Rapid Communication

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 as 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 β_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 ■ macrophages ■ oxidized LDL ■ CD11b/CD18 ■ 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 Lusis[1]). 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_2O_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 ≈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-

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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$^{-/-}$,[3] CD36$^{-/-}$,[4] and CD18$^{-/-}$[5] mice are described elsewhere.

Cells

Mouse mononuclear cells were obtained by lavage of the peritoneal cavity and used in experiments immediately thereafter.[6] Twenty-nine percent (±3%) of the cells harvested from the peritoneum of knockout mice and background-appropriate wild-type mice were macrophages, as indicated by their expression of nonspecific esterase and endocytosis of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–labeled acetylated LDL (not shown).[7] 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,[7] 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 multispot 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 LDL per spot (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) (KRBGA solution) containing 2.5×10^4 mouse peritoneal mononuclear cells or human macrophages for 45 minutes at 37°C in a 5% CO_2/95% air atmosphere. Slides were washed with KRBGA, 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 H_2O_2 as a representative ROS in the supernatant was measured with the Amplex Red™ H_2O_2 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 KRBGA containing 20 μmmol/L Amplex Red™ (Molecular Probes), 0.2 U/mL horseradish peroxidase (Molecular Probes), and 3×10^5 mouse peritoneal mononuclear cells or human macrophages for 2 hours at 37°C in a 5% CO_2/95% air atmosphere. Fluorescence of the product formed by the reaction between Amplex Red™ and H_2O_2 was assayed with a fluorescent plate reader.

Antibodies

Mouse peritoneal mononuclear cells or human macrophages were preincubated with KRBGA containing monoclonal rat anti-mouse CD18 $IgG_{1;k}$ (Pharmingen) or control rat $IgG_{1;k}$ (Pharmingen) at 0.4, 2, or 10 μg/mL; monoclonal mouse anti-human CD18 IgG_{1b} (Ancell Corp) or control mouse IgG_{1b} (AnCell) at 0.4, 2, or 10 μg/mL; or monoclonal rat anti-mouse/human CD11b IgG_{2b} (Serotec) or control rat IgG_{2b} (Pharmingen) at 0.04, 0.2, or 1 μg/mL for 15 minutes at 37°C in a 5% CO_2/95% air atmosphere where indicated.

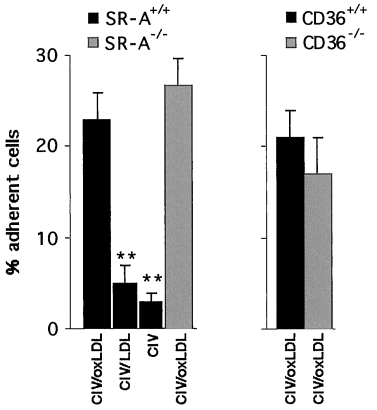

Figure 1. Adhesion of peritoneal macrophages from knockout mice (gray bars) lacking SR-A (SR-A$^{-/-}$) or CD36 (CD36$^{-/-}$) and background-appropriate wild-type mice (black bars) to surfaces coated with CIV, CIV/LDL, or CIV/oxLDL.

Statistical Analysis

All experiments were performed with triplicate samples for 3 lots of macrophages. Student's *t* test was used to pairwise compare adhesion (Figures 1 and 3b) and accumulation of H_2O_2 (Figures 2 and 3a) by wild-type mouse macrophages plated on CIV/oxLDL with wild-type macrophages plated on CIV or CIV/LDL, or by knockout macrophages (SR-A$^{-/-}$, CD36$^{-/-}$, or CD18$^{-/-}$) plated on CIV/oxLDL. Student's *t* test was used to compare adhesion (not shown) and accumulation of H_2O_2 (Figure 3c) by human or wild-type mouse macrophages plated on CIV/oxLDL in the absence of antibody or EDTA (leftmost cluster of bars) with macrophages preincubated with anti-CD11b, anti-CD18, control antibodies (not shown), or EDTA. Data are presented as mean±SEM, with ** indicating $P\leq0.01$.

Results

Less than 4% of total mononuclear cells (≈13% of macrophages) from wild-type mice, SR-A$^{-/-}$ mice, and CD36$^{-/-}$ mice adhered to surfaces coated with CIV or CIV/LDL (Figure 1), whereas ≈23% of total mononuclear cells (≈78% of macrophages) from wild-type, SR-A$^{-/-}$, and CD36$^{-/-}$ mice adhered to surfaces coated with CIV/oxLDL (Figure 1). These findings indicate that neither SR-A nor CD36 is required for adhesion of murine peritoneal macrophages to oxLDL-containing matrixes and that other adhesion-

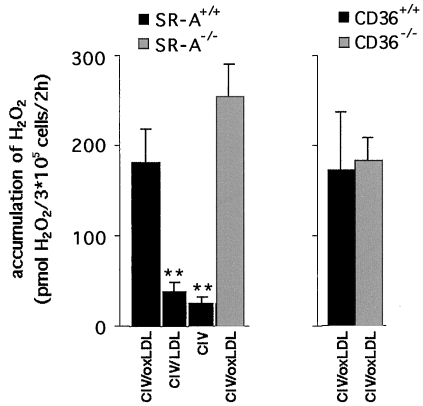

Figure 2. Accumulation of H_2O_2 in the supernatant on plating of peritoneal macrophages from knockout mice (gray bars) lacking SR-A (SR-A$^{-/-}$) or CD36 (CD36$^{-/-}$) and background-appropriate wild-type mice (black bars) on surfaces coated with CIV, CIV/LDL, or CIV/oxLDL.

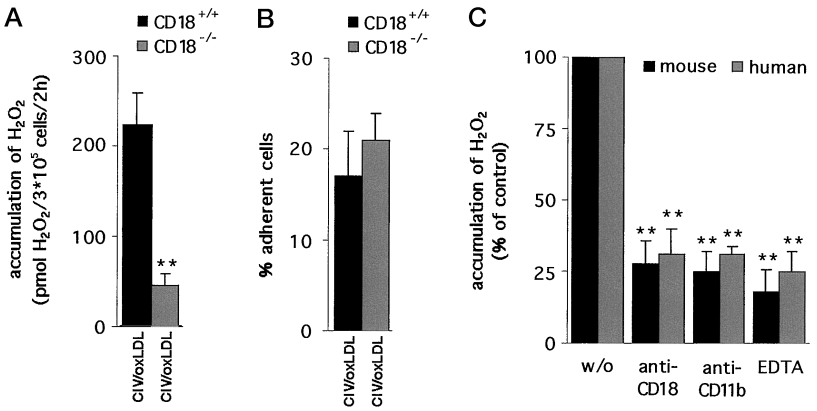

Figure 3. a, Accumulation of H_2O_2 in the supernatant on plating of peritoneal macrophages from knockout mice (gray bar) lacking CD18 ($CD18^{-/-}$) and background-appropriate wild-type mice (black bar) on surfaces coated with CIV/oxLDL. b, Adhesion of peritoneal macrophages from knockout mice (gray bar) lacking CD18 ($CD18^{-/-}$) and background-appropriate wild-type mice (black bar) to CIV/oxLDL. c, Effect of anti-CD11b and anti-CD18 IgG and of EDTA on accumulation of H_2O_2 in the supernatant on plating of mouse (black bars) or human (gray bars) macrophages on surfaces coated with CIV/oxLDL compared with macrophages plated on this surface in the absence of antibodies or EDTA (control).

mediating receptors, eg SR-BI,[7] may mediate macrophage binding to ligands provided by oxLDL.

To assess whether adhesion to CIV, CIV/LDL, or CIV/oxLDL stimulates ROS production by $SR\text{-}A^{-/-}$, $CD36^{-/-}$, or wild-type murine macrophages, we incubated these cells on matrixes containing these proteins and assayed for accumulation of H_2O_2 in the supernatant as a representative ROS. Consistent with the findings of Maxeiner et al,[2] wild-type mouse peritoneal macrophages produced very little ROS on surfaces coated with CIV or CIV/LDL (≈33 pmol H_2O_2 per 3×10^5 cells/2 hours; Figure 2) but produced significantly more ROS on surfaces coated with CIV/oxLDL (≈185 pmol H_2O_2 per 3×10^5 cells/2 hours; Figure 2). However, in contrast to the results predicted by Maxeiner et al,[2] macrophages from $SR\text{-}A^{-/-}$ and $CD36^{-/-}$ mice produced at least as much ROS as did wild-type macrophages (≈247±36 and ≈188±47 pmol H_2O_2 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 matrixes and ROS production by these cells when they adhere to such matrixes.

Interestingly, neutrophils also have been shown to produce ROS on interaction with oxLDL (see Maeba et al[8] 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[9] and J.H., unpublished observations, 2000) but do express high levels of the β_2-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 (eg, fibrinogen, iC3b[10]). This suggested a role for CD11b/CD18 in ROS production by macrophages. As anticipated, resident peritoneal macrophages from mice genetically deficient in the β_2-chain of CD11b/CD18 ($CD18^{-/-}$ mice) adhered as efficiently to matrixes 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 H_2O_2 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 H_2O_2 per 3×10^5 cells/2 hours; Figure 1). These results indicate that CD18 plays a hitherto unanticipated role in oxLDL-stimulated ROS production by macrophages.

To further explore this finding, we assessed the effect of antibodies against mouse and human CD18 or CD11b on ROS production by mouse and human macrophages adherent to surfaces containing CIV/oxLDL. These antibodies almost completely inhibited ROS production by mouse or human macrophages. Neither anti-CD18 nor anti-CD11b IgG had any effect on adhesion of these cells to surfaces containing CIV/oxLDL (not shown). The same concentration of isotype-matched control IgG had no effect on mouse or human macrophage adhesion to or ROS production on CIV/oxLDL (not shown).

β_2-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 matrixes. Because antibodies against CD11b/CD18 are as effective in inhibiting ROS production as is the absence of all 4 β_2-integrins in $CD18^{-/-}$ macrophages, it appears that CD11b/CD18 is the β_2-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 CD18, along with chemoattractants such as 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,[11] P-selectin,[11] or MCP-1[12] of endothelial cells or of CD18[11] or CCR2, the receptor for MCP-1 on macrophages,[13] 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 subendothelial space mature into macrophages that interact with and ingest oxLDL via scavenger receptors (eg, SR-A, SR-BI, CD36), thereby becoming foam cells.[1] Interactions with matrix-bound oxLDL may also stimulate these macrophages to produce ROS in vivo, as they do in vitro (eg, Figures 1 through 3 and References 2 and 14). The NADPH oxidase and the 12/15-lipoxygenase pathways are thought to play important roles in the atherogenic effects of macrophages. NADPH oxidase is upregulated in macrophages in atherosclerotic lesions.[15] Inhibitors of NADPH oxidase slow the appearance of vascular lesions in a rabbit model of atherosclerosis.[16] In mouse models, however, genetic disruption of 2 subunits of NADPH oxidase ($gp91^{phox}$[17] and $p47^{phox}$[18]) had no significant effect on lesion development. There are no reports of atherosclerosis in patients whose monocytes are deficient in NADPH oxidase (eg, chronic granulomatous disease). Macrophages from such patients retain the ability to produce ROS through the 15-lipoxygenase pathway,[19,20] especially after treatment with cytokines[21] (eg, γ-interferon[22]). Furthermore, chronic granulomatous disease macrophages oxidize LDL in vitro, though with reduced efficiency.[14]

15-Lipoxygenase colocalizes with epitopes of oxLDL in human and rabbit atherosclerotic lesions.[23] Genetic disruption of 12/15-lipoxygenases in a mouse model of atherosclerosis[24] or inhibition of 15-lipoxygenase in a rabbit model[25] of atherosclerosis has been reported to limit the progression of atherosclerotic lesions. These results suggest that macrophage production of ROS via both the NADPH and lipoxygenase pathways contributes to the development of atherosclerosis. The finding that the absence or inhibition of CD11b/CD18 reduces ROS production by macrophages adherent to a matrix containing oxLDL by ≈80% suggests that CD11b/CD18 plays a role in atherosclerosis. Nageh et al[11] observed an ≈50% reduction in fatty streaks in mice whose CD18 genes had been knocked out.

Anti-CD18 antibodies reduce migration of monocytes across unstimulated human umbilical vein EC monolayers by ≈75%.[26] 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.[26] 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[11] may reflect decreased production of ROS and other proinflammatory substances within the subendothelial 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, β_2-integrins are not required for adhesion to oxLDL-containing matrixes (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.

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.[2] 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 vascular disease by oxidizing extracellular matrix proteins, lipoproteins, and lipids and stimulating cell death. Inhibition of ROS or nitric oxide production[27] has been demonstrated to slow progression of atherosclerosis. The findings reported here raise the possibility that agents that disrupt the participation of CD11b/CD18 in intracellular signaling pathways could slow the onset and/or progression of atherosclerosis and other diseases involving macrophage ROS production.

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