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LOX-1 Supports Adhesion of Gram-Positive and Gram-Negative Bacteria

Takeshi Shimaoka,* Noriaki Kume,† Manabu Minami,† Kazutaka Hayashida,† Tatsuya Sawamura,‡ Toru Kita,† and Shin Yonehara*

Adhesion of bacteria to vascular endothelial cells as well as mucosal cells and epithelial cells appears to be one of the initial steps in the process of bacterial infection, including infective endocarditis. We examined whether lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), a member of scavenger receptor family molecules with C-type lectin-like structure, can support adhesion of Gram-positive and Gram-negative bacteria. Chinese hamster ovary-K1 (CHO-K1) cells stably expressing LOX-1 can support binding of FITC-labeled Staphylococcus aureus and Escherichia coli, which was suppressed by poly(I) and an anti-LOX-1 mAb. Adhesion of these bacteria to LOX-1 does not require divalent cations or serum factors and can be supported under both static and nonstatic conditions. Cultured bovine aortic endothelial cells (BAEC) can also support adhesion of FITC-labeled S. aureus, which was similarly suppressed by poly(I) and an anti-LOX-1 mAb. In contrast, binding of FITC-labeled E. coli to BAEC was partially inhibited by the anti-LOX-1 mAb, and poly(I) did not block FITC-labeled E. coli adhesion to BAEC, but, rather, enhanced it under a static condition. TNF-α increased LOX-1-dependent adhesion of E. coli, but not that of S. aureus, suggesting that S. aureus adhesion to BAEC may require additional molecules, which cooperate with LOX-1 and suppressed by TNF-α. Taken together, LOX-1 can work as a cell surface receptor for Gram-positive and Gram-negative bacteria, such as S. aureus and E. coli, in a mechanism similar to that of class A scavenger receptors; however, other unknown molecules may also be involved in the adhesion of E. coli to BAEC, which is enhanced by poly(I).

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Adhesion of bacteria to vascular endothelial cells has been suggested as one of the initial steps in the invasion of blood-borne bacteria to other organs. In particular, adhesion of Staphylococcus aureus may be important, because S. aureus often causes bacterial endocarditis in the absence of pre-existing vascular injury (1). A previous report has suggested that 50-kDa (2) and 130-kDa (3) cell surface molecules on cultured vascular endothelial cells can support binding of S. aureus; however, molecular identification has not been accomplished. In addition, adhesion and subsequent engulfment of Gram-positive and Gram-negative bacteria by macrophages appear crucial for host defense and immune responses after bacterial infection.

Scavenger receptor family molecules have been implicated in bacterial adhesion to macrophages. Scavenger receptor class A (SR-A)2 can bind Gram-positive (4) and Gram-negative (5) bacteria, including S. aureus and Escherichia coli, as well as lipoteichoic acid from Gram-positive bacteria (4) and LPS from Gram-negative bacteria (6). Studies with SR-A knockout mice revealed that SR-A plays important roles in host defense against bacterial infection; it enhances sensitivities for S. aureus (7) and Listeria infection (8) as well as for LPS-mediated septic shock (9).

MARCO, another member of SR-A, can also adhere to Gram-positive and Gram-negative bacteria (10–12).

We have identified lectin-like oxidized low density lipoprotein receptor 1 (LOX-1), a 48- to 50-kDa type II membrane glycoprotein with C-type lectin-like structure in the extracellular domain, which acts as an endocytosis receptor for oxidized low density lipoprotein in cultured bovine aortic endothelial cells (BAEC) (13). LOX-1 is synthesized as a 40-kDa precursor protein with N-linked high mannose carbohydrate chains and is subsequently further glycosylated and processed into a 48-kDa mature form (14). LOX-1 has a broad spectrum of physiological ligands, including oxidized or aged erythrocytes, apoptotic cells (15), and activated platelets (16). In vivo, LOX-1 is highly expressed in endothelial cells, macrophages, and smooth muscle cells in atherosclerotic lesions of humans (17) and hypercholesterolemic rabbits (18). Interestingly, LOX-1 can be induced by proinflammatory stimuli, such as TNF-α (19), TGF-β (20), and LPS (21), as well as by fluid shear stress (21, 22), suggesting its roles in the settings of inflammatory diseases and vascular injury.

In the present study, we studied whether LOX-1 can support adhesion of bacteria, such as S. aureus and E. coli, in both cultured vascular endothelial cells and Chinese hamster ovary-K1 (CHO-K1) cells stably expressing LOX-1.

Materials and Methods

Reagents

DMEM and Ham’s F-12 medium were obtained from Nissui (Tokyo, Japan). FCS were purchased from Sanko Junyaku (Tokyo, Japan). Poly(I) and poly(C) were purchased from Sigma (St. Louis, MO).

Cell culture

BAEC were isolated from bovine aortas by scraping the inner surface with glass cover slips and were cultured in DMEM containing 10% heat-inactivated FCS in an atmosphere of 95% air/5% CO2 at 37°C. Wild-type CHO-K1 cells were maintained in F-12/10% FCS. CHO-K1 cells stably...
expressing bovine LOX-1 (BLOX-1-CHO) were maintained in F-12/10% FCS supplemented with 10 μg/ml of blasticidin S (Funakoshi, Tokyo, Japan) as previously described (13).

DNA transfection into COS-7 cells

COS-7 cells were prepared by cotransfection of expression vectors encoding LOX-1 and truncated Aic2A with extracellular and transmembrane regions (23), and cells expressing Aic2 were collected using the panning method with anti-Aic2 mAb (24). Finally, LOX-1 was expressed in >80% of the collected cells.

Flow cytometry

BLOX-1-CHO or BAEC (1 × 10^6 cells/50 μl of buffer A; PBS with 5% FCS and 0.05% sodium azide) were incubated with anti-LOX-1 mAb (JTX20, 30 μg/ml) or control IgG (20 μg/ml) on ice for 1 h. After washing with buffer A, the cells were incubated with FITC-conjugated anti-mouse IgM mAb (20 μg/ml; PharMingen, San Diego, CA) for 1 h on ice, washed, and applied to EPICS Elite (Coulter, Hialeah, FL).

Bacterial adhesion assay

After BLOX-1-CHO or BAEC were incubated with FITC-labeled S. aureus or E. coli (3 × 10^6 cells/ml unless otherwise indicated; Molecular Probes, Eugene, OR) in the culture medium at 37°C for 1 h, BLOX-1-CHO or BAEC were washed with the culture medium twice and thereafter with PBS twice to remove unbound bacteria, then subjected to fluorescence microscopy (Axioskop2; Zeiss, New York, NY). Cells were treated with trypsin for 5 min, and then detached cells were subjected to flow cytometry by use of EPICS Elite (Coulter). Numbers of BLOX-1-CHO or BAEC that bound FITC-labeled bacteria were calculated. In some experiments cells were pretreated with pol(I), pol(C) (100 or 500 μg/ml), a neutralizing anti-LOX-1 mAb (JTX20, 30 μg/ml) (16), or control IgG (30 μg/ml) for 15 min before addition of FITC-labeled bacteria. After BLOX-1-CHO or BAEC were incubated with FITC-labeled S. aureus or E. coli (3 × 10^6 cells/ml unless otherwise indicated; Molecular Probes) in the culture medium at 37°C for 1 h, BLOX-1-CHO or BAEC were washed with the culture medium twice and thereafter with PBS twice to remove unbound bacteria, then treated with trypsin for 5 min. After detached cells were chilled to 4°C, the cells were incubated with rhodamine-labeled Con A (20 μg/ml; Vector Laboratories, Burlingame, CA) for 60 min at 4°C to visualize the plasma membrane. After washing three times with PBS, the cells were immediately fixed with 10% (v/v) formaldehyde and observed under a confocal laser microscope (Bio-Rad, Hercules, CA).

To examine adhesion of bacteria to LOX-1 under nonstacion conditions, FITC-labeled bacteria (250-μl suspension in DMEM/10% FCS) were incubated with BLOX-1-CHO or BAEC cultured in 12-well plates on a see-saw shaker (Biocraft, Elmond Park, NY) at a frequency of 36 cycles/min at 37°C for 1 h. In some experiments, BAEC had been pretreated with or without TNF-α (10 ng/ml) for 24 h before the bacteria adhesion assay was performed.

Results

S. aureus and E. coli are bound to cells expressing LOX-1

We first examined levels of LOX-1 expression in BLOX-1-CHO and BAEC. As shown in Fig. 1, both BLOX-1-CHO and BAEC expressed significant levels of LOX-1 on the cell surface, which was shown by flow cytometry, although the level of LOX-1 expression was slightly higher and was heterologous in BLOX-1-CHO. In addition, as we have shown previously (19), a proinflammatory cytokine, TNF-α, induced cell surface expression of LOX-1 (Fig. 1, A and D).

To determine whether LOX-1 can bind Gram-positive and Gram-negative bacteria, we examined FITC-labeled S. aureus and E. coli binding to BLOX-1-CHO and control CHO-K1 cells under usual culture conditions. As shown in Fig. 2, A and D, BLOX-1-CHO showed prominent adhesion of FITC-labeled S. aureus and E. coli. In contrast, untransfected CHO-K1 cells did not show any significant binding of FITC-labeled S. aureus or E. coli (Fig. 2, A and C). Confocal microscopy showed that FITC-labeled S. aureus (Fig. 2E) and E. coli (Fig. 2F) were engulfed by BLOX-1-CHO. The numbers of cell that bound FITC-labeled S. aureus were quantified by flow cytometry (Fig. 2, G–J). Bar graphs indicate that ~10% of BLOX-1-CHO bound both FITC-labeled S. aureus and E. coli, although these bacteria adhered to <3% of untransfected CHO-K1 cells (Fig. 2, K and L).

In addition to BLOX-1-CHO, COS-7 cells transiently transfected with human LOX-1 cDNA similarly bound both FITC-labeled S. aureus and E. coli (data not shown). Furthermore, BLOX-1-CHO did not significantly bind zymosan (data not shown) in the case of MARCO (10). To explore the requirement of serum factors and divalent cation, adhesion assay was performed in PBS without magnesium, calcium, or serum. As shown in Fig. 3, the results were almost identical with those observed in complete culture medium with 10% serum (Fig. 2, K and L), indicating that neither divalent cations nor serum factors are necessary for the adhesion of S. aureus and E. coli to LOX-1.

Poly(I) inhibits S. aureus and E. coli binding to LOX-1

Previous studies have shown that poly(I) inhibits bacterial adhesion to MARCO, a member of class A scavenger receptors (12). In addition, poly(I) blocked binding of oxidized low-density lipoprotein to LOX-1 (25). We, therefore, examined whether poly(I) can inhibit binding of S. aureus and E. coli to BLOX-1-CHO. As shown in Fig. 4, A and B, adhesion of FITC-labeled S. aureus and E. coli to BLOX-1-CHO was remarkably suppressed by poly(I), but not by poly(C). An mAb directed to bovine LOX-1 also blocked binding of FITC-labeled S. aureus and E. coli to BLOX-1-CHO (Fig. 4, C and D).
BAEC was inhibited by poly(I), but not by poly(C) (Fig. 6B), as shown in BLOX-1-CHO (B and D) or untransfected CHO-K1 cells (A and C), for 1 h, washed, and subjected to fluorescence microscopy. Confocal microscopy shows that FITC-labeled *S. aureus* (E) and *E. coli* (F) were engulfed by BLOX-1-CHO. The numbers of cells binding FITC-labeled *S. aureus* (G and H) and *E. coli* (I and J) in BLOX-1-CHO (H and J) and untransfected CHO-K1 cells (G and I) were measured by flow cytometry. The numbers of BLOX-1-CHO (percentage of total) that bind FITC-labeled *S. aureus* (K) and *E. coli* (L) are also indicated as bar graphs.

**FIGURE 2.** Adhesion of FITC-labeled *S. aureus* and *E. coli* to LOX-1. FITC-labeled *S. aureus* (A and B) and *E. coli* (C and D) was incubated with BLOX-1-CHO (B and D) or untransfected CHO-K1 cells (A and C), for 1 h, washed, and subjected to fluorescence microscopy. Confocal microscopy shows that FITC-labeled *S. aureus* (E) and *E. coli* (F) were engulfed by BLOX-1-CHO. The numbers of cells binding FITC-labeled *S. aureus* (G and H) and *E. coli* (I and J) in BLOX-1-CHO (H and J) and untransfected CHO-K1 cells (G and I) were measured by flow cytometry. The numbers of BLOX-1-CHO (percentage of total) that bind FITC-labeled *S. aureus* (K) and *E. coli* (L) are also indicated as bar graphs.

Effects of TNF-α on *S. aureus* and *E. coli* adhesion to BAEC

TNF-α can induce cell surface expression of LOX-1 (Fig. 1, C and D). Therefore, we determined whether TNF-α-treated BAEC show enhanced adhesion of *S. aureus* or *E. coli*. As shown in Fig. 6C, total adhesion of *E. coli* as well as LOX-1-dependent *E. coli* adhesion (none minus anti-LOX-1) was significantly enhanced by TNF-α treatment. In contrast, neither total adhesion nor LOX-1-dependent adhesion (none minus anti-LOX-1) of *S. aureus* was significantly enhanced by TNF-α treatment. These results suggest that LOX-1 alone can support the adhesion of *E. coli*, but may not support that of *S. aureus*. Other molecules that can be down-regulated or functionally suppressed by TNF-α may act in cooperation with LOX-1 to support adhesion of *S. aureus* in BAEC, although further studies are needed to clarify this point. These unidentified molecules might be constitutively expressed and functionally active in CHO-K1 cells.

**FIGURE 2.** Continued

**LOX-1 can support adhesion of *S. aureus* and *E. coli* under nonstatic conditions**

To determine whether LOX-1 can support adhesion of bacteria only under static conditions, adhesion assay was performed under...
nonstatic conditions using a seesaw shaker. Both FITC-labeled *S. aureus* (Fig. 7A) and *E. coli* (Fig. 7B) were bound to BLOX-1-CHO, but not control CHO-K1 cells, on a seesaw shaker. Poly(I), but not poly(C), inhibited adhesion of *S. aureus* and *E. coli* to

**FIGURE 3.** Adhesion of FITC-labeled *S. aureus* and *E. coli* to LOX-1 in PBS without calcium or magnesium. FITC-labeled *S. aureus* (A) and *E. coli* (B) were incubated with BLOX-1-CHO or untransfected CHO-K1 cells for 1 h in PBS with 0.25% BSA and washed, and the numbers of BLOX-1-CHO (percentage of total) binding FITC-labeled *S. aureus* (A) or *E. coli* (B) are indicated as bar graphs.

**FIGURE 4.** Poly(I) and an anti-LOX-1 Ab inhibit adhesion of *S. aureus* and *E. coli* to LOX-1. FITC-labeled *S. aureus* (A) and *E. coli* (B) were incubated with BLOX-1-CHO in the presence or the absence of poly(I) or poly(C) (100 µg/ml) as indicated. Relative numbers of bacteria adherent cells were calculated compared with those without poly(I) or poly(C). FITC-labeled *S. aureus* (C) and *E. coli* (D) were incubated with BLOX-1-CHO in the presence or the absence of an anti-LOX-1 Ab or control IgG (30 µg/ml) as indicated. Relative numbers of bacteria adherent cells were calculated compared with those without the Ab.

**FIGURE 5.** Adhesion of *S. aureus* and *E. coli* to BAEC. FITC-labeled *S. aureus* (A) and *E. coli* (B) were incubated with BAEC for 1 h, washed, and subjected to fluorescence microscopy. Confocal microscopy indicates that FITC-labeled *S. aureus* (C) and *E. coli* (D) were engulfed by BAEC. The numbers of BAEC that bind FITC-labeled *S. aureus* (E) and *E. coli* (F) were measured by flow cytometry. Bar graphs indicate the numbers (percentage of total cells) of BAEC that bind FITC-labeled bacteria as indicated (G).
A neutralizing anti-LOX-1 Ab significantly reduced the number of BAEC that bind both FITC-labeled *S. aureus* and *E. coli* under nonstatic conditions (Fig. 7, E and F). These results are in parallel with those found under static conditions, as demonstrated in Fig. 4. Taken together, LOX-1 appears to support adhesion of *S. aureus* and *E. coli* under both static and nonstatic conditions.

Bacterial adhesion to BAEC was also examined under nonstatic conditions. As shown in Fig. 8C, both total and LOX-1-dependent (none minus anti-LOX-1) adhesion of FITC-labeled *E. coli* was significantly increased after TNF-α treatment. In contrast, TNF-α-induced increases in both total and LOX-1-dependent (none minus anti-LOX-1) adhesion of FITC-labeled *S. aureus* were modest (Fig. 8A). Poly(I), but not poly(C), inhibited adhesion of *S. aureus*, but did not significantly inhibit that of *E. coli*, to BAEC under nonstatic conditions (Fig. 8, B and D), which appeared similar to that observed under the static condition (Fig. 6B).

**Discussion**

Adhesion of bacteria to vascular endothelial cells has been suggested as one of the crucial steps in the dissemination of bacteria to other organs via the bloodstream, including infective endocarditis. In macrophages, adhesion and subsequent engulfment of bacteria appear to play important roles in self-defense and immune responses after bacterial infection. The present study for the first time provides evidence that LOX-1, one of the scavenger receptor family members, expressed by vascular endothelial cells and macrophages, can support adhesion of Gram-positive and Gram-negative bacteria, such as *S. aureus* and *E. coli*. In addition to bacteria, bacterial endotoxin, LPS, is a ligand of SR-A, a member of the scavenger receptor family; however, LOX-1 did not significantly bind LPS (data not shown).

As previously shown in SR-A, poly(I) suppressed adhesion of *S. aureus* and *E. coli* to LOX-1, suggesting a similar ligand specificity of LOX-1 to these scavenger receptors, although LOX-1 does not significantly bind acetylated low-density lipoprotein (25), which is a high-affinity ligand for SR-A. In BAEC, in contrast, poly(I) inhibited binding of *S. aureus*, but not *E. coli*, suggesting that molecules other than LOX-1 which can specifically bind *E. coli* but not *S. aureus*, through some interactions with poly(I), may also be involved, although this point remains to be further clarified.
Although bacteria can bind to multiple molecules, including extracellular matrix glycoproteins (26–29), binding of bacteria to endothelial membrane proteins at the primary infection site might prevent bacteria from dissemination to the bloodstream and other organs. This appears to be an alternative hypothesis concerning the roles of LOX-1 in bacterial infection; however, studies with suitable animal experimental models may be required to elucidate the exact roles of LOX-1.

As well as MARCO and SR-A, LOX-1 is also expressed by tissue macrophages (20, 30, 31). Interestingly, proinflammatory stimuli dramatically induced the expression of LOX-1 (20, 30), although these stimuli down-regulate SR-A expression (32, 33) in macrophages. Adhesion and subsequent phagocytosis of bacteria in macrophages appear to play important roles in Ag presentation and immune responses after bacterial infection. In fact, in CHO-K1 cells stably expressing LOX-1 and BAEC, bacteria can be engulfed after the adhesion to the cell surface LOX-1. Therefore, bacteria may also be engulfed or phagocytosed in macrophages after the adhesion to LOX-1 as well as class A scavenger receptors on their cell surface.

In summary, the present report for the first time provides evidence that LOX-1, alone or in cooperation with other molecules, can support adhesion of Gram-positive and Gram-negative bacteria. Further studies would elucidate the pathophysiological roles of LOX-1 in bacterial infection in vivo.

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