AcLDL is considered to bind SRs for uptake into macrophages.5,6) However, the elucidation of receptor for AcLDL has been insufficient, and the identification of a receptor might facilitate a more comprehensive understanding of tissue homeostasis and atherosclerosis.

SRs consist of classes A to I and are expressed as cell surface receptors on macrophages, dendritic cells, and endothelial cells.7) SRs play a key role in the removal of a broad range of discarded elements and were initially identified by their ability to bind and remove modified or damaged endogenous and exogenous molecules, including maldated protein, fibril amyloid-β, AcLDL, OxLDL, oxidized phosphatidylserine, apoptotic cells, bacteria, bacterial lipopolysaccharides, and viral components.7) The affinity of SRs for multiple ligands is involved in the pathogenesis of multiple diseases, including atherosclerosis, Alzheimer’s disease, and microbial infections.7) Nucleolin is a multifunctional shuttling protein that is present in the nucleus, cytoplasm, and on the surface of macrophages.8,9) We previously identified nucleolin as a scavenger receptor for apoptotic cells9) and modified proteins such as fibril amyloid-β,10) advanced glycosylation end products,11) and maldated-proteins.12) In addition, nucleolin is a scavenger receptor for microbes and their substrates, including lipopolysaccharide,13) enterohemorrhagic Escherichia coli (E. coli) O157:H7,14) and human parainfluenza virus type 3.15)

The similar functions between nucleolin and SRs in scavenging capacity suggest that nucleolin expressed on the surface of phagocytes might have a general scavenger-like ability and thereby recognize modified LDL. Here, we investigated whether or not nucleolin is a receptor for AcLDL on macrophages.

MATERIALS AND METHODS

Materials Normal LDL and 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocyanine (DiI)-labeled AcLDL were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). PKH76 Green Fluorescent Cell-linker Kit, nucleolin-specific oligonucleotide aptamer antineoplastic guanine-rich oligonucleotide (AGRO), and cytosine-rich oligonucleotide (CRO) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Alexa Fluor 488-labeled anti-CD11b antibody was purchased from Becton Dickinson (Tokyo, Japan). rNUC284, a truncated recombinant human nucleolin containing residues 284 to 710 and corresponding to the C-terminal-facing two-thirds of the molecule, was transfected into E. coli and purified as previously described.9) Anti-nucleolin antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, AL, U.S.A.). Control rabbit immunoglobulin G (IgG) was purchased from Santa Cruz Biotechnology (Delaware, CA, U.S.A.).

AcLDL Preparation AcLDL was prepared by reacting normal LDL with acetic anhydride, as described by Basu et al.17) Normal LDL was fluorescently labeled using a DiI probe, as previously described.6) After dialysis, protein concentrations were measured using the Lowry method,18) and fluorescence intensities were measured using a VARIOSKAN microspec-
trometer (excitation: 488 nm, emission: 585 nm). The strengths of DiI-labeled normal LDL and DiI-labeled AcLDL (Cosmo Bio) fluorescence per unit dosage were each adjusted by adding unlabeled LDL.

**Surface Plasmon-Resonance (SPR) Assay** Binding of normal LDL or AcLDL to immobilized nucleolin was quantitatively analyzed using a Biacore 2000X (GE Healthcare, Tokyo, Japan), which directly measures the binding of a recombinant protein to its natural biological ligand in real time and in a highly reproducible manner. Briefly, HBS running buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM sodium chloride (NaCl), 3.4 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% Tween 20, pH 7.4) was used for sample dilution and analysis. The CM5 dextran sensor chip was immobilized by rNUC284 produced in *E. coli* and purified as previously described. To evaluate binding, each type of LDL was passed over the sensor chip. An activated and blocked flow cell without immobilized rNUC284 was used to evaluate nonspecific binding. Results were calculated using BIAevaluation 4.0 software (Biacore; GE Healthcare, Cleveland, OH, U.S.A.).

**Binding of Normal LDL and AcLDL to Murine Macrophages** The protocol was approved by the Ethics Committee for Animal Experiments of Tokyo University of Pharmacy and Life Sciences ( Permit Number: P14–15). Surgery was performed under anesthesia, and all efforts were made to minimize the suffering of mice. Thioglycollate-elicited murine macrophages were isolated from male ddY mice (Japan SLC, Inc., Shizuoka, Japan), as previously described. Macrophages were suspended at 5×10^5 cells/mL in RPMI-1640 medium buffered with 20 mM HEPES (pH 7.2) and then incubated for 10 min and gentle pipetting. HEK cells were then incubated with 10 µg/mL DiI-labeled normal LDL or AcLDL at 37°C for 1 h and immediately analyzed using a flow cytometer with CELLQUEST software and gating for FSC and SSC regions of intact HEK cells.

**Confocal Images of Phagocytosed Normal LDL and AcLDL** Following the incubation of macrophages with or without DiI-labeled normal LDL or AcLDL as above, macrophages were stained using the PKH76 Green Fluorescent Cell-linker Kit (Sigma-Aldrich). LDL taken up by macrophages was identified using confocal laser-scanning fluorescence microscopy (FV1000D; Olympus, Tokyo, Japan).

**Statistical Analysis** Data are presented as the mean ± standard deviation (S.D.) of at least three independent experiments. Student’s *t*-test was used for statistical analysis, and *p*<0.05 was considered significant.

**RESULTS AND DISCUSSION**

**Binding of AcLDL to Immobilized Nucleolin** First, we investigated the potential role of nucleolin as an AcLDL receptor (Fig. 1). To determine whether or not AcLDL directly binds to nucleolin, we analyzed interactions between nucleolin and AcLDL using SPR assay. Normal LDL and AcLDL were applied to a CM5 dextran sensor chip, which was immobilized with rNUC284. The sensorgram demonstrated that affinity of AcLDL was dose-dependent, that it was not easily dissociated by washing (Fig. 1B), and that normal LDL had no affinity for rNUC284 (Fig. 1A). These results indicate that AcLDL bound to nucleolin whereas normal LDL did not.

**Binding of AcLDL to Nucleolin on Macrophages** Figure 2A shows the binding affinities of normal LDL and AcLDL to thioglycollate-elicited murine macrophages. AcLDL had a higher affinity for murine macrophages than normal LDL, although both AcLDL and normal LDL bound to macrophages in a dose-dependent manner. We next investigated whether or not AcLDL binds to cell surface-expressed nucleolin on macrophages. As shown in Fig. 2B, binding of AcLDL to macrophages was inhibited by anti-nucleolin antibody but not

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**Fig. 1. Binding of Normal LDL and AcLDL to Immobilized Nucleolin**

Normal LDL (A) and AcLDL (B) were applied at 10, 50 or 100 µg/mL and passed over rNUC284-immobilized CM5 dextran sensor chips. An activated and blocked flow cell without immobilized ligands was used to evaluate nonspecific binding.
by control rabbit IgG. In contrast, the binding of normal LDL to macrophages was not inhibited by either of the antibodies (Fig. 2B). The nucleolin-specific oligonucleotide aptamer AGRO binds to RNA binding domain of nucleolin and G-quadruplex structure of AGRO is important for the specific binding. As shown in Fig. 2C, AGRO inhibited the binding of AcLDL to macrophages, but the control compound CRO did not. The binding of normal LDL was not inhibited by AGRO (Fig. 2C). In addition, recombinant nucleolin was expressed on the surface of non-macrophage HEK cells by transient transfection. Although AcLDL had a higher affinity for HEK cells transfected with nucleolin than those without, the binding of normal LDL was not altered following nucleolin transfection (Fig. 2D). These results suggest that AcLDL, but not normal LDL, binds to nucleolin expressed on macrophages.

**Phagocytosis of AcLDL by Macrophages** We observed a significant increase in the intracellular accumulation of AcLDL compared to normal LDL in murine macrophages (Figs. 3A–D), suggesting that AcLDL may undergo preferential phagocytosis and accumulation in macrophages. In addition, we investigated whether or not cell surface-expressed nucleolin is involved in the phagocytosis of AcLDL. As shown in Fig. 3E, phagocytosis of AcLDL by macrophages was inhibited by anti-nucleolin antibody but not by control rabbit IgG. These results suggest that nucleolin expressed on macrophages is a receptor for AcLDL, but not for normal LDL.

The results of the present study indicate that, in contrast to normal LDL, AcLDL directly binds immobilized nucleolin (Fig. 1). Compared to normal LDL, AcLDL has a higher affinity for the surface of macrophages (Fig. 2), resulting in a higher level of intracellular accumulation (Fig. 3). The binding of AcLDL to macrophages was inhibited by anti-nucleolin antibody (Fig. 2B) and AGRO (Fig. 2C). In addition, AcLDL has a higher affinity for nucleolin-transfected HEK cells than non-transfected HEK cells (Fig. 2D). Furthermore, in the presence of anti-nucleolin antibody, the binding and phagocytosis levels of AcLDL by macrophages were reduced by about 40 and 46%, respectively (Figs. 2B, 3E), suggesting that nucleolin may contribute approximately 40% of AcLDL uptake capacity by macrophages. Taken together, these results clearly indicate that nucleolin is an AcLDL receptor on macrophages. We recently reported that nucleolin is a phagocytic receptor that initiates the removal of a variety of discarded elements. These previous observations and the present study support our hypothesis that cell surface-expressed nucleolin on phagocytes has a general scavenger-like ability.

Although AcLDL does not bind to LDL receptors, it does bind to SRs, which generally have an affinity for polyamionic molecules. Chemically-induced acetylation of the lysine or arginine residues of apoproteins of LDL induces an excess negative charge, causing it to become a ligand for SRs on macrophages. In addition to AcLDL in the present study, nucleolin also binds to negatively charged molecules such as...
Taken together, these findings suggest that the polyanionic nature of AcLDL might play a major role in its binding to nucleolin. However, as the binding of rNUC284 and AcLDL did not dissociate with NaCl treatment in SPR analysis (data not shown), factors other than negative charge might also contribute to the binding affinity of nucleolin for AcLDL. In contrast, the binding of rNUC284 and AcLDL was dissociated by treatment with the protein-denaturing agent guanidine (data not shown). The same observations were also made regarding the binding of nucleolin with other anionic molecules, including glycosylated protein and maleylated protein. These findings suggest that not only an ionic bond but also superstructural modifications are required for the binding of AcLDL to nucleolin. At present, however, no definitive explanation has been given for this binding mechanism.

Nucleolin can be detected on the cell surface, otherwise its lack of a transmembrane domain. Song et al. reported that integrin α5β1 has a transmembrane domain, and works a definitive role during the nucleolin-mediated internalization process. Although the mechanism of nucleolin-mediated AcLDL-internalizing process remains unknown, we speculate that nucleolin, as well as other SRs, on macrophages may scavenge modified LDL including oxidized LDL to maintain homeostasis, while excessive phagocytosis of modified LDL via nucleolin may induce foam cell formation.

In the present study, we observed that nucleolin acts as a receptor for AcLDL but not for normal LDL, suggesting that nucleolin mediates a novel pathway of foam cell formation. Nucleolin expressed on macrophages might therefore be a promising target for the prevention and treatment of atherosclerosis. Further studies are required to clarify the action of macrophages following nucleolin-mediated phagocytosis of AcLDL.

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Conflict of Interest The authors declare no conflict of interest.

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