Lectin-like oxidized LDL receptor-1 (LOX-1) supports adhesion of mononuclear leukocytes and a monocyte-like cell line THP-1 cells under static and flow conditions

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Abstract Adhesion of mononuclear leukocytes to vascular endothelial cells appears one of the initial steps in the process of atherogenesis and inflammation. We examined if LOX-1, an endothelial scavenger receptor with C-type lectin-like structure, can support adhesion of mononuclear leukocytes. Under a static condition, CHO-K1 cells stably expressing LOX-1 showed more prominent adhesion of human peripheral blood mononuclear leukocytes and THP-1 cells than untransfected CHO-K1 cells, in a temperature-independent fashion. Mononuclear leukocytes also adhered to plastic plates precoated with recombinant soluble LOX-1 extracellular domain. A neutralizing anti-LOX-1 monoclonal antibody, as well as oxidized low-density lipoprotein, significantly blocked adhesion of THP-1 cells to CHO-K1 cells overexpressing LOX-1 and bovine aortic endothelial cells. Under a flow condition, increased numbers of THP-1 cells showed rolling with reduced velocities on LOX-1-expressing CHO-K1 cells, compared with those on untransfected CHO-K1 cells. Taken together, LOX-1 can work as a cell surface receptor for mononuclear leukocytes under both static and flow conditions.

Key words: Endothelial cell; Mononuclear leukocyte; Adhesion molecule; Oxidized low-density lipoprotein; Scavenger receptor; Rolling

1. Introduction

Adhesion of mononuclear leukocytes to vascular endothelial cells has been suggested as one of the initial steps in atherogenesis as well as inflammatory responses [1]. Previous reports have suggested that multiple molecules appear to be involved at the different stages of endothelial–leukocyte adhesion, including rolling and firm adhesion [2]. These molecules include selectins, such as E-selectin, P-selectin, and L-selectin, and immunoglobulin gene superfamily molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [2]. Studies with mice deficient in genes encoding these adhesion molecules, as well as functional blocking antibodies, have revealed the crucial roles of these adhesion molecules in leukocyte recruitment in the settings of atherogenesis as well as inflammation [3–5]; however, involvement of other adhesion mechanisms also has been suggested [5].

Oxidized low-density lipoprotein receptor-1 (LOX-1) is a 50 kDa type II membrane glycoprotein with C-type lectin-like structure in the extracellular domain, which acts as an endocytosis receptor for atherogenic oxidized low-density lipoprotein (LDL) [6]. LOX-1 is synthesized as a 40 kDa precursor protein with minimal *N*-linked high mannose carbohydrate chains, and subsequently further glycosylated and processed into a 48–50 kDa mature form [7]. Interestingly, LOX-1 can be induced by proinflammatory stimuli, such as tumor necrosis factor-α [8], transforming growth factor-β [9], oxidized LDL [10] and fluid shear stress [11], suggesting its roles in the settings of inflammatory diseases and vascular injury. In vivo, LOX-1 is highly expressed in endothelial cells covering early atherosclerotic lesions of humans [12] and hypercholesterolemic rabbits [13].

Like other scavenger receptor family molecules [14,15], LOX-1 has a broad spectrum of physiological ligands, including oxidized or aged erythrocytes [16], apoptotic cells [16], activated platelets [17], bacteria [18], and fibronectin [19]. Therefore, in the present study, we sought to determine if LOX-1 can support adhesion of mononuclear leukocytes.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium and Ham's F12 medium were obtained from Nissui (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Sanko Junyaku (Tokyo, Japan). BCECF-AM was from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

Wild-type CHO-K1 cells (wild-type CHO) were maintained in F12 medium containing 10% (v/v) heat-inactivated FCS. CHO-K1 cells stably expressing human LOX-1 (hLOX-1-CHO) were established by transfection with a pME vector containing the full-length human LOX-1 and screening the ability to accumulate DiI-labeled oxidized LDL [6,10]. Cloned cells were maintained in F12/10% fetal bovine serum (FBS) supplemented with G418 (0.8 mg/ml). CHO-K1 cells stably expressing bovine LOX-1 (bLOX-1-CHO) were maintained in F12/10% FCS supplemented with 10 μg/ml of blasticidin S (Funakoshi, Tokyo, Japan) as previously described [6,20]. Cultured bovine aortic endothelial cells (BAEC) were isolated and cultured as previously indicated [6,9,11,18,19]. Human peripheral blood mononuclear leukocytes were isolated from healthy donors by Ficoll density gradient centrifugation [21,22]. THP-1, U-937, and Jurkat cells were obtained from American Type Culture Collection (Rockville, MD, USA), and cultured in RPMI 1640/10% FCS.

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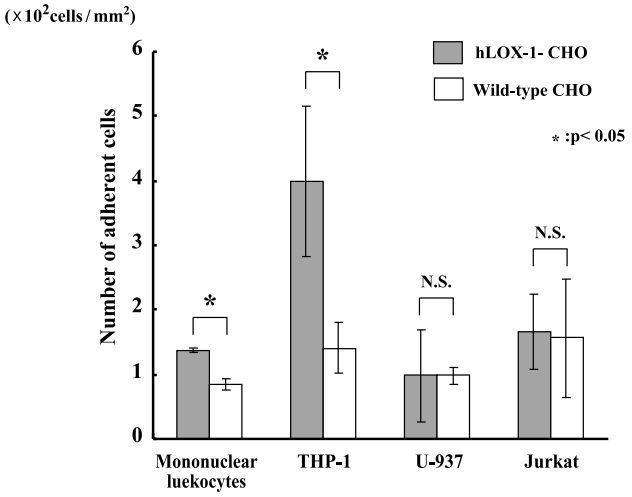

Fig. 1. Adhesion of human peripheral mononuclear leukocytes and the related cell lines to CHO-K1 cells stably expressing human LOX-1 (hLOX-1-CHO). hLOX-1-CHO were incubated with the indicated leukocytes labeled with BCECF-AM to allow them to adhere for 30 min at 37°C. Non-adherent cells were detached by centrifugation at 270×*g* for 5 min. Adherent cells were lysed and the fluorescence was measured (filled bars). Untransfected wild-type CHO-K1 cells (wild-type CHO) served as negative controls (blank bars). The graph indicates the numbers of adherent leukocytes. Mean values from 10 wells and S.D. are indicated by columns and bars respectively. A representative figure from four independent experiments is shown.

2.3. Leukocyte adhesion assay under a static condition

Static leukocyte adhesion assays were performed as previously described with minor modifications [21]. In brief, after hLOX-1-CHO, bLOX-1-CHO, wild-type CHO, or BAEC.6 grown in 96 well plates were incubated with BCECF-labeled human peripheral blood mononuclear leukocytes, THP-1 cells, U937 cells or Jurkat cells (1×10^6 cells/ml) suspended in RPMI 1640/10% FBS at 4, 25 or 37°C for the indicated time periods, wells were filled with the assay medium, sealed, inverted, and centrifuged at low speed (270×*g* for 25°C and 37°C assays, and 50×*g* for the 4°C assay) for 5 min. After non-adherent leukocytes were removed, cells were dissolved with a lysis buffer consisting of 50 mM Tris pH 8.4 and 0.1% SDS, and the fluorescence was measured in Titertek Fluoroscan II (Flow Laboratories) [21]. In some experiments, cells were pretreated with a neutralizing anti-LOX-1 monoclonal antibody (20 μg/ml) [23], control IgG (20 μg/ml), oxidized LDL (20 or 40 μg/ml), or native LDL (20 or 40 μg/ml) for 10 min before addition of BCECF-labeled leukocytes.

2.4. Leukocyte adhesion assay to plates precoated with recombinant human LOX-1 extracellular domain (rhLOX-1 ECD)

rhLOX-1 ECD was produced in *Escherichia coli* by transfecting a cDNA fragment corresponding to the extracellular domain (amino acids 84–273) of human LOX-1 which had been subcloned into pQE vector (Qiagen). After precoating with the indicated concentrations of rhLOX-1 ECD for 30 min at 37°C, cell culture plates were blocked by incubation with 1% (w/v) heat-denatured (57°C, 25 min) bovine serum albumin (BSA) for 1 h at room temperature followed by washing with phosphate-buffered saline. BCECF-labeled human peripheral blood mononuclear leukocytes were allowed to adhere to rhLOX-1 ECD-coated plates by incubation at 37°C for 30 min. After non-adherent cells were removed by low speed centrifugation (270×*g*, 5 min), adherent cells were dissolved with a lysis buffer consisting of 50 mM Tris pH 8.4 and 0.1% SDS, and the fluorescence was measured in Titertek Fluoroscan II.

2.5. Leukocyte rolling in a flow chamber

Confluent wild-type CHO and hLOX-1-CHO were cultured in a parallel plate flow chamber (5 mm in width, 0.254 mm in depth). The flow chamber was perfused with BCECF-labeled THP-1 cells (3×10^5 cells/ml) suspended in RPMI 1640/10% FCS at a flow rate corresponding to the indicated shear stress at 22°C. The leukocyte movement was recorded by a fluorescence microscope (Zeiss) equipped with a fluorescence digital videocamera (Hamamatsu). The rolling velocity of THP-1 cells was determined by capturing two different time frames with a 33 ms delay and measuring lengths of tracks

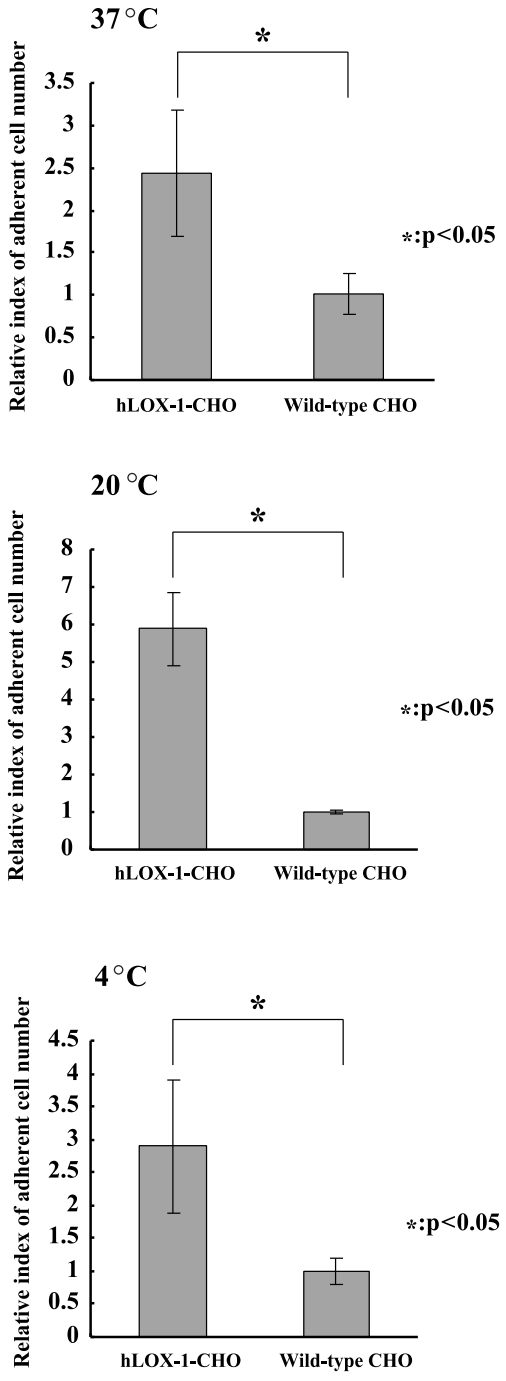

Fig. 2. Adhesion of THP-1 cells to hLOX-1-CHO at different temperatures. After hLOX-1-CHO were incubated with BCECF-labeled THP-1 cells at the indicated temperature (4, 20, or 37°C) for 30 min, non-adherent cells were removed by low speed centrifugation and the cell-associated fluorescence was measured as described in Section 2. Untransfected wild-type CHO-K1 cells served as negative controls. THP-1 cell numbers adhered to wild-type CHO were 171 ± 41, 25.5 ± 1.5, and 12.6 ± 2.5 cells/mm² at 37, 20, and 4°C, respectively. Relative adherent cell numbers are indicated. Columns and bars indicate mean values and S.D. calculated from 10 wells for each experiment. Representative figures from four independent experiments are shown.

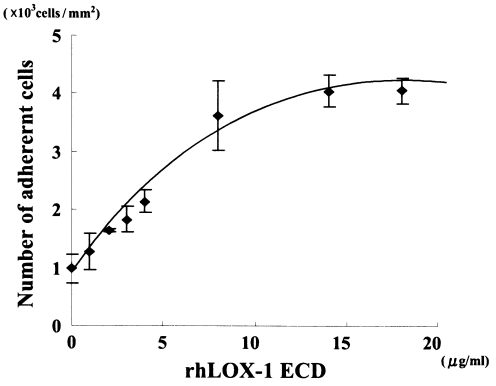

Fig. 3. Adhesion of THP-1 cells and human peripheral blood mononuclear leukocytes to plates precoated with rhLOX-1 ECD. After precoating with the indicated concentrations of rhLOX-1 ECD, followed by blocking with heat-denatured BSA, adhesion of BCECF-labeled human peripheral blood mononuclear leukocytes was measured as described in Section 2. Relative adherent cell numbers (mean values and the S.D.) calculated from 10 wells for each rhLOX-1 ECD concentration are indicated. Dots indicate means and bars indicate S.D. A representative figure from four independent experiments is indicated.

of THP-1 cells on the merged images created with IMAGE 1.62 (National Institutes of Health, Bethesda, MD, USA). Slowly moving THP-1 cells at velocities less than one tenth of the centerline flow velocity were defined as rolling cells.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviations (S.D.). Statistical significance of the differences between two groups was analyzed by two-tailed unpaired Student's *t*-test. Statistical comparison between more than two groups was performed by one-factorial ANOVA. Post-hoc test was Fischer's PLSD test. The null hypothesis was considered to be rejected at $P < 0.05$.

3. Results

3.1. Adhesion of peripheral blood mononuclear leukocytes and THP-1 cells to CHO-K1 cells stably expressing LOX-1 under a static condition

To examine if LOX-1 can support adhesion of mononuclear leukocytes, a static adhesion assay was performed in both hLOX-1-CHO and untransfected CHO-K1 cells. As shown in Fig. 1, hLOX-1-CHO showed significant enhancement of mononuclear leukocyte and THP-1 cell adhesion, but not U-937 or Jurkat cell adhesion, when compared to untransfected wild-type CHO-K1 cells. A 1.5-fold increase was observed in mononuclear leukocytes, whereas a 3.5-fold increase was seen in THP-1 cells. Adhesion of THP-1 cells to hLOX-1-CHO was time-dependently increased for the initial 30 min, and reached the maximal level after 30 min (data not shown). To explore temperature dependence in leukocyte–LOX-1 interactions, a static adhesion assay was carried out in THP-1 cells at 4, 22, and 37°C. Enhancement of THP-1 cell adhesion to hLOX-1-CHO, when compared to that to wild-type CHO, was similarly observed at 4, 22, and 37°C (Fig. 2), suggesting that temperature-independent interactions between THP-1 cells and LOX-1 may be involved. These results are quite different from the integrin-mediated adhesion of leukocytes to VCAM-1 or ICAM-1, in which temperature-dependent activation of integrins is necessary.

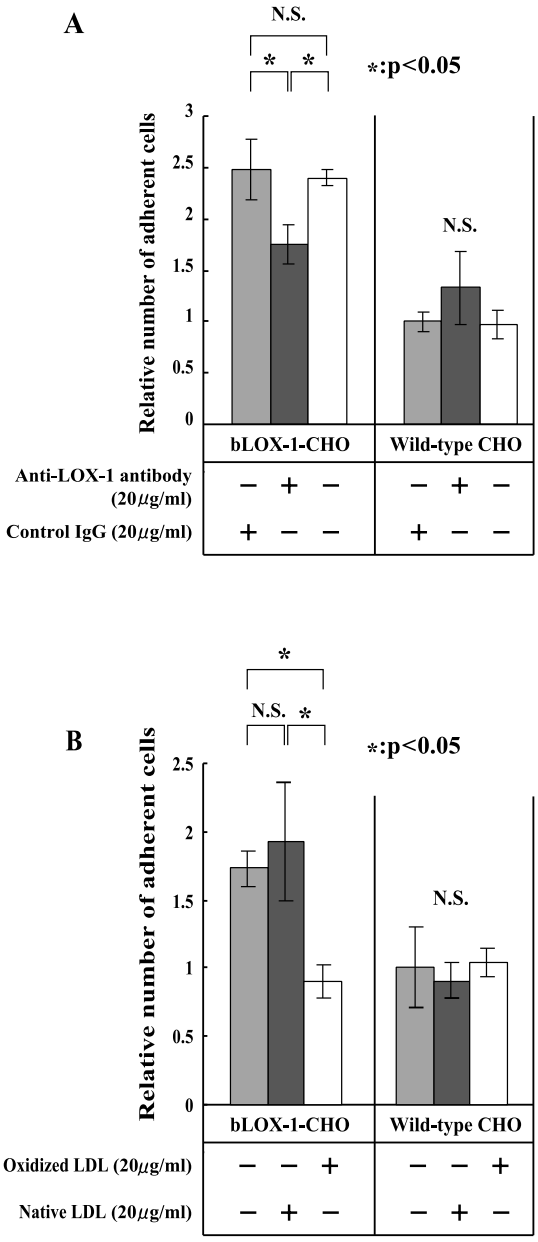

Fig. 4. A: Effects of a neutralizing anti-LOX-1 antibody on THP-1 cell adhesion to LOX-1. After bLOX-1-CHO cells cultured in 96 well plates were incubated with BCECF-labeled THP-1 cells at 37°C for 30 min in the presence of a neutralizing anti-bovine LOX-1 monoclonal antibody or subclass-matched control IgG, non-adherent cells were removed by low speed centrifugation, and the fluorescence was measured as described in Section 2. THP-1 cell numbers adherent to bLOX-1-CHO and wild-type CHO were 267.1 ± 32.1 and 107.9 ± 10.3 cells/mm^2, respectively, in the absence of antibodies. Relative adherent cell numbers are indicated in the bar graph. Mean values (columns) and the S.D. (bars) calculated from five wells for each adhesion condition are indicated. B: Effects of oxidized LDL, a ligand for LOX-1, on THP-1 cell adhesion to LOX-1. After hLOX-1-CHO cells cultured in 96 well plates were incubated with BCECF-labeled THP-1 cells at 4°C for 30 min in the presence of oxidized LDL (20 μg/ml) or native LDL (20 μg/ml), non-adherent cells were removed by low speed centrifugation and the fluorescence was measured as described in Section 2. Wild-type CHO-K1 cells served as negative controls. THP-1 cell numbers adherent to bLOX-1-CHO and wild-type CHO were 46.3 ± 3.7 and 26.8 ± 7.9 cells/mm^2, respectively, in the absence of lipoproteins. Relative adherent cell numbers are indicated. Mean values (columns) and the S.D. (bars) calculated from five wells for each adhesion condition are indicated. Representative figures from four similar experiments are shown.

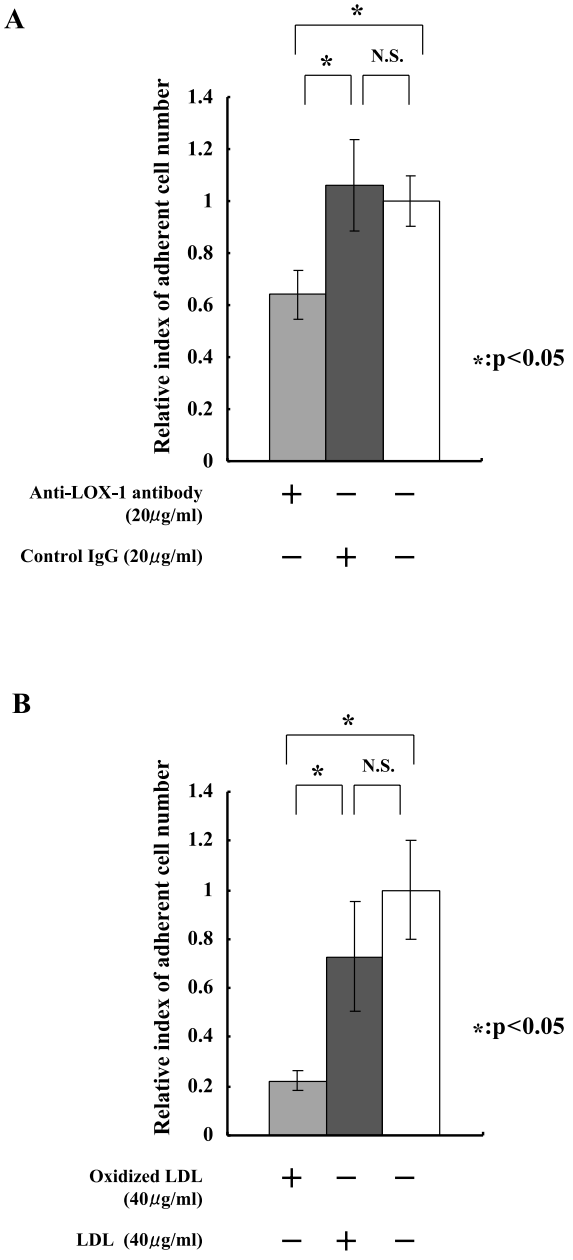

Fig. 5. A: Effects of a neutralizing anti-LOX-1 monoclonal antibody on THP-1 cell adhesion to BAEC. After confluent monolayers of BAEC in 96 well plates were incubated with BCECF-labeled THP-1 cells in the presence or absence of a neutralizing anti-LOX-1 monoclonal antibody or control IgG at 37°C, non-adherent cells were removed by low speed centrifugation and the fluorescence was measured as indicated in Section 2. Adherent THP-1 cell numbers were 134.2 ± 13.1 cells/mm^2 in the absence of antibodies. Relative adherent cell numbers are indicated. Mean values (columns) and the S.D. (bars) calculated from five wells for each adhesion condition are indicated. B: Effects of oxidized LDL on THP-1 cell adhesion to BAEC. Confluent monolayers of BAEC in 96 well plates were incubated with BCECF-labeled THP-1 cells in the presence or absence of 20 μg/ml of oxidized LDL or native LDL at 4°C. Non-adherent cells were removed by low speed centrifugation and the fluorescence was measured as indicated in Section 2. Adherent THP-1 cell numbers were 59.1 ± 11.7 cells/mm^2 in the absence of lipoproteins. Relative adherent cell numbers are indicated. Mean values (columns) and the S.D. (bars) calculated from five wells for each adhesion condition are indicated. Representative figures from four similar experiments are indicated.

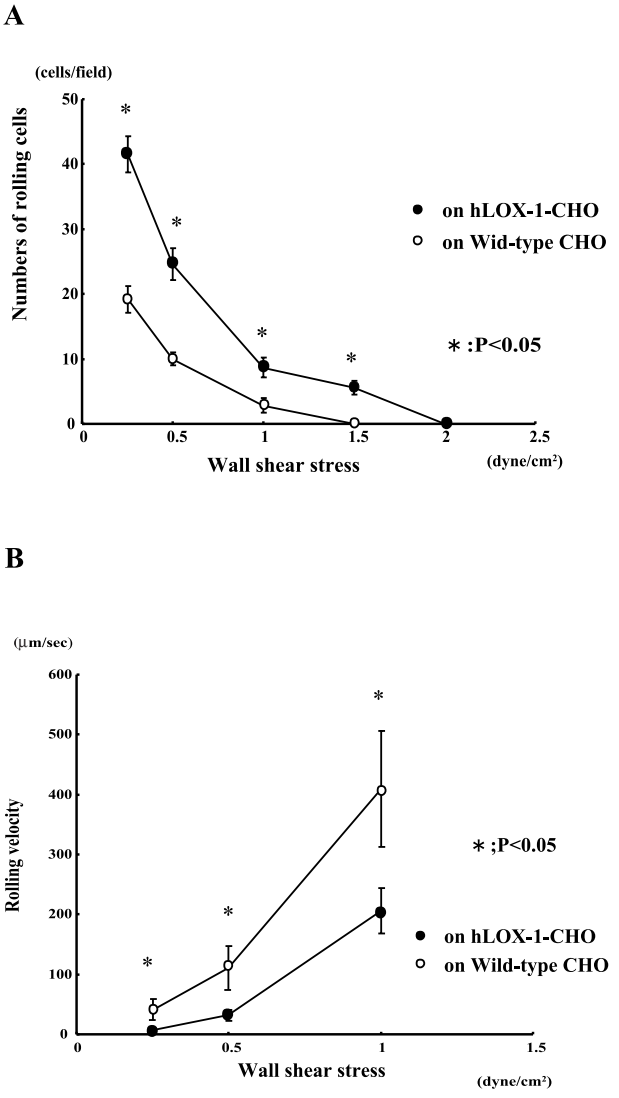

Fig. 6. A: Numbers of rolling THP-1 cells on hLOX-1-CHO cells and untransfected wild-type CHO-K1 cells. BCECF-labeled THP-1 cells were perfused over hLOX-1-CHO cells or wild-type CHO-K1 cells cultured in a flow chamber at the indicated shear stress. Numbers of rolling THP-1 cells were counted as described in Section 2. Numbers of rolling cells were counted in 10 different merged images with time differences of more than 5 s. Mean values (columns) and the S.D. (bars) calculated from 10 independent merged images are indicated. B: Rolling velocities of THP-1 cells on hLOX-1-CHO cells and untransfected wild-type CHO-K1 cells. BCECF-labeled THP-1 cells were perfused over hLOX-1-CHO cells or wild-type CHO-K1 cells cultured in a flow chamber at the indicated shear stress. Velocities of rolling THP-1 cells were measured as described in Section 2. Columns indicate means and bars indicate S.D. of rolling velocities calculated from 50 independent THP-1 cells. Representative figures from four independent experiments are shown.

3.2. Adhesion of THP-1 cells and human peripheral blood mononuclear leukocytes to rhLOX-1 ECD

To obtain direct evidence that mononuclear leukocytes and THP-1 cells adhere to LOX-1, adhesion assays were also performed in plates precoated with rhLOX-1 ECD without cells. As shown in Fig. 3, THP-1 cells and human peripheral blood mononuclear leukocytes adhered to plates precoated with rhLOX-1 ECD in a concentration-dependent manner. Maxi-

mal adhesion was observed at 10 μg/ml of rhLOX-1 ECD and the adhesion was saturated at 20 μg/ml.

3.3. Effects of a neutralizing anti-LOX-1 monoclonal antibody and oxidized LDL on THP-1 cell adhesion to LOX-1

A neutralizing anti-LOX-1 monoclonal antibody, which inhibits binding of oxidized LDL to LOX-1, partly (by 72% of the LOX-1-dependent adhesion) but significantly inhibited adhesion of THP-1 cells to CHO-K1 cells stably expressing bovine LOX-1 (bLOX-1-CHO). A subclass-matched control IgG did not significantly affect THP-1 cell adhesion to bLOX-1-CHO. In wild-type CHO-K1 cells, in contrast, the neutralizing anti-LOX-1 antibody did not inhibit adhesion of THP-1 cells (Fig. 4A). THP-1 cell adhesion to bLOX-1-CHO was also significantly (by almost 100% of the LOX-1-dependent adhesion) inhibited by oxidized LDL, but not native LDL (Fig. 4B), suggesting that binding sites on LOX-1 for THP-1 cells and oxidized LDL overlap.

3.4. Effects of a neutralizing anti-LOX-1 monoclonal antibody and oxidized LDL on THP-1 cell adhesion to BAEC

To explore if LOX-1 on the cell surface of BAEC can support adhesion of leukocytes, an adhesion assay was similarly carried out in BAEC by use of the neutralizing anti-LOX-1 monoclonal antibody. As shown in Fig. 5A, THP-1 cell adhesion to BAEC was significantly (by 40%) inhibited by the anti-LOX-1 monoclonal antibody but not control IgG. In addition, oxidized LDL, but not native LDL, suppressed THP-1 adhesion to BAEC by 78% (Fig. 5B). These results thus indicate that LOX-1 plays a significant role in THP-1 cell adhesion to BAEC.

3.5. LOX-1 supports rolling of THP-1 cells under a flow condition

To explore if LOX-1 can support rolling under a flow condition, we examined rolling velocity of THP-1 cells on both bLOX-1-CHO and wild-type CHO by use of a flow chamber (Fig. 6A). As shown in Fig. 6B, the velocity was reduced by 43% in THP-1 cells rolling on hLOX-1-CHO when compared with those rolling on wild-type CHO. Velocities of non-interacting THP-1 cells were not significantly different between bLOX-1-CHO and wild-type CHO (data not shown).

4. Discussion

LOX-1 has a wide variety of pathophysiological ligands, such as aged/apoptotic cells [16], activated platelets [17], bacteria [18], and fibronectin [19], in addition to oxidized LDL [6,20]. The present study demonstrates, for the first time, that LOX-1 can support adhesion of mononuclear leukocytes and related cell lines under both static and flow conditions.

Endothelial–leukocyte adhesion can be supported by multiple molecular interactions, including selectins–carbohydrates and immunoglobulin gene superfamily molecules–integrins [2]. In addition to these molecular interactions, LOX-1 may also be involved in adhesion of mononuclear leukocytes. As shown in Fig. 2B, LOX-1 can support cell adhesion at 4°C, suggesting that integrins may not be the counter receptor of LOX-1. In addition, LOX-1 can support rolling of THP-1 cells under low shear flow conditions (less than 1 dyne/cm^2). Previous studies with selectins have shown that selectins support rolling most effectively at the threshold levels of shear rates, and lower shear rates do not support rolling interactions sufficiently [24]. In contrast to selectins, LOX-1 appears to support rolling of THP-1 cells more effectively under slower flow conditions. LOX-1 may be able to support rolling and adhesion of leukocytes at the later stages of leukocyte–endothelial interactions, after the selectin-mediated rolling. Interestingly, LOX-1 expression is drastically induced by proinflammatory stimuli [8,9], as well as oxidized LDL [10], which are related to vascular dysfunction including atherogenesis [1]. In addition, endothelial expression of LOX-1 is upregulated in cells covering early phases of atherosclerotic lesions [12,13]. Recruitment of mononuclear leukocytes is the prominent feature of both atherogenesis and inflammation. LOX-1 may, thus, play a role in leukocyte recruitment in these pathological settings, in cooperation with selectins and immunoglobulin gene superfamily molecules [2].

Because oxidized LDL can inhibit LOX-1–THP-1 interactions, binding sites for oxidized LDL and THP-1 cells on the LOX-1 molecule appear overlapped. Counter receptors for LOX-1 on THP-1 cells and mononuclear leukocytes remain to be identified; however, they should be abundantly expressed on the cell surface of THP-1 cells, as well as mononuclear leukocytes, but not U-937 or Jurkat cells. Previous studies have indicated that LOX-1 may transmit biological signals after ligation with its ligands, such as oxidized LDL [25] and activated platelets [17]. It remains to be determined whether binding of leukocytes to LOX-1 can transmit biological signals to vascular endothelial cells.

In summary, the present report provides evidence, for the first time, that LOX-1 can support adhesion of mononuclear leukocytes and THP-1 cells, the related cell line, under both static and flow conditions. Further studies related to functional blockade and overexpression of this unique scavenger receptor may provide novel insights into the roles of LOX-1 in the pathogenesis of vascular dysfunction.

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