Oxidized LDL–Induced NF-κB Activation and Subsequent Expression of Proinflammatory Genes Are Defective in Monocyte-Derived Macrophages From CD36-Deficient Patients

Mohamed Janabi, Shizuya Yamashita, Ken-ichi Hirano, Naohiko Sakai, Hisatayo Hiraoka, Kengo Matsumoto, Zhongyan Zhang, Shuichi Nozaki, Yuji Matsuzawa

Abstract—CD36 is 1 of the class B scavenger receptor expressed on monocytes, monocyte-derived macrophages (Mφ), platelets, and adipocytes. In our previous studies, we reported that the uptake of oxidized low density lipoproteins (OxLDLs) is reduced by ∼50% in Mφ from CD36-deficient patients compared with that in control subjects. Recently, we have shown that CD36 is highly expressed in human atherosclerotic aorta. Possibilities have been raised that besides the wide distribution and multifunctional characteristics of CD36, this molecule may also be involved in the mediation of intracellular signaling. The aim of the present study was to elucidate the role of CD36 in cytokine secretion and to investigate the CD36-mediated intracellular signaling stimulated by OxLDL. On addition of OxLDL or thrombospordin-1, the Mφ from CD36-deficient patients secreted significantly less amounts of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) compared with those from controls. RNase protection assay with multiprobe template sets demonstrated that after incubation with OxLDL, the mRNAs of a variety of cytokines, including genes encoding IL-1Ra, IL-1β, IL-6, TNF-α and -β, and interferon (IFN)-γ and -β, were significantly lower in the Mφ of patients. The addition of antibody against CD36 attenuated this OxLDL-induced response in controls. We also observed a reduced response in nuclear factor-κB (NF-κB) activity in OxLDL-stimulated Mφ from CD36-deficient patients. Unlike OxLDL, stimulation by lipopolysaccharide induced an increase in NF-κB activity in Mφ from CD36-deficient patients, suggesting that lipopolysaccharide-mediated signaling was conserved. These results demonstrate that in addition to the reduced OxLDL uptake that we reported previously, CD36-deficient patients may also have an impaired response of OxLDL-induced NF-κB activation and subsequent cytokine expression. (Arterioscler Thromb Vasc Biol. 2000;20:1953-1960.)

Key Words: atherosclerosis ■ CD36 ■ monocyte-derived macrophages ■ oxidized LDL ■ cytokines

Hypercholesterolemia is 1 of the major and most extensively studied risk factors of atherosclerosis.1 Recent studies have contributed to our understanding of the role of oxidatively modified LDLs (OxLDLs) in the process of atherosclerosis. Furthermore, administration of antioxidants such as probucol inhibits the development of atherosclerosis in some animal models, which also supports the role of OxLDL in the pathogenesis of atherosclerosis.2–3 CD36 is 1 of the family members of class B scavenger receptors that are widely expressed in human tissues and that bind and take up OxLDLs. A variety of effects of this lipoprotein on different cell functions has been demonstrated, including the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β).4 Previous data have shown that scavenger receptors by function are competent signal transduction molecules, although individual receptors that mediate this effect have not been well defined.5 Moreover, much less is known about the effects of uptake of the various forms of modified LDL on the gene expression, synthesis, and secretion of molecules in human monocyte–derived macrophages (Mφ).

Several lines of evidence indicate that nuclear factor-κB (NF-κB) transcription factors may play an important role in atherosclerosis. Homodimeric or heterodimeric combinations of NF-κB family proteins, such as p50, p52, p65, c-Rel, and Rel B, mediate NF-κB activity. NF-κB is found in an inactive form (I-κB) in the cytoplasm. On stimulation, I-κB is phosphorylated, and thereby NF-κB is released from I-κB, is translocated to the nucleus, and binds to the promoter DNA.6 Activation of NF-κB by inflammatory stimuli has been demonstrated in Mφ, endothelial cells, smooth muscles cells, and T cells. All of these cell types have been reported to play a major role in atherogenesis and display a distinct pattern of gene expression in this condition.7 Recently, OxLDLs have been implicated in modulating the expression of several
cytokines, growth factors, and lipopolysaccharide (LPS)-induced molecules, which are regulated by NF-κB.³⁸ The secretion of TNF-α and IL-1β by isolated Mφ in normal subjects has been measured with contradicting results, but no such reports on human CD36–deficient Mφ have been available yet.

In view of the potential importance and unresolved issues regarding the role of human CD36 in cytokine expression, this study was designed to analyze the in vitro effect of OxLDL on the release of cytokines from CD36-deficient Mφ in comparison with that from control Mφ and to clarify the CD36-mediated intracellular signaling stimulated by OxLDL. To approach this problem, we used human Mφ obtained from CD36-deficient patients and CD36-positive control subjects and showed that CD36 is an important mediator for activation of NF-κB when stimulated with OxLDL.

Methods

Study Subjects

Blood was obtained from 6 healthy, unrelated, CD36-positive control subjects and 6 different unrelated, CD36-deficient patients, identified by screening with specific antibodies and immunofluorescent flow cytometry, who were matched for age and risk factors. Their monocytes as well as platelets lacked CD36 on cell surfaces (data not shown).

Materials

Thrombospondin-1 (TSP-1) and monoclonal antibody to human platelet TSP-1 used an ELISA that was obtained from Hematologic Technologies, Inc.

Lipoprotein Preparation

LDLs (d=1.019 to 1.063 g/mL) were isolated by sequential ultracentrifugation from the sera of 5 normolipidemic volunteers who had fasted overnight for 12 to 14 hours as described previously.⁰ The protein content of LDL was determined by the method of Lowry et al with bovine serum albumin (BSA) as a standard. This represents the native LDL used in the current study. Oxidation of LDL was performed in a cell-free system. In brief, native LDL was diluted to 1 mg of protein per milliliter with PBS and dialyzed at 4°C for 24 hours against PBS in the absence of EDTA. The LDL was sterilized by ultrafiltration through a 0.45-μm filter (Millipore), diluted to a concentration of 400 μg protein per milliliter with PBS, and incubated at 37°C in the presence of 5 μmol/L CuSO₄ for 8 hours unless otherwise specified. Oxidation of LDL was confirmed by agarose gel electrophoresis. The OxLDL was stored under nitrogen at 4°C and used within 48 hours. LDL was acetylated with repeated additions of acetic anhydride. Freshly prepared LDL, OxLDL, and acetylated LDL were used for each experiment. To assess the optimal secretion levels of cytokines in the presence or absence of OxLDL, time course experiments were performed. (Please see Figure I, published online and available at http.) These peak levels were evident after 6 hours of incubation.

Mφ Isolation and Culture

Mixed mononuclear cells from blood samples of fasting, healthy, CD36-positive volunteers and CD36-deficient patients were isolated by a density gradient centrifugation method and lymphocyte separation solution (Nacalai Tesque). Twenty milliliters of blood (anti-coagulated with 10 U/mL sodium heparin) was layered over 15 mL of Ficoll-Paque and centrifuged at 10 g for 30 minutes. The mixed mononuclear cell band was removed by aspiration, and the cells were washed twice with serum-free RPMI-1640 medium. The cells were resuspended and seeded at a density of 10⁵ in 10-cm cell-culture dishes (Primaria Labware, Becton Dickinson and Co) in serum-free medium with 0.1% low-endotoxin BSA. Monocytes were incubated for 2 hours at 37°C in 5% CO₂, and then adherent cells were washed twice to remove loosely adherent cells. All experiments were performed in serum-free medium supplemented with 0.1% BSA. These cells represented monocytes in an early stage of differentiation and therefore are referred to as monocyte-Mφ in the text. Freshly isolated mononuclear cells from whole blood were used in each experiment.

Immunofluorescent Flow Cytometry

FITC-conjugated (Sigma Chemical Co) mouse monoclonal antibodies against human CD36, OKM5 (Ortho Diagnostic Systems Inc) and FA6-152 (Cosmo Bio Inc), were used for immunofluorescent flow cytometric analysis. Mononuclear cells were isolated by density gradient centrifugation as described above. Mononuclear cells (2×10⁵) were incubated with IgG1 or FITC-conjugated OKM5 (final concentration 2.5 μg/mL) for 30 minutes at 4°C. Then the cells were washed twice with PBS and assayed by flow cytometry (FACScan, Becton Dickinson and Co). Data were analyzed by using the Cell Quest software program, and cell debris was excluded by appropriate 2-dimensional gating methods. Specific fluorescence intensity was calculated by subtracting FITC-IgG1 intensity from the mean fluorescent intensity of FITC–monoclonal antibodies.

Cytokine Analysis

ELISA was used for analysis of TNF-α and IL-1β levels. The assay is sensitive and reproducible and has been applied for the measurement of TNF-α and IL-1β in plasma and tissue culture. One milliliter of the medium was centrifuged for 5 minutes at 10⁴ rpm. An aliquot (200 μL) from 3 independent samples of the supernatant was then added to TNF-α and IL-1β immunoassay plates (Amersham). The plates were processed according to the manufacturer’s protocol and read within 30 minutes at 450 nm.

Preparation of Total Cellular RNA and Ribonuclease Protection Assay

For ribonuclease (RNase) protection assay experiments, the Mφ from CD36-deficient and control subjects were isolated as described above. The cells were incubated overnight in serum-free medium supplemented with 0.1% BSA, subsequently washed in Dulbecco’s PBS, and incubated for an additional 6 hours in the presence or absence of 40 μg/mL OxLDL in serum-free medium supplemented with 0.1% BSA. For neutralizing experiments, the Mφ from control subjects were stimulated with 40 μg/mL OxLDL in serum-free medium; human-specific anti-CD36 antibody FITC-OKM5 or non-specific mouse antibody FITC-IgG1 (final concentration 1 μg/mL) was included in each assay, and OxLDL without addition of antibodies served as a control. Total RNA was extracted with TRIzol reagent (GBCO). An antisense cRNA was transcribed by using T7 RNA polymerase in the presence of [³²P]UTP (3000 Ci/mmol, New England Nuclear). Five micrograms of total RNA from the CD36-deficient and control Mφ was hybridized with the probe at 56°C and incubated for 16 hours. Annealed products were digested with ribonuclease T1 at 37°C for 30 minutes. The protected fragments were precipitated and subjected to 6% polyacrylamide/urea gel. Results were analyzed by autoradiography and quantified by laser densitometric scanning (FUJI BAS 200).

Electrophoretic Mobility Shift Assay

Mφ were incubated with either 10 μg/mL bacterial LPS (Escherichia coli 0111:B4, Sigma) or 40 μg/mL OxLDL or in their absence in serum-free RPMI-1640 medium supplemented with 0.1% BSA. Nuclear extracts were prepared according to Mufson et al with the additional step of washing the nuclear pellets in low-salt buffer before high-salt buffer extraction of nuclear proteins to remove any residual cytosolic contamination. Protein concentration was measured by the method of Lowry et al. The NF-κB binding protein detection system (Life Technologies, Inc) was used according to the manufacturer’s instructions. In brief, end labeling of a double-stranded oligonucleotide containing 2 NF-κB–binding elements was performed by using [³²P]ATP and T4 polynucleotide kinase. Nuclear extracts (5 μg of protein) were incubated with 10⁴ counts per minute of the labeled oligonucleotide at room temperature for 20 minutes. The specificity of protein-DNA complexes was verified by competition assays with excess unlabeled oligonucleotides. Nuclear ex-
tracts from HeLa cells were used as a positive control. For supershift analysis, 2 μg of the indicated antibody, p50 or p65 (Santa Cruz Biotechnology), was incubated with nuclear extracts for 30 minutes before addition of the labeled probe. The samples were separated on a 6% non-denaturing polyacrylamide gel.

Statistical Analysis
Values are given as mean ± SD. Statistical difference between groups was performed with an unpaired t test. A value of \( P < 0.05 \) was considered significant.

Results
To assess the optimal secretion of TNF-α and IL-1β into the medium in the presence or absence of OxLDL, time course experiments were performed. The peak levels of TNF-α and IL-1β concentration were evident after 6 hours of incubation. The secretion of TNF-α and IL-1β declined progressively thereafter, and by 24 hours after stimulation, secretion had returned to baseline levels. To establish the range of concentrations over which lipoproteins are effective, we performed a dose titration of lipoprotein-induced cytokine expression. OxLDL stimulated Mφ in a dose-dependent manner, with a maximal effect at a concentration of 40 μg/mL (data not shown).

To address the relevance of CD36 in the release of cytokines into the medium and mRNA expression in Mφ exposed to OxLDL, secretion of these cytokines was analyzed by ELISA with the use of conditioned medium from cultured Mφ. The secretion of TNF-α from the control Mφ treated with 40 μg/mL OxLDL almost doubled after 6 hours of incubation, whereas that of CD36-deficient Mφ was remarkably low \( (P < 0.01; \text{Figure 1A}) \). Furthermore, the IL-1β levels in conditioned medium from the CD36-deficient Mφ after the addition of 40 μg/mL OxLDL were also significantly lower than those from the control Mφ \( (P < 0.01; \text{Figure 1B}) \). There was no significant difference in the amounts of TNF-α and IL-1β released from the unstimulated Mφ from CD36-deficient and normal control subjects 6 hours after replacement of the medium (Figures 1A and 1B). By contrast, acetylated LDL and native LDL under the same experimental condition had no significant effect on TNF-α and IL-1β secretion in both groups (data not shown). The close correspondence between TNF-α and IL-1β values in both stimulated and unstimulated conditioned media of Mφ from the controls and patients suggests a common mechanism in releasing these cytokines. To exclude the possibility of the effect of bacterial LPS as a potent inducer of cytokine release from Mφ, the concentrations of LPS were measured in all preparations. When the lipoprotein preparations contained >0.006 ng/mL LPS, they were not used in the study.

We further showed that the expression of CD36 was mandatory for mediating the enhanced secretion of cytokines into the conditioned medium by coincubating the control Mφ stimulated by OxLDL with anti-human CD36 monoclonal antibody OKM5 or nonspecific mouse IgG1 (final concentration 1 μg/mL). The Mφ incubated with OKM5 showed a significant reduction of TNF-α and IL-1β secretion, whereas those incubated with mouse IgG1 had no significant change \( (P < 0.05 \) and \( P < 0.01 \), respectively; Figures 2A and 2B).

To determine the effect of another CD36 ligand, TSP-1, on cytokine release, we cultured Mφ from the controls and CD36-deficient subjects in the absence or presence of varying concentrations of TSP-1 for 2 hours before collection of the conditioned media. Incubation with TSP-1 for 2 hours was chosen on the basis of previous reports.\(^\text{13}\) The secretion of TNF-α from the control Mφ treated with TSP-1 (concentrations of 0.4 to 1 μg/mL) was significantly higher than that from the CD36-deficient Mφ. (Please see Figure II, available online at http.) When control Mφ were cultured in the presence of neutralizing antibodies against human CD36,
OKM5, or TSP-1, the activation of TNF-α was significantly abrogated (data not shown).

Next we investigated whether the induced release of TNF-α and IL-1β secretion from OxLDL-stimulated, monocyte-derived Mφ. To determine whether CD36 was involved in mediating the OxLDL-induced upregulation of TNF-α and IL-1β secretion into the medium, freshly prepared monocyte-Mφ from control subjects were stimulated with 40 μg/mL OxLDL and incubated at 37°C with the specific anti-human CD36 monoclonal antibody FITC-OKM5 or the nonspecific mouse antibody FITC-IgG1. A, TNF-α, B, IL-1β. Serum-free medium served as the control. Similar results were obtained in 4 independent experiments. All experiments were performed in triplicate. Error bars represent mean±SD. Brackets indicate statistically significant differences between groups as shown and their P values.

Figure 2. Effect of CD36 antibodies (OKM5) on TNF-α and IL-1β secretion from OxLDL-stimulated, monocyte-derived Mφ. To determine whether CD36 was involved in mediating the OxLDL-induced upregulation of TNF-α and IL-1β secretion into the medium, freshly prepared monocyte-Mφ from control subjects were stimulated with 40 μg/mL OxLDL and incubated at 37°C with the specific anti-human CD36 monoclonal antibody FITC-OKM5 or the nonspecific mouse antibody FITC-IgG1. A, TNF-α, B, IL-1β. Serum-free medium served as the control. Similar results were obtained in 4 independent experiments. All experiments were performed in triplicate. Error bars represent mean±SD. Brackets indicate statistically significant differences between groups as shown and their P values.

Figure 3. Effect of Ox-LDL on expression of cytokine mRNAs: analysis of a variety of cytokine mRNAs from CD36-deficient and CD36-positive control Mφ stimulated with 40 μg/mL OxLDL in serum-free medium. Total RNA (5 μg/lane) was analyzed. An antisense cRNA was transcribed by using T7 RNA polymerase in the presence of [32P]UTP. Autoradiograms from this analysis are shown in A and B. A, Controls (lanes 1 through 3) and CD36-deficient subjects (lanes 4 through 6). B, The human cytokine (hCK)-2 multiprobe set templates not treated with RNases (lane 1), control (lane 2), and CD36-deficient subjects (lanes 3 through 5). L32 = housekeeping gene. Samples were derived from different, unrelated, CD36-deficient and CD36-positive subjects. Total RNA per lane was normalized by comparison with total RNA hybridized with a human (h) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Similar results were obtained in 3 independent experiments.

Cytokines were significantly lower from CD36-deficient patients than those from control Mφ (Figures 3A and 3B). These observations were highly reproducible in repeated experiments. To determine whether changes in mRNA levels can be ascribed, at least in part, to the difference in CD36
Interestingly, when the control Mφes were incubated with either the anti-CD36 antibody OKM5 or IgG1. 

Expression, control Mφes stimulated with OxLDL were incubated with either the anti-CD36 antibody OKM5 or IgG1. Interestingly, when the control Mφes stimulated with OxLDL were treated with OKM5, the intensity of the TNF-α-retracted band was significantly diminished. By contrast, when incubated with nonspecific mouse IgG1, no significant change in the intensity of the OxLDL-enhanced band was noted (Figure 4). A similar result was observed with IL-1β (data not shown). This finding suggests that the expression of CD36 might be a key factor in the release of these cytokine mRNAs after stimulation with OxLDL. Resting Mφes expressed little to no mRNA of human TNF-α and human IL-1β in both control and CD36-deficient subjects (data not shown).

Because NF-κB is involved in the control of a variety of genes that are activated on inflammation, NF-κB may play a central role in atherogenesis. Other reports have shown that a rapid activation of NF-κB involving dissociation from its inhibitor and translocation to the nucleus occurs in response to a variety of agents that include OxLDL, LPS, and cytokines. To further search for the role of CD36 in NF-κB activation, we used nuclear proteins harvested from the Mφes of unrelated CD36-deficient and control subjects, incubated with or without either OxLDL or LPS, and explored the possibility that activation of NF-κB represents an important step in the regulation of cytokine expression by an electrophoretic mobility shift assay (EMSA) with a radiolabeled oligonucleotide containing the NF-κB binding sequence. The EMSA revealed that NF-κB activities were increased after incubation with OxLDL in the control Mφes, whereas the Mφes from CD36-deficient patients showed a defective response to OxLDL. Interestingly, the addition of bacterial LPS to the medium induced a significant stimulative response in Mφes from both the controls and patients, suggesting that LPS-mediated signaling was conserved in the patients’ Mφes. The EMSA reaction specificity was verified by competition assays with a 20-fold excess of unlabeled oligonucleotide, which resulted in the disappearance of the bands established by OxLDL in control Mφes (Figure 5A). To confirm the specificity of the protein-DNA interaction, we performed a gel supershift assay with antibodies for the p50 and p65 subunits of NF-κB. When incubated with the nuclear extracts, a specific DNA binding complex of NF-κB interacted with antisense p65 (Figure 5B). Thus, NF-κB complex was likely to contain dominantly homodimers of p65. We took a step further to determine whether CD36 was directly mediating the OxLDL-enhanced NF-κB DNA binding activity by coincubating OxLDL-treated Mφes with human-specific anti-CD36 OKM5 (1 μg/mL) or mouse IgG. When incubated with OKM5, the intensity of the gel-retarded band was significantly diminished, although a degree of activation was observed. In contrast, the Mφs incubated with control IgG, no change in the intensity of the OxLDL-enhanced band was observed (Figure 5C). Although the
precise molecular mechanism involving NF-κB–OxLDL activation has yet to be fully established, the principal effects seem to involve CD36. These findings confirm previous reports implicating the involvement of CD36 in OxLDL-induced NF-κB activation and hence, cytokine regulation.

Discussion
This study examined the role of CD36 in cytokine secretion by using Mφ from CD36-deficient patients and CD36-positive control individuals. We also evaluated the CD36-mediated intracellular signaling stimulated by OxLDL. CD36 is a cell surface receptor that has been shown to interact with a variety of ligands, including TSP, collagen, Plasmodium falciparum–infected erythrocytes, apoptotic neutrophils, modified LDLs, anionic phospholipids, and long-chain fatty acids. A number of these CD36 ligands elicit the transduction of intracellular signals involved in cell activation and internalization of bound ligands.16,17 Recently, we have reported that this molecule is expressed on Mφ of atherosclerotic lesions from human aorta.18 Furthermore, we demonstrated that in monocyte-derived Mφ from CD36-deficient patients, the uptake of OxLDL was reduced by about 50% compared with that in control Mφ, suggesting that CD36 may also play a significant role in OxLDL uptake.19 The present findings provide additional insight that in monocyte-derived Mφ from CD36-deficient subjects, neither TNF-α nor IL-1β secretion into the medium was significantly induced by OxLDL. Furthermore, CD36-deficient Mφ stimulated with OxLDL had significantly reduced levels of a variety of cytokine mRNAs, including genes encoding IL-1Ra, IL-1β, IL-6, TNF-α and -β, and IFN-1γ and -β, in comparison with normal control Mφ.

Our assumption that CD36 plays a principal role in the release of cytokines was reinforced by the fact that the Mφ from CD36-deficient subjects stimulated by TSP-1, a natural ligand for CD36, also secreted a lesser amount of TNF-α into the culture medium in comparison with controls. (Please see Figure II, available online at http.) Further confirmation that the association of OxLDL or TSP-1 with CD36 on Mφ was vital in the activation of TNF-α and IL-1β was the finding that when OKM5 or TSP-1 antibody was added to the medium in the presence of TSP-1, there was significant attenuation of the effects of TSP-1 in activating Mφ to release TNF-α or IL-1β (data not shown). These findings led us to seriously consider the possibility that activation occurs at the cell surface of activated Mφ and requires the expression of CD36.

Our data show that CD36-deficient patients have significantly lower levels of CD36 mRNA expression and release of TNF-α and IL-1β in response to OxLDL in comparison with control subjects; however, CD36 OxLDL-dependent induction of TNF-α and IL-1β release into the medium and mRNA expression were in part inhibited by incubation of control Mφ with the human CD36–specific antibody OKM5 (Figures 2 and 4). The high levels of mRNA seen in the control Mφ indicate that these cells were in an activated state. Previous studies have shown that monocyte adhesion is associated with the activation of cytokine mRNA production.20 Hence, in the intima, Mφ will release cytokines on their adhesion to the extracellular matrix, and this might be further induced if Mφ are exposed to OxLDL trapped within the matrix. We have observed that the Mφ obtained from CD36-deficient patients adhere poorly to collagen-coated culture dishes (M.J. et al, unpublished observations, 2000). Furthermore, OxLDL significantly enhanced NF-κB–like activity in nuclear extracts from controls, whereas no significant activation was observed in the patients’ nuclear extracts. The activation of NF-κB DNA binding activity was manifested, at least in part, by the p65 subunit, which was detected with a supershift gel assay. Besides p65 and p50, other proteins may bind NF-κB target DNA, whose electrophoretic mobility would not be affected by p65 or p50 subunit–specific antibody. This concept supports our earlier studies that OxLDL itself upregulates the expression of CD36 in human Mφ and that this action occurs at the transcription level.21

We observed some degree of induction of NF-κB in the presence of CD36 antibody (Figure 5C), but the mechanism of induction was not very clear. There are several possible explanations for this phenomenon: (1) In this series of experiments, Mφ were coincubated for 6 hours in the culture medium in the presence of OKM5. The incubation for 6 hours was possibly sufficient for antibody-bound CD36 to internalize and for de novo synthesis of CD36. (2) The role of other receptors such as scavenger receptor type A was not accounted for in the present series of studies. (3) Presumably sequestration of TNF-α and IL-1β occurs on the cell surface via CD36; hence, the presence of antibody may lead to the displacement of cytokines from the cell surface and proceed to the activation of transcription factor NF-κB. (4) The interaction of CD36 with its specific antibodies has been reported to lead to an oxidative burst in Mφ.22 Studies to determine whether any of these events result in activation of NF-κB after stimulation of CD36 by OxLDL are underway.

Above all, the low LPS concentrations in the test samples led us to speculate that the Mφ activation observed in the present study was possibly in part mediated through CD36 and could not be attributed to LPS contamination, suggesting a distinct signaling pathway and the growing influence that CD36, as an individual receptor, has on the process of foam cell formation. However, in a separate series of experiments, stimulation of Mφ by LPS led to an increase in NF-κB activity in both controls and patients, suggesting that LPS-mediated signaling was conserved in the patients’ Mφ, most likely via CD14.23 These results indicate that the LPS-dependent activation pathway of cytokine secretion is different from that of the CD36-dependent activation pathway.

We did not evaluate specific CD36 features required for the OxLDL-mediated enhancement of NF-κB DNA binding activity. However, it has been shown that OxLDL-mediated enhancement of NF-κB DNA binding activity after activation of CD36-producing Chinese hamster ovary cells required an intact carboxyl-terminal cytoplasmic segment of CD36.24 Furthermore, Yehualaeishet al25 showed that activation of rat alveolar Mφ transforming growth factor-β1 required interaction of CD36 ligands and its cell surface receptor, CD36. We are therefore confident that the OxLDL activation of NF-κB binding in Mφ observed herein required expression of CD36. This was further confirmed by the finding that NF-κB activity was inhibited in part by coincubation of Mφ with OKM5, suggesting a direct effect of OxLDL on CD36 but not on other receptors.
The physiological function of CD36 has not been well characterized, although this molecule has been proposed to mediate cytoadherence of *P. falciparum*–parasitized erythrocytes. It will be important to know whether such adherence could also transduce a transmembrane signal that is able to modulate transcription factor(s) in a similar way to what we observed here. Tontonoz et al.\(^\text{26}\) showed that expression of CD36 was directly induced by the transcription factor peroxisome proliferator–activated receptor-\(\gamma\), which has a role in atherogenesis, adipogenesis, and insulin signaling. Furthermore, CD36 functions in a feedback loop as a receptor for OxLDL, in which the lipid components of OxLDL activate peroxisome proliferator–activated receptor-\(\gamma\), thereby inducing CD36 expression. Therefore, it will also be of great interest to determine whether this link is involved in CD36-mediated signal transduction by OxLDL, hence modulating NF-\(\kappa B\) activity and leading to cytokine regulation.

We and others have extensively clarified the pathophysiological significance of CD36 as a major receptor for OxLDL. Since we discovered the CD36-deficient subjects, we have identified 3 types of gene mutations.\(^\text{19}\) Some of our CD36-deficient patients in this study have not shown any clinical symptoms of coronary artery disease (CAD), while others have signs and symptoms of it. It remains an interesting question whether such low levels of cytokines are protective against atherosclerosis and/or whether they delay the process of atheroma formation. Previous reports have shown evidence that the circulating levels of cytokines, under different conditions, can exhibit favorable or unfavorable effects. The unfavorable effects may occur when concentrations are high or when low concentrations persist for longer periods.\(^\text{27}\)

Recently, we have found that the human CD36 deficiency is relatively frequent in the Japanese general population and appears proatherogenic in association with the clustering of multiple risk factors. We analyzed 26 patients with type 1 CD36 deficiency and clarified the clinical characteristics of these patients, especially with respect to atherogenicity. These patients had multiple risk factors for CAD, such as hyperlipidemia, high blood pressure, and insulin resistance. Furthermore, we found that the frequency of type 1 CD36 deficiency was 3-fold higher (0.9%) in patients with CAD than in the general population (0.3%; K. Miyaoka, personal communication, 1999).

In addition, it has recently been reported that \(\approx 17\%\) of Japanese patients with CAD have CD36 deficiency, suggesting a significant relative risk attributable to this genetic defect.\(^\text{28}\) Furthermore, Hwang et al.\(^\text{28}\) showed that of 7 patients with CD36 deficiency who underwent biochemical tests, 3 had type 2 diabetes (1 with insulin resistance and 1 with hypertriglyceridemia), 1 had hypertension, and 1 had CAD. In summary, the biological plausibility of CD36 and the phenotype of patients with CD36 deficiency suggest that CD36 may play an important part in the pathogenesis of CAD and the human insulin resistance syndrome. Taken together, this study demonstrates that in addition to reduced OxLDL uptake, CD36-mediated intracellular signaling is altered in CD36-deficient patients, leading to a defective response of OxLDL-induced NF-\(\kappa B\) activation and subsequent cytokine secretion.

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Secretion of TNF-α and IL-1β from monocyte-derived macrophages into the medium.

For ELISA analysis of TNF-α and IL-1β levels in the medium, an aliquot (200 μl) from the conditioned medium that had been harvested after culturing Mφ was added to high-sensitivity human TNF-α and IL-1β immunoassay plates and processed according to the manufacturer's protocol. Time course experiments in the presence or absence of OxlDL.
Activation of monocyte-derived macrophages by thrombospondin-1.

For ELISA analysis of TNF-α levels in the medium, an aliquot (200 μl) from the conditioned medium that had been harvested after culturing Mφ 2 h in the presence or absence of human platelet thrombospondin-1 of varying concentrations was added to high-sensitivity human TNF-α immunoassay plates and processed according to the manufacturer's protocol. All experiments were performed in triplicate. NS denotes no significance and error bars represent the mean ± S.D. Similar results were obtained in three independent experiments. Error bars represent the mean ± S.D.