LDL activates signaling pathways leading to an increase in cytosolic free calcium and stimulation of CD11b expression in monocytes

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Abstract **In the present study, we investigated the mechanisms by which plasma lipoproteins modulate the integrin-dependent adhesion properties of monocytes. LDL induced the expression of the monocyte CD11b in vitro as well as in vivo via intracellular signaling mechanisms involving calcium transients. The effect on CD11b transcription was specific for native LDL and was blocked by a neutralizing anti-LDL receptor antibody. Neither oxidized LDL nor HDL had any effect on CD11b expression. Although LDL stimulated CD11b surface expression, the integrins were not activated. To initiate the CD11b-specific adhesion to the endothelium, the engagement of chemokine receptor CCR2 and intact chemokine-to-integrin signaling was necessary. However, the activation of CCR2 with monocyte chemoattractant protein-1 not only stimulated the integrins preexisting on the cell surface, but also increased the number of CD11b molecules on the cell surface. This was particularly pronounced in THP-1 cells after treatment with LDL. In a previous study, we showed that LDL induces the expression of CCR2 in monocytes. We conclude that this may be the underlying cause of the enhanced chemokine effect on CD11b expression and activation observed with these cells.**—Han, K. H., Y. Chen, M. K. Chang, Y. C. Han, J-H. Park, S. R. Green, A. Boullier, and O. Quehenberger. **LDL activates signaling pathways leading to an increase in cytosolic free calcium and stimulation of CD11b expression in monocytes.** ***J. Lipid Res.*** **2003.** 44: **1332–1340.**

Supplementary key words adhesion protein • atherosclerosis • CCR2 • integrin • monocyte recruitment

The earliest visible lesion in human and experimental models of atherosclerosis is the arterial fatty streak characterized by the presence of lipid-laden foam cells (1). Most of the foam cells in the fatty streak are derived from circulating monocytes, as shown by immunochemical techniques (2). Although monocyte recruitment commences early in lesion development, it persists throughout all stages of plaque development, consistent with a chronic inflammatory process. Various mediators of inflammation including adhesion molecules, cytokines, and chemoattractant factors have been shown to initiate the extravasation of monocytes (3, 4). The initial interaction between monocytes and endothelium is mediated by selectins that recognize a sialylated carbohydrate determinant on their counterreceptor (5, 6). Selectins are believed to be importantly involved in a variety of chronic inflammatory reactions, and the interruption of the leukocyte-endothelial adhesion cascade has been a major focus in atherosclerosis research. Results from recent studies with mice deficient in E-selectin and P-selectin supported the critical involvement of these adhesion molecules in atherosclerosis (7, 8).

Although rolling appears to be a prerequisite for firm adherence of monocytes to blood vessels, the selectin-mediated adherence is transient and reversible unless followed by a second event. For firm adhesion, the engagement of G-protein-coupled chemokine receptors is required, which leads to the activation of integrins, resulting in a strong and sustained adhesion (9, 10). Monocytes express both the β1 integrin CD49d/CD29 and the β2 integrins CD11a/CD18, CD11b/CD18, and CD11c/CD18 (11–13). The strong attachment to the endothelium is mediated by interaction with a class of ligands that belong to the immunoglobin superfamily, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (14). The critical involvement of these adhesion molecules in monocyte recruitment and atherosclerosis was initially demonstrated in animal models of

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Abbreviations: Bt_2cAMP, dibutyryl-cAMP; $[Ca^{2+}]_i$, intracellular calcium concentration; HUVEC, human umbilical vein endothelial cell; MCP-1, monocyte chemoattractant protein-1; PE, phycoerythrin; TBARS, thiobarbituric acid-reactive substances.

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hypercholesterolemia (15–17). Additional studies with neutralizing antibodies blocking integrin function or with animals deficient in ICAM-1 demonstrated reduced monocyte entry and fatty streak formation, further establishing the central role of this integrin system for monocyte recruitment in experimental atherosclerosis (18, 19).

The transmigration of the adherent monocytes through the endothelial layer into the intima is governed by chemotactic factors (20), including monocyte chemoattractant protein-1 (MCP-1), a well-characterized member of the CC family of chemotactic cytokines (21, 22). It is expressed in substantial amounts in a number of pathological conditions characterized by persistent monocyte infiltration, including atherosclerotic lesions (23–25). Like all chemokine receptors, the receptor for MCP-1, CCR2, is a heptahelical receptor that signals through heterotrimeric G-proteins (26). A causal function for the CCR2/MCP-1 chemokine system in atherosclerosis was established in in vivo studies showing that the formation of atherosclerotic lesions was significantly reduced in mice deficient in either CCR2 or MCP-1 (27, 28).

Recently, we identified LDL as a positive regulator for monocyte CCR2 expression and demonstrated that CCR2 expression and the chemotactic response to MCP-1 were several-fold higher in monocytes isolated from hypercholesterolemic patients (29, 30). A similar increase in CCR2 expression was also observed with monocytes from normal donors after treatment with LDL ex vivo at concentrations characteristic of hypercholesterolemia. In addition to chemotaxis, MCP-1 initiates a number of responses in monocytes that are physiologically important in inflammation. It is well established that ligation of chemokine receptors, including CCR2, induces surface expression and activation of integrins (9, 10, 31). We therefore hypothesized that an increase in CCR2 expression as seen in monocytes from hypercholesterolemic patients may enhance the adhesion properties of monocytes and accelerate the recruitment process.

In the present study, we introduce a potential mechanism for the excessive recruitment of monocytes to the arterial wall associated with atherogenesis. We show that a causal link exists between plasma LDL levels, monocyte CCR2 expression, CD11b expression, and the adhesion and transmigration properties of monocytes. These results demonstrate that leukocyte functions pertinent to recruitment are affected by hypercholesterolemia and may represent potential therapeutic targets for the treatment of chronic inflammatory disorders, including atherosclerosis.

MATERIALS AND METHODS

Preparation of lipoproteins

Human LDL (d = 1.019–1.063 g/ml) and HDL (d = 1.063–1.21 g/ml) were isolated by ultracentrifugation as described (32). Mildly modified LDL was prepared by incubation of LDL (2 mg protein/ml) with 2 μM $CuSO_4$ in EDTA-free PBS for 4 h at 37°C, and fully oxidized LDL was prepared by incubation in Ham's F-10 medium with 10 μM $CuSO_4$ for 24 h at 37°C (33). Native LDL and HDL contained less than 0.3 nmol of thiobarbituric acid-reactive substances (TBARS) per mg protein, as determined by fluorometric assay (34). Minimally modified LDL contained 4 nmol of TBARS per mg protein, and fully oxidized LDL contained 55 nmol of TBARS per milligram of protein. All lipoprotein preparations contained less than 0.05 ng/ml of endotoxin, measured with the timed-gel formation kit (Sigma).

Cell culture, animals, and isolation of circulating monocytes

The THP-1 monocytes (American Type Culture Collection) were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 20 μM mercaptoethanol (Life Technologies, Inc.). Human umbilical vein endothelial cells (HUVECs), obtained from Cell Applications Inc., were cultured with Earl's salts 199 medium (Irvine Scientific) containing 20% fetal bovine serum, antibiotics, bovine brain extract (20 μg/ml), insulin (20 μg/ml), hydrocortisone (1 μg/ml), and human endothelial growth factor (20 ng/ml) obtained from Cambrex Corp. For the in vivo experiments, C75/BL6 mice (The Jackson Laboratory) were divided into two groups. The control group received regular chow and the experimental group received a high-fat diet containing 1.25% cholesterol (Harlan-Teklad). The animals received 3–4 g of food per mouse per day for 3 weeks.

Circulating mouse monocytes were isolated as described (35). Briefly, whole blood in 3 mM EDTA was layered onto 3 ml of Histopaque 1.077 (Sigma), and mononuclear cells were separated by centrifugation at 600 *g* for 15 min. Further purification depended on the intended usage. For flow cytometry, contaminating platelets were removed by two washes with PBS containing 0.1% BSA and 0.2% EDTA (400 *g*, 4°C, 15 min), and the monocytes were identified and gated with phycoerythrin (PE)-labeled antibody against mouse CD80 (Pharmingen). For RNA isolation, monocytes were purified by plating for 30 min to achieve a purity of >85%.

In vitro incubation conditions

THP-1 monocytes were incubated for 24 h with the various lipoproteins in the presence of 5% human lipoprotein-deficient serum. Cholesterol loading was achieved by adding an ethanolic solution of free cholesterol (25 μg/ml) to cultures of THP-1 monocytes for 24 h. The neutralizing anti-LDL receptor IgG C7 was purchased from American Type Culture Collection and was used at a 1:200 dilution. To inhibit protein synthesis, cycloheximide (CHX) (Sigma) was used at 1 μM, and the intracellular calcium antagonist TMB-8 (Calbiochem) was used at 100 nM. All these reagents were added to the THP-1 monocytes 3 h prior to the lipoproteins and were present in the media during the various incubation periods.

Analysis of integrin expression

The expression levels of CD11a, CD11b, and CD49d were estimated by semiquantitative PCR. Total RNA was isolated by guanidium thiocyanate-phenol-chloroform extraction (36), and cDNA was generated by reverse transcription using Superscript II (Invitrogen). For PCR amplification, oligonucleotides specific for CD11a (5′-GTGGCCTGTGTTACCTCTTCCG-3′ and 5′-TCACGGTGTAACCCAAATAGCC-3′), CD11b (5′-TCGGCGGATGAAGGAGTTTG-3′ and 5′-CTTTGCACCCGGTTCCGTAAG-3′), and CD49d (5′-GGTGATGCTGTTGCTGTGCC-3′ and 5′-GATGGTGCTCTGCATGGGTG-3′) were used. As the internal standard, glyceraldehyde-3-phosphate dehydrogenase was amplified and analyzed under identical conditions using the specific primer pair 5′-GACCCCTTCATTGACCTC-3′ and 5′-GCTAAGCAGTTGGT-

GGTG-3′. The primers were annealed to the template cDNA at 56°C for 1 min and extended at 72°C for 2 min for 28 cycles. The amplified DNA was analyzed by agarose gel electrophoresis, and the intensity of each band was measured by densitometric scanning. The linearity of the amplification was established with serial dilutions of the template DNA.

Flow cytometry

THP-1 monocytes were washed twice with 3 ml of ice-cold PBS containing 0.1% BSA and 0.01% sodium azide (Buffer A), and 10^5 cells were resuspended in 100 μl of Buffer A. Fc receptors were blocked with unlabeled nonspecific mouse IgG (5 μg). To estimate the β-integrin surface expression, 0.5 μg of PE-labeled mouse IgG against CD11a, CD11b, or CD49d (Pharmingen) was added, and the cells were incubated for 30 min at 4°C. To measure total (surface and intracellular) expression, THP-1 cells were permeabilized with 0.2% saponin for 5 min prior to addition of the antibodies. After the labeling procedure, the cells were washed with ice-cold Buffer A and the fluorescence was measured by flow cytometry. To estimate CD11b expression on mouse monocytes, peripheral blood mononuclear cells were isolated as described above, and the monocytes were identified and gated by the expression of the surface marker CD80 with PE-conjugated anti-CD80 IgG. CD11b expression on the gated monocytes was determined with FITC-conjugated anti-mouse CD11b IgG (Pharmingen). All experiments were repeated in at least three independent studies and analyzed with CELLQUEST software.

Calcium mobilization assay

Cells were washed once with PBS, suspended to 5×10^6 cells/ml in phenol red-free RPMI 1640 medium containing 10 mM HEPES (Sigma) and 0.1% BSA, and labeled with 5 μM indo-1 AM (Molecular Probes) at 37°C for 30 min in the dark. Following brief washes with PBS, the cells were resuspended at a density of 1.5×10^6 cells/ml in Hank's balanced salt solution containing 1.25 mM calcium chloride (Irvine Scientific). Changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$) in response to various concentrations of LDL were monitored as described (37). Fluorescence was simultaneously recorded at 400 nm and 490 nm with the excitation set at 360 nm on an LS50B Luminescence Spectrophotometer (Perkin Elmer). The change in $[Ca^{2+}]_i$ was expressed as the fluorescence ratio at 400 nm/490 nm. The data are expressed as a percentage of maximal fluorescence achieved with treatment of the cells with 10 μM of the ionophore A23187 (Sigma).

Adhesion assay

HUVECs were grown to confluence in 96-well plates. ICAM-1 expression in these cells was tested before and after stimulation with cytokines by RT-PCR and flow cytometry using labeled mouse anti-human ICAM-1 IgG (R&D Systems). Although cytokines, including tumor necrosis factor α, induce ICAM-1 expression in HUVECs, the basal expression was already substantial and sufficient to support THP-1 adhesion. This was especially true for the static adhesion protocol employed in this study, and all adhesion experiments were performed with nonstimulated HUVECs. THP-1 monocytes were preincubated with 50 μg/ml of LDL for 24 h. After the incubation period, the THP-1 monocytes were washed with PBS containing 0.1% BSA and labeled with 5 μM of Calcein-AM (Molecular Probe) at 37°C for 15 min. The labeled THP-1 monocytes were suspended in phenol red-free RPMI 1640 medium containing 0.1% BSA and stimulated with 10 nM MCP-1 for 30 min. To establish the baseline adhesion independent of MCP-1-mediated activation, a neutralizing mouse anti-MCP-1 IgG (R&D Systems) was added at 10 μg/ml. Dibutyryl-cAMP (Bt_2cAMP), the permeable analog of cAMP, was used at 50 μM for 30 min prior to stimulation with MCP-1. The THP-1 monocytes (10^5 cells in 200 μl) were then added to the HUVEC monolayer and incubated at 37°C in the absence or presence of anti-human CD11b IgG (10 μg/ml; R&D Systems). In control experiments, a nonspecific isotype IgG (10 μg/ml; R&D Systems) was used. After 30 min, nonadherent THP-1 monocytes were removed by gentle washes (2×) with PBS, the remaining cells were lysed with 200 μl of 0.2 N NaOH solution, and the fluorescence intensity at 530 nm was measured with the excitation set at 485 nm. The number of bound THP-1 monocytes was calculated from standard curve.

Transmigration assay

The transmigration assay was performed in 24-well Transwell plates and polycarbonate membranes with an 8 μm pore size (Falcon). HUVECs (20,000 cells) were seeded into each Transwell and grown for 72 h to allow formation of tight monolayers. The Transwells were transferred to a 24-well plate containing 600 μl of prewarmed RPMI 1640 medium with 0.1% BSA, and Calcein-labeled THP-1 monocytes (5×10^5 cells in 200 μl) were added to the upper compartment. After a 15 min equilibrium period, MCP-1 (10 nM) was added to the lower compartment. After 2 h at 37°C under a 5% CO_2 atmosphere, the cells that transmigrated to the lower compartment were pelleted and lysed with 200 μl of 0.2 N NaOH. The fluorescence intensity was measured and the corresponding number of migrated cells was calculated as described for the adhesion assay. Spontaneous transendothelial migration in the absence of MCP-1 or presence of neutralizing anti-MCP-1 IgG was insignificant. To assess the role of CD11b, anti-CD11b IgG (10 μg/ml) was added to the incubation medium.

RESULTS

Stimulation of monocyte CD11b expression by native LDL

To determine potential regulatory functions of lipoproteins on monocyte integrin expression, THP-1 cells were incubated with native LDL and the surface expression of β-integrins was estimated by flow cytometry. As shown in **Fig. 1**, LDL increased the surface expression of CD11b, but not that of CD11a or CD49d. The stimulation of CD11b expression was further established by analysis of the steady-state level of CD11b mRNA. Native LDL enhanced CD11b transcripts in a dose-dependent fashion, and the presence of 50 μg/ml of LDL in the culture medium increased CD11b transcripts in THP-1 monocytes about 2.5-fold in 24 h (**Fig. 2**). The mRNA levels for CD11a and CD49d remained unchanged (data not shown). The effect on CD11b expression was specific for native LDL. Minimally modified LDL, fully oxidized LDL, and HDL did not produce any significant changes in CD11b mRNA levels (Fig. 2).

The positive regulatory effect of LDL on CD11b expression was not limited to cells in culture but was also seen in monocytes in vivo. Several genetically engineered mouse models of hypercholesterolemia are available. However, in these animals a Western diet increases plasma cholesterol to levels that are not seen in humans, often exceeding 1,500 mg/dl. To achieve plasma cholesterol levels that reflect more the human conditions, we used C57/BL6 mice

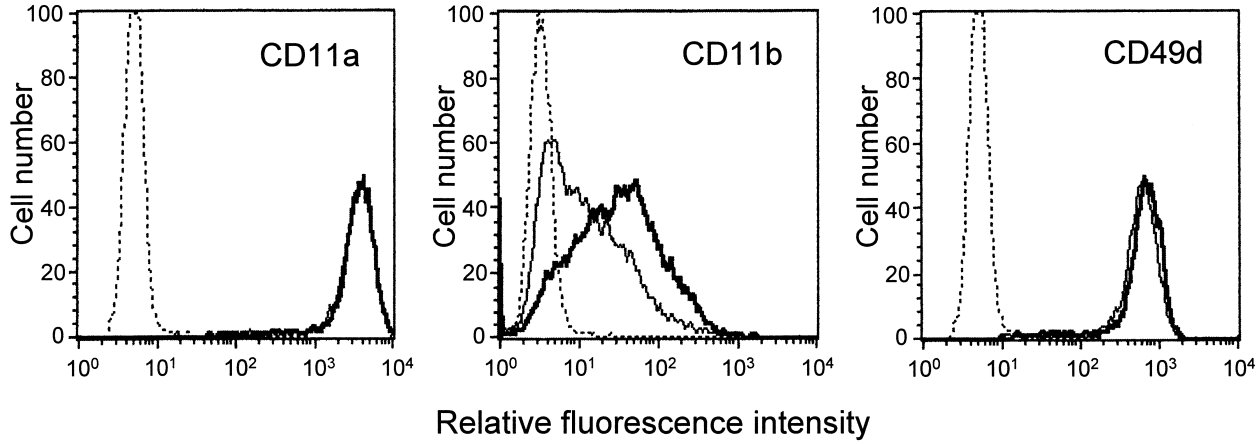

Fig. 1. The effect of LDL on β-integrin surface expression. Cultures of THP-1 monocytes were treated for 24 h with human LDL (50 μg protein/ml), and 10^5 cells in 100 μl buffer A were labeled with phycoerythrin (PE)-conjugated mouse IgG specific for CD11a, CD11b, and CD49d as described under Materials and Methods. Fc receptors were blocked by preincubation of the cells with nonspecific mouse IgG. The integrin surface expression was analyzed by flow cytometry (bold lines) and compared with untreated control cells cultured in the absence of human LDL but labeled with the antibodies under identical conditions (fine lines). PE-conjugated nonspecific mouse IgG was used to measure nonspecific binding (dashed lines).

with intact lipoprotein clearance capabilities. After 3 weeks on a Western diet (1.25% cholesterol), total plasma cholesterol of C57/BL6 mice was 369 ± 15 mg/dl, and that of the control group on normal chow was 158 ± 17 mg/dl. Both CD11b mRNA and protein levels were on average 2-fold higher in the monocytes from the high-cholesterol group compared with monocytes from the control group (**Fig. 3**). This observation confirmed the in vitro data obtained with THP-1 cells, and establishes THP-1 cells as a valid system for the study of CD11b expression induced by LDL.

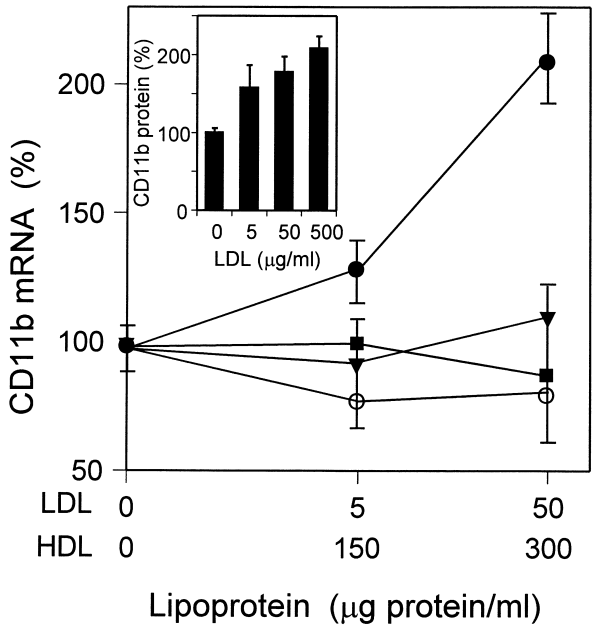

Fig. 2. Stimulation of CD11b expression by lipoproteins. THP-1 cells were incubated for 24 h with 0 μg, 5 μg, and 50 μg protein/ml of native LDL (closed circle), minimally modified LDL (closed triangle), or fully oxidized LDL (closed square), or with 0 μg, 150 μg, and 300 μg protein/ml of HDL (open circle). The steady-state level of CD11b mRNA was estimated by PCR amplification of reverse-transcribed cDNA and normalized to glyceraldehyde-3-phosphate dehydrogenase analyzed under identical conditions. The inset shows the effect of LDL on total CD11b expression estimated by flow cytometry using permeabilized THP-1 cells. The data shown represent the mean ± SD of three experiments.

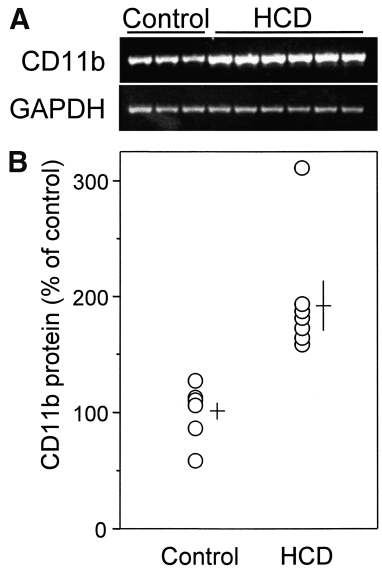

Fig. 3. CD11b expression in circulating monocytes from mice fed a high-fat diet. C57/BL6 mice were placed on a diet containing 1.25% cholesterol (HCD) and control animals (Control) were fed a normal chow diet. After 3 weeks, circulating mononuclear leukocytes were isolated, and the effect of the diet on monocyte CD11b expression was determined. A: Analysis of CD11b transcripts. The monocytes were purified by plating, and CD11b mRNA was estimated by semi-quantitative PCR of reverse-transcribed cDNA. B: Analysis of CD11b protein by flow cytometry. Monocytes were identified with anti-CD80 antibody, and the CD11b expression of the CD80-positive cells was estimated using FITC-labeled anti-mouse CD11b IgG. $P < 0.01$, determined by unpaired Student's *t*-test; n = 7.

Activation of intracellular signaling pathways by LDL

In a previous study, we found that LDL increases the expression of the monocyte chemokine receptor CCR2 and identified cholesterol as the driving force (29). To examine if similar cholesterol-dependent mechanisms were also responsible for the increase of monocyte CD11b expression, we treated THP-1 monocytes with free cholesterol. In contrast to the intact LDL particle, free cholesterol did not enhance CD11b expression (**Fig. 4**). The positive regulatory effect of native LDL was clearly receptor dependent, and the blocking anti-LDL receptor antibody IgG-C7 inhibited the expression of CD11b protein. A nonspecific isotype control IgG had no effect.

To determine if the stimulation of CD11b expression required the activation of intracellular signaling pathways, we estimated LDL-induced calcium transients in THP-1 cells. The cells responded rapidly (within seconds) to native LDL with an increase in the $[Ca^{2+}]_i$ (**Fig. 5A**). A significant attenuation of the calcium response was observed when the phosphoinositide signaling pathway was inhibited. TMB-8, an antagonist of the inositol 1,4,5-trisphosphate (IP_3) receptor, very potently reduced the $[Ca^{2+}]_i$ (Fig. 5A). A similar reduction was also observed with the blocking anti-LDL receptor IgG C7, whereas an isotype control IgG had no effect (data not shown). To establish a causal link between the calcium transients and CD11b expression, we estimated CD11b protein. The treatment of cells with TMB-8 not only blunted the effect of LDL on $[Ca^{2+}]_i$, but also significantly reduced CD11b protein levels, similar to CHX (Fig. 5B).

Effect of LDL on chemokine-stimulated adhesion and transmigration

Chemokines can stimulate the translocation of CD11b from intracellular pools to the plasma membrane (31). Consistent with this, we found that activation of CCR2 with MCP-1 (20 nM for 4 h) increased CD11b surface expression on THP-1 monocytes (**Fig. 6**). The increase, although significant, was relatively small. In contrast, MCP-1

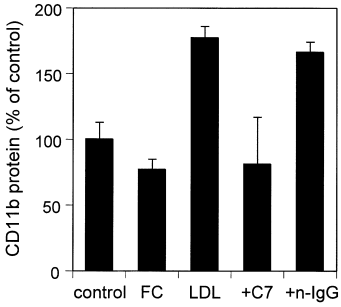

Fig. 4. The effect of LDL on CD11b expression is receptor mediated. THP-1 cells were treated for 24 h with no addition (control), 25 μg/ml of free cholesterol (FC), 50 μg/ml of LDL (LDL), and with 50 μg/ml of LDL in the presence of either the blocking anti-LDL receptor IgG C7 at a dilution of 1:200 (+C7) or 10 μg/ml nonspecific control IgG (+n-IgG). Total cellular CD11b expression was determined on permeabilized cells by flow cytometry. Data are the mean ± SD of three independent experiments.

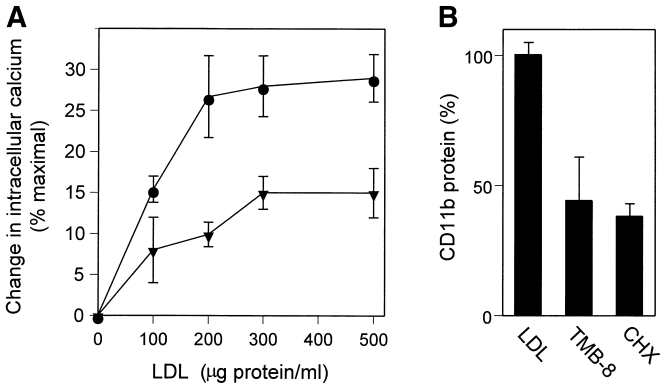

Fig. 5. Stimulation of CD11b expression by LDL is mediated by signaling pathways involving calcium transients. A: THP-1 monocytes were labeled with Indo-1AM and stimulated with the indicated concentrations of LDL, and intracellular calcium transients were measured as described under Materials and Methods in the absence (closed circle) or presence (closed triangle) of 100 nM of TMB-8, which was added 3 h prior to the stimulation with LDL. The change in intracellular calcium is expressed as the fraction of the maximal response achieved with the ionophore A23187. Data are the mean ± SD of three independent experiments. B: Parallel with the calcium measurements, CD11b protein expression was determined by flow cytometry using permeabilized cells as described under Materials and Methods. THP-1 cells were incubated for 24 h with either 50 μg/ml of LDL only (LDL) or with a combination of LDL and 100 nM TMB-8 (TMB-8) or 1 μM cycloheximide (CHX). Data are the mean ± SD of three independent experiments.

stimulated CD11b surface expression very effectively in LDL-treated cells. As expected, native LDL by itself increased the basal CD11b surface expression substantially. Subsequent stimulation of these cells with MCP-1 further enhanced the surface density of CD11b about 4-fold, and was then about 8-fold higher than that of control cells (Fig. 6).

A substantial body of evidence implicates integrins, including CD11b, in chemokine-mediated monocyte adhesion and recruitment to the vascular wall in atherogenesis (38). Consistent with this, the treatment of THP-1 cells with LDL profoundly increased their CD11b-dependent adhesion to endothelial monolayers, but only after stimulation with MCP-1 (**Fig. 7**). Activation of CCR2 was essential, and no difference in adhesion between control and LDL-treated THP-1 cells was observed in the absence of MCP-1 (data not shown). These results suggested that, although LDL increases the surface density of CD11b, it does not directly activate the integrin. This was further examined by blocking the established chemokine-to-integrin signaling pathway. Chemokines stimulate the nucleotide exchange on the small G-protein RhoA, a critical mediator for fast integrin-dependent leukocyte adhesion (39). The fast integrin-dependent adhesion triggered by chemokines, including interleukin 8 and formyl peptide, is inhibited by cAMP through its effector protein kinase A, a negative regulator of the RhoA GDP/GTP exchange activity (40). To further define the role of CCR2 in CD11b activation and to explore whether MCP-1 activates CD11b through similar RhoA-dependent signaling pathways, we

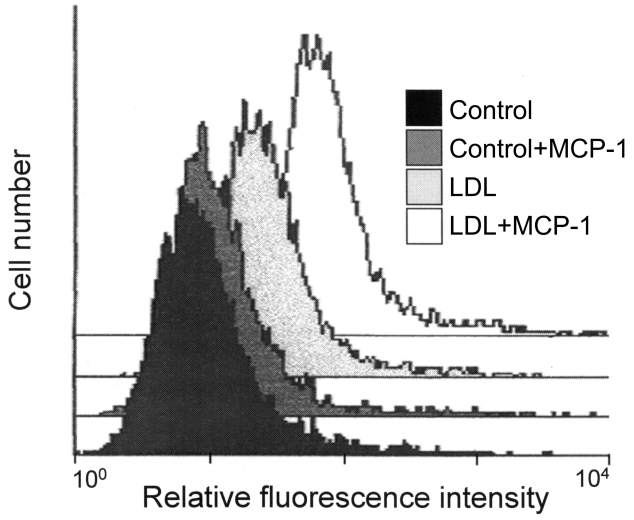

Fig. 6. Monocyte chemoattractant protein-1 (MCP-1)-stimulated surface expression of CD11b is increased by LDL. THP-1 monocytes were treated for 24 h with 50 μg/ml of LDL (LDL) and then stimulated for an additional 4 h with 20 nM of MCP-1 (LDL+MCP-1). Control cells were maintained under identical conditions in the absence of LDL before (Control) and after (Control+MCP-1) stimulation with MCP-1. The surface expression of CD11b was determined by flow cytometry as described in Fig. 1.

pretreated THP-1 cells with Bt_2cAMP, a permeable analog of cAMP. Bt_2cAMP inhibited the MCP-1-triggered adhesion to endothelial cells, suggesting a role for RhoA in CCR2-to-integrin signaling (Fig. 7). These results further indicated that, although LDL stimulates CD11b surface expression, this is by itself not sufficient to increase monocyte adhesion. Intact chemokine-to-intregrin signaling is an absolute requirement for CD11b activation.

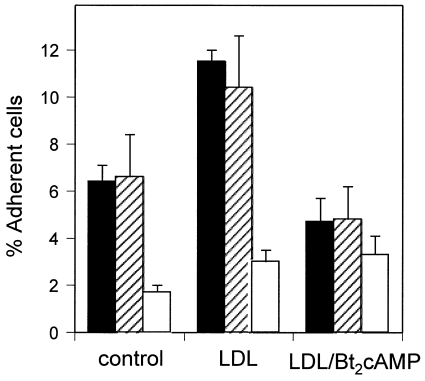

Fig. 7. Inhibition of MCP-1-dependent adhesion of THP-1 cells by cAMP. Untreated THP-1 monocytes (control) and THP-1 monocytes treated for 24 h with 50 μg/ml of LDL (LDL), or with LDL followed by additional 30 min with 50 μM dibutyryl-cAMP (LDL-Bt_2cAMP) were added to a monolayer of human umbilical vein endothelial cells (HUVECs). The effect of the treatments on CD11b-dependent adhesion was determined in the absence (closed bars) or presence (open bars) of a blocking anti-CD11b antibody (10 μg/ml). Nonspecific isotype IgG (10 μg/ml) was included as a control (hatched bars). Shown is the chemokine-dependent adhesion of the THP-1 cells determined after stimulation with 10 nM MCP-1 for 30 min and subtraction of the background adhesion in the absence of the chemokine. Data are the mean ± SD of three independent experiments.

Like adhesion, transendothelial migration was also enhanced (about 4- to 5-fold) by treatment of THP-1 monocytes with LDL (**Fig. 8**). The movement of the THP-1 cells across the endothelial monolayer in response to MCP-1 was CD11b dependent and was blocked by a neutralizing antibody against CD11b. No effect was observed with nonspecific IgG isotype control. A similar degree of inhibition was also seen after pretreatment of the THP-1 cells with Bt_2cAMP, indicating that chemokine-mediated activation of integrins was essential for monocyte adhesion and diapedesis.

DISCUSSION

Pathological studies demonstrated that blood-derived monocytes/macrophages play a key role in atherogenesis and constitute a significant cellular component of the atherosclerotic plaque (41). Tissue infiltration of monocytes is a complicated process and involves the ability of the leukocytes to interact with the cells of the vessel wall and to respond to the microenvironment at all stages of extravasation. Reduction of monocyte recruitment is expected to be athero-protective, and genes involved in the critical stages of extravasation represent potential therapeutic targets. Thus, intensive research efforts have been directed toward understanding the mechanisms of monocyte entry into the arterial wall and their subsequent transformation to foam cells. A previous study has demonstrated that monocytes in hypercholesterolemic animals are more readily recruited to the vessel wall compared with normal controls, but the mechanism of this remained obscure

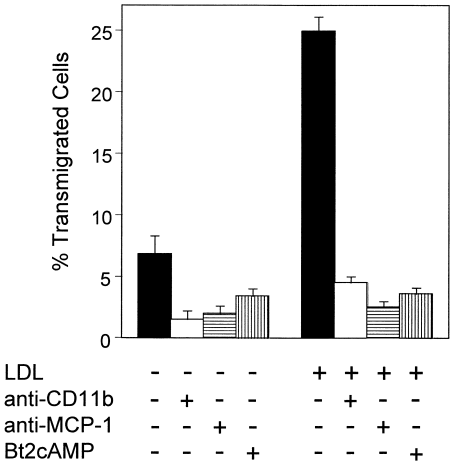

Fig. 8. The effect of the LDL treatment on THP-1 monocyte migration across HUVEC monolayers. THP-1 monocytes were treated for 24 h with 50 μg/ml of LDL and added to HUVEC monolayers grown in Transwell plates. The transmigration was initiated with 10 nM MCP-1 added to the lower compartment and analyzed as described under Materials and Methods. The anti-CD11b antibody (anti-CD11b) and the anti-MCP-1 antibody (anti-MCP-1) were added at 10 μg/ml. Bt_2cAMP was present at 50 μM. Data are the mean ± SD of three independent experiments.

(42). We postulated that the excessive accumulation of monocytes in atherosclerotic lesions is in part caused by an enhanced chemotactic response of monocytes due to elevated CCR2 gene expression (29, 30). These results suggested that, in addition to the well-studied effects on the cells of the vasculature, LDL may also alter gene expression in blood leukocytes, which may modulate their response to inflammatory stimuli.

The results from the present study provide support for a positive regulatory role of plasma LDL on CD11b expression in monocytes. The increase in CD11b expression was not associated with a change in size or granularity of the monocytes, suggesting that the induction of monocyte gene expression by LDL is not the result of a general stimulation of the cell growth program, but rather an independent and specifically regulated event. Our findings are in agreement with data from a recent report that showed that leukocyte adherence in mesenteric venules was increased in hypercholesterolemic rats and was reduced by lipid-lowering treatment (43). Similar results were also obtained in a study on hypercholesterolemic human patients (44, 45).

There are several lines of evidence indicating that activation of the LDL receptor and stimulation of signaling pathways are necessary for the effects of LDL on CD11b expression. First, the stimulation of CD11b expression was inhibited when the binding of LDL to the receptor was blocked by a neutralizing anti-LDL receptor antibody. Second, other lipoproteins, including oxidized LDL and HDL that bind to monocytes through receptors distinct from the LDL receptor, did not affect CD11b levels. Cholesterol, which is known to stimulate the expression of monocyte CCR2 (29), was ruled out as the active component, and increasing monocyte cholesterol levels by incubation with free cholesterol did not induce CD11b expression. Finally, a previous report demonstrated that the expression levels of β2 integrins, including CD11b, were identical in monocytes that were isolated either from patients with homozygous familial hypercholesterolemia or from normal controls (46). Monocyte CD11b expression remained unchanged despite high plasma LDL levels, most likely because the known functional defects of the LDL receptor in these patients prevented adequate lipoprotein binding and LDL receptor signaling. These results agree with our observations suggesting that stimulation of CD11b expression by LDL involves the LDL receptor and LDL receptor-mediated activation of signaling pathways.

A signaling role for LDL and its receptor has been demonstrated in a number of cell models, including all cellular constituents of the vessel wall. In smooth muscle cells and endothelial cells, LDL induced the phosphoinositide-dependent signaling pathway leading to an increase in cytosolic-free calcium through pertussis toxin-sensitive GTP-binding protein (47–49). In our study, monocytes responded similarly, and LDL induced a rapid and transient increase of the $[Ca^{2+}]_i$. Although the exact mechanisms remain unclear, the concomitant increase of CD11b expression suggests an association between these two events. A causal link is further indicated by our inhibition studies, in which TMB-8, an antagonist of the IP_3 receptor, proved to be a very potent inhibitor of both calcium release and CD11b expression.

For the sustained interaction of mononuclear cells with the endothelium, not only surface expression but also activation of the integrins present on the cell surface are necessary (9, 10). Chemokines are important modulators of integrin adhesiveness, and MCP-1 represents a powerful trigger for firm adhesion of monocytes to the vascular endothelium (50). Our findings indicate that, although LDL can stimulate the surface expression of CD11b, it does not directly activate the integrin. For firm adhesion, the engagement of chemokine receptors, including CCR2, and intact chemokine-to-integrin signaling are required.

In addition to the effect on integrin activation, chemokines may also contribute to monocyte adhesion by increasing the integrin surface expression. A previous study showed that stimulation of freshly isolated human monocytes with MCP-1 transiently increased the number of CD11b molecules on the cell surface (31). A detailed analysis of the mechanism performed by the same group demonstrated that up-regulation of CD11b is associated with mobilization from intracellular pools rather than gene transcription.

We found that the MCP-1-stimulated surface expression of CD11b was particularly pronounced in THP-1 cells that were treated with LDL. From our recent work, we know that these conditions also stimulate the expression of CCR2, and we hypothesize that elevated expression levels of CCR2 not only intensify the activation trigger for the integrins preexisting on the cell surface, but may also stimulate CD11b surface expression. Together with the direct effects of LDL on CD11b expression, these events may constitute a mechanistic basis for the accumulation of monocytes in the vessel wall associated with atherogenesis.

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