CD11b/CD18 Mediates Production of Reactive Oxygen Species by Mouse and Human Macrophages Adherent to Matrixes Containing Oxidized LDL

Jens Husemann, Amrom Obstfeld, Maria Febbraio, Tatsuhiko Kodama, Samuel C. Silverstein

Abstract—Production of reactive oxygen species (ROS) and other proinflammatory substances by macrophages adherent to matrix proteins that contain or have been modified by oxidized LDL (oxLDL) may play an important role in atherogenesis. In vitro, human macrophages adhere to matrixes containing oxLDL via scavenger receptors and are signaled to produce ROS partly by interactions of the class B scavenger receptor (SR-B) CD36 with ligands on the matrix. In this report, we show that macrophages from mice genetically deficient in SR-A or CD36 adhered equally well and produced equal amounts of ROS on interaction with matrix-associated oxLDL. In contrast, macrophages from mice genetically deficient in the CD18 chain of $\beta_2$-integrins produced insignificant amounts of ROS on interaction with oxLDL-containing matrixes, even though they adhered to these matrixes as efficiently as did macrophages from wild-type mice. Antibodies against CD18, CD11b, or EDTA, the last of which chelates divalent cations required for integrin function, had no effect on adhesion of normal mouse or human macrophages to matrixes containing oxLDL but almost completely inhibited ROS production by macrophages adherent to this matrix. Thus, CD11b/CD18 plays an important role in regulating production of ROS by mouse and human macrophages adherent to matrixes containing oxLDL. It may play a hitherto-unsuspected role in regulating macrophage signaling pathways involved in inflammation and atherogenesis. (Arterioscler Thromb Vasc Biol. 2001;21:1301-1305.)

Key Words: reactive oxygen species n macrophages n oxidized LDL n CD11b/CD18 n scavenger receptors

Accumulation of oxidatively modified LDL (oxLDL) in and subsequent recruitment of monocytes to the subendothelial space of arteries are early events in atherogenesis (reviewed in Lusis1). Monocytes trapped at this site via interactions of their scavenger receptors (eg, SR-A, CD36) with oxLDL and with matrix proteins modified by oxidized lipids mature into macrophages. Endocytosis of modified lipids and lipoproteins by these macrophages transforms them into foam cells. Foam cells form fatty streaks, which contribute to the development of atherosclerotic lesions.

Like macrophages, endothelial cells (ECs) and smooth muscle cells (SMCs) express scavenger-type receptors that interact with ligands expressed on oxLDL and on matrix proteins modified by oxidized lipids.1 These interactions stimulate macrophages, vascular SMCs, and vascular ECs to produce reactive oxygen species (ROS).1 eg, superoxide, which spontaneously reacts with water to generate other ROS, eg, H$_2$O$_2$. These ROS cause further oxidative modification of lipids and lipoproteins. Macrophages are thought to play the most prominent role in these processes owing to their high levels of ROS-generating enzymes, eg, NADPH oxidase and 5/12-lipoxygenase.1 We have reported that although SR-A plays a major role in promoting adhesion of human macrophages to oxLDL-containing matrixes, it appears to play little or no role in signaling ROS production.2 In contrast, antibodies that block interactions of the class B scavenger receptor CD36 with oxLDL have no effect on macrophage adhesion to oxLDL-containing matrixes but do inhibit ROS production by human macrophages adherent to these matrixes by $\approx$60%.2 To further explore these findings, we compared adhesion to and ROS production by resident peritoneal macrophages from mice genetically deficient in SR-A (SR-A$^{-/-}$), CD36 (CD36$^{-/-}$), or CD18 (CD18$^{-/-}$) plated on oxLDL-containing matrixes with that of macro-
phages from background-appropriate wild-type mice plated on this matrix.

**Methods**

**Reagents**

All reagents were from Sigma Chemical Co unless indicated otherwise.

**Mice**

All mice used in these experiments were 6 to 8 weeks of age. SR-A−/−, CD36−/−, and CD18−/− mice are described elsewhere.

**Cells**

Mouse mononuclear cells were obtained by lavage of the peritoneal cavity and used in experiments immediately thereafter. Twenty-nine percent (±3%) of the cells harvested from the peritoneum of knockout mice were wild-type macrophages, as indicated by their expression of nonspecific esterase and endocytosis of 1,1′dioctadecyl-3,3,3′,3′-tetramethyldiocarbocyanine perchlorate–labeled acetylated LDL (not shown). Human monocytes were isolated from fresh buffy coats (New York Blood Center, New York, NY) and cultured for 3 to 5 days as described before being used in experiments.2

**Adhesion**

To compare adhesion of wild-type, SR-A−/−, CD36−/−, and CD18−/− mouse peritoneal mononuclear cells, we used surfaces coated with collagen IV alone (CIV), collagen IV and native LDL (CIV/LDL), or collagen IV and oxLDL (CIV/oxLDL). Because macrophages do not adhere efficiently to collagen IV, surfaces coated with this protein facilitate detection of adhesion-promoting ligands, such as oxLDL, absorbed to it.

To measure adhesion, 6-mm-diameter spots of multipotential slides were incubated with 30 μL per spot of 50 μg/mL collagen IV (Fluka) in double-distilled water for 1 hour at 37°C, washed once with double-distilled water, air-dried, coated with 10 μg/mL collagen IV (Intracell) or oxLDL as described,2 air-dried again, and overlaid with 50 μL Krebs-Ringer buffer containing 1 mmol/L glucose (Sigma) and 0.1% bovine serum albumin (Sigma) (KR HBOA solution) containing 2.5×104 mouse peritoneal mononuclear cells or human macrophages for 45 minutes at 37°C in a 5% CO2/95% air atmosphere. Slides were washed with KR HBOA, and the number of adherent cells was determined by using CyQuant GR cell proliferation assay (Molecular Probes) according to the manufacturer’s instructions.

**ROS Production**

Accumulation of H2O2 as a representative ROS in the supernatant was measured with the Amplex Red™ H2O2 assay (Molecular Probes) according to the manufacturer’s instructions. The wells of 96-well, flat-bottomed microtiter plates were coated with CIV, CIV/LDL, or CIV/oxLDL as described above and overlaid with 150 μL KR HBOA containing 20 μmol/L Amplex Red™ (Molecular Probes), 0.2 U/mL horseradish peroxidase (Molecular Probes), and 3×104 mouse peritoneal mononuclear cells or human macrophages for 2 hours at 37°C in a 5% CO2/95% air atmosphere. Fluorescence of the product formed by the reaction between Amplex Red™ and H2O2 was assayed with a fluorescent plate reader.

**Antibodies**

Mouse peritoneal mononuclear cells or human macrophages were preincubated with KR HBOA containing monochelical anti-mouse CD18 IgG1 (Pharmingen) or control rat IgG1 (Pharmingen) at 0.4, 2, or 10 μg/mL; monoclonal mouse anti-human CD18 IgG1, (Ancell Corp) or control mouse IgG1 (AnCell) at 0.4, 2, or 10 μg/mL; or monoclonal anti-mouse/human CD11b IgG2a (Serotec) or control rat IgG2a (Pharmingen) at 0.04, 0.2, or 1 μg/mL for 15 minutes at 37°C in a 5% CO2/95% air atmosphere as indicated.
mediating receptors, e.g., SR-BI, may mediate macrophage binding to ligands provided by oxLDL.

To assess whether adhesion to CIV, CIV/LDL, or CIV/oxLDL stimulates ROS production by SR-A−/−, CD36−/−, or wild-type murine macrophages, we incubated these cells on matrices containing these proteins and assayed for accumulation of H2O2 in the supernatant as a representative ROS. Consistent with the findings of Maxeiner et al., wild-type mouse peritoneal macrophages produced very little ROS on surfaces coated with CIV or CIV/LDL (~33 pmol H2O2 per 3 × 10^5 cells/2 hours; Figure 2) but produced significantly more ROS on surfaces coated with CIV/oxLDL (~185 pmol H2O2 per 3 × 10^5 cells/2 hours; Figure 2). However, in contrast to the results predicted by Maxeiner et al., macrophages from SR-A−/− and CD36−/− mice produced at least as much ROS as did wild-type macrophages (~247 ± 36 and ~188 ± 47 pmol H2O2 per 3 × 10^5 cells/2 hours, respectively; Figure 2). These findings show that receptors other than SR-A and CD36 can mediate adhesion of resident macrophages to oxLDL-containing matrices and ROS production by these cells when they adhere to such matrices.

Interestingly, neutrophils also have been shown to produce ROS on interaction with oxLDL (see Maeba et al. and J.H., unpublished observations, 2000). Neutrophils are not found in atherosclerotic lesions and do not express detectable levels of SR-A or CD36 (Naito et al. and J.H., unpublished observations, 2000) but do express high levels of the β2-integrin CD11b/CD18 (also known as Mac-1, complement receptor 3, CR3). Neutrophils and macrophages can be induced to produce ROS when they adhere to surfaces containing ligands for CD11b/CD18 (e.g., fibrinogen, iC3bβ). This suggested a role for CD11b/CD18 in ROS production by macrophages. As anticipated, resident peritoneal macrophages from mice genetically deficient in the β2-chain of CD11b/CD18 (CD18−/− mice) adhered as efficiently to matrices containing CIV/oxLDL as did wild-type murine macrophages (Figure 3b). However, macrophages from CD18−/− mice produced ~20% as much ROS as did wild-type macrophages on adhesion to this matrix (Figure 3a). The amount of ROS produced by CD18−/− macrophages adherent to CIV/oxLDL (~39 pmol H2O2 per 3 × 10^5 cells/2 hours; Figure 3a) was not significantly different from the amount secreted by wild-type macrophages adherent to surfaces containing CIV or CIV/LDL (~33 pmol H2O2 per 3 × 10^5 cells/2 hours; Figure 1). These results indicate that the β2-integrin CD11b/CD18, along with chemoattractants such as monocyte—derived macrophage adhesion to or ROS production on CIV/oxLDL (not shown).

β2-Integrins require divalent cations to bind ligands. To determine whether the divalent cation—dependent, ligand-binding domain of CD11b/CD18 is involved in this system, we tested the effect of 5 mmol/L EDTA on adhesion and ROS production by mouse and human macrophages plated on CIV/oxLDL. EDTA had no effect on macrophage adhesion but caused an ~80% decrease in ROS production by wild-type mouse and human macrophages plated on CIV/oxLDL (Figure 3c). To confirm that EDTA did not block ROS production per se, we tested ROS production by resident mouse peritoneal macrophages and by human blood monocyte—derived macrophages stimulated with 100 ng/mL phorbol myristate acetate or 10 mg/mL zymosan. The presence of EDTA had no effect on ROS production induced by these stimuli (data not shown).

Thus, by genetically eliminating expression of CD18, use of antibodies that masked the ligand-binding domain(s) of CD18, and chelation of divalent cations required for CD11/CD18 functions, we have disrupted a required step in ROS production by macrophages adherent to oxLDL-containing matrices. Because antibodies against CD11b/CD18 are as effective in inhibiting ROS production as is the absence of all four β2-integrins in CD18−/− macrophages, it appears that CD11b/CD18 is the β2-integrin involved in this signaling pathway.

**Discussion**

To enter the subendothelial space, blood monocytes must cross the vascular endothelium. Leukocyte and endothelial cell adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1 (VCAM-1), very late—acting antigen-4 (VLA-4), P-selectin, L-selectin, and intercellular adhesion molecule-1 are expressed at high densities on the surface of monocyte...
chemotactic protein-1 (MCP-1), are involved in this process to differing degrees, depending on the circumstances. Genetic disruption of intercellular adhesion molecule-1, P-selectin, or MCP-1 of endothelial cells or of CD18 or CCR2, the receptor for MCP-1 on macrophages, does not affect plasma lipids but results in decreased macrophage numbers in fatty streaks and reduced lesion size in murine models of atherosclerosis. These results indicate that monocyte recruitment plays an important role in the initiation of atherosclerosis. There are insufficient data to determine whether such a decrease in monocyte recruitment and lesion size would translate into reduced long-term morbidity and mortality in atherosclerosis in humans.

Monocytes that enter the sub endothelial space mature into macrophages that interact with and ingest oxLDL via scavenger receptors (eg, SR-A, SR-BI, CD36), thereby becoming foam cells. Interactions with matrix-bound oxLDL may also engender receptors (eg, SR-A, SR-BI, CD36), thereby becoming foam cells that interact with and ingest oxLDL via scavenger receptors. However, CD36 is not required for this response (Figure 2). ROS production by neutrophils and macrophages remains slow in a rabbit model of atherosclerosis. In humans, CD11b/CD18 may cooperate with CD36 in signaling ROS production on macrophage interaction with oxLDL. In macrophages from CD36−/− mice, however, CD36 is not required for this response (Figure 2). ROS and possibly other proinflammatory substances (eg, nitric oxide) produced by macrophages interacting with oxLDL may contribute to the initiation and progression of atherosclerotic lesions. The findings reported here indicate a central and hitherto-unsuspected role for CD18 in regulating oxidant production by macrophages in atherosclerotic lesions.

In conclusion, CD11b/CD18 may be a significant player in regulating macrophage signaling pathways involved in atherogenesis. In humans, CD11b/CD18 may cooperate with CD36 in signaling ROS production on macrophage interaction with oxLDL. In macrophages from CD36−/− mice, however, CD36 is not required for this response (Figure 2). ROS and possibly other proinflammatory substances (eg, nitric oxide) produced by macrophages interacting with oxLDL may contribute to the initiation and/or progression of atherosclerotic lesions. The findings reported here indicate a central and hitherto-unsuspected role for CD18 in regulating oxidant production by macrophages in atherosclerotic lesions.

Anti-CD18 antibodies reduce migration of monocytes across unstimulated human umbilical vein EC monolayers by 75%. However, when human umbilical vein EC monolayers are pretreated with proinflammatory substances (eg, lipopolysaccharide, tumor necrosis factor-α, or interleukin-1β), migration is no longer inhibited by anti-CD18 antibodies but is blocked by antibodies against VLA-4 and VCAM-1. This suggests that monocyte migration across “inflamed” endothelium is CD18 independent. The findings reported here raise the possibility that the reduction in atherosclerotic lesion size observed by Nageh et al may reflect decreased production of ROS and other proinflammatory substances within the sub endothelial compartment rather than reduced monocyte migration across the vascular endothelium into this compartment.

The mechanism by which CD11b/CD18 participates in ROS production by neutrophils and macrophages remains unresolved. At present, there is no evidence that CD11b/CD18 interacts directly with oxLDL. As documented here, β2-integrins are not required for adhesion to oxLDL-containing matrices (Figure 3b). It is possible that activation of CD11b/CD18 by signals generated by the interactions of specific cell surface receptors with matrix-bound oxLDL capacitates this receptor to organize/assemble the NADPH oxidase. Whatever mechanism is responsible, the findings reported here indicate a central and hitherto-unsuspected role for CD18 in regulating oxidant production by macrophages in atherosclerotic lesions.


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