# Reduced Uptake of Oxidized Low Density Lipoproteins in Monocyte-derived Macrophages from CD36-deficient Subjects

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#### **Abstract**

To clarify the physiological roles of CD36 as an oxidized low density lipoprotein (OxLDL) receptor, we analyzed the monocyte-derived macrophages from normal and two CD36-deficient subjects, since we identified the molecular abnormalities (Kashiwagi, H., Y. Tomiyama, Y. Kosugi, M. Shiraga, R. H. Lipsky, Y. Kanayama, Y. Kurata, and Y. Matsuzawa. 1994. Blood. 83:3545-3552; and Kashiwagi, H., Y. Tomiyama, S. Honda, S. Kosugi, M. Shiraga, N. Nagao, S. Sekiguchi, Y. Kanayama, Y. Kurata, and Y. Matsuzawa. 1995. J. Clin. Invest. 95:1040-1046). Scatchard analysis of 125I-OxLDL binding showed a linear plot and the maximum binding was lower by  $\sim 40\%$  in the macrophages from subjects with CD36 deficiency than those from normal controls. Competition studies showed that the uptake of 125I-OxLDL was suppressed by OKM5, an antibody against CD36, by 53% in normal control macrophages, but not in the CD36deficient macrophages. After incubation with OxLDL for 24 h, cholesteryl ester mass accumulation was reduced by  $\sim 40\%$  in the macrophages from CD36-deficient subjects than those from normal controls. These results suggest that CD36 is one of the physiological receptors for OxLDL. Since specific binding of OxLDL was only reduced by  $\sim 40\%$ in spite of the complete deficiency of CD36, several other receptors also may have some role in OxLDL uptake. Further studies will be needed to assess the quantitative role of CD36 in foam cell formation in vivo. (J. Clin. Invest. 1995. 96:1859-1865.) Key words: macrophage • oxidized LDL receptor • CD36 deficiency • acetyl LDL receptor • foam cell formation

## Introduction

Foam cell formation is a characteristic feature of atherosclerotic lesions. Previous studies suggested that macrophages play an

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important role in foam cell formation (1-4). Goldstein et al. first reported that LDL modified chemically by acetylation is incorporated into macrophages through scavenger receptors, resulting in the formation of lipid-laden foam cells (5). Kodama et al. identified one of the scavenger receptors by using acetyl-LDL (AcLDL)<sup>1</sup> as a ligand (6, 7). However, it seems unlikely that acetylation of LDL would occur in vivo. Steinberg et al. found that the incubation of LDL with endothelial cells produced modified LDL, which was later found to be composed of oxidized LDL (OxLDL) (8, 9). OxLDL is now considered to be one of the modified LDLs that may exist in vivo. A number of recent studies have established the role of OxLDL in atherogenesis (10-12). OxLDL was reported to bind to the AcLDL receptor, while several investigators suggested that the OxLDL receptors other than AcLDL receptor found by Kodama et al. may be present (13-15).

CD36 is a glycoprotein of 88 kD and is expressed on platelets, monocytes, and capillary endothelial cells (16). The physiological function of CD36 has not been well characterized although it was proposed to be a receptor for both thrombospondin and collagen or to mediate cytoadherence of plasmodium falciparum parasitized erythrocytes (17–20). From the research on the subjects refractory to HLA-matched platelets transfusion (21-24), Kashiwagi et al. have identified the subjects whose platelets and monocytes lack CD36, and clarified three types of gene abnormalities (substitution of Pro<sup>90</sup> by Ser, deletion of two bases in exon 5, and insertion of one base in exon 10; Kashiwagi, H., Y. Tomiyama, S. Nozaki, S. Honda, H. Mizutani, S. Kosugi, M. Shiraga, N. Nagao, Y. Kanakura, Y. Kurata, and Y. Matsuzawa, manuscript in submission) (25, 26). All these abnormalities result in the absence of CD36 expression on the cell surface. Recently, Endemann et al. reported (27) that CD36 may be a novel receptor for OxLDL based on the results of experiments using CD36-transfected cells. In the current study, we aimed to clarify the physiological role of CD36 as an oxidized LDL receptor by analyzing the binding capacity of monocyte-derived macrophages from subjects with CD36 deficiency in comparison with those from normal controls. We demonstrate that the uptake of OxLDL is markedly reduced in macrophages from CD36-deficient subjects and that CD36 may be one of the physiological receptors for OxLDL in vivo.

#### **Methods**

Subjects. Two female subjects (case 1 and case 2) with anti-Nak<sup>a</sup> antibodies were identified by screening with platelet-specific antibodies

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<sup>1.</sup> Abbreviations used in this paper: AcLDL, acetylated LDL; CE, cholesteryl ester; OxLDL, oxidized LDL.

at the Osaka Red Cross Blood Center and the Department of Blood Transfusion at Kinki University Hospital (Osaka, Japan). Case 1 is 50-yr-old and case 2 39-yr-old, respectively. They are apparently healthy and have neither suffered from coronary heart disease nor presented any symptoms of easy bleeding. They do not have any family history of coronary heart disease or other atherosclerotic cardiovascular diseases. Immunofluorescent flow cytometry demonstrated that their platelets and monocytes lacked cell surface CD36 (see Fig. 1). Their gene abnormalities were the same and consisted of compound heterozygosity for a two-base deletion in exon 5 and one base insertion in exon 10 (Kashiwagi, H., Y. Tomiyama, S. Nozaki, S. Honda, H. Mizutani, S. Kosugi, M. Shiraga, N. Nagao, Y. Kanakura, Y. Kurata, and Y. Matsuzawa, manuscript in submission) (26).

Immunofluorescent flow cytometry. Immunofluorescent flow cytometric analysis was performed by using a mouse monoclonal antibody against CD36, (OKM5); (Ortho Diagnostic System Inc., Raritan, NJ) (24). Mononuclear cells were isolated by density gradient centrifugation of heparinized peripheral blood using Ficoll-paque. 20 ml of blood (anticoagulated with 10 U/ml heparin) was layered over 10 ml of Ficoll-paque and centrifuged at 1,000 g for 30 min. Mononuclear cells (2  $\times$  10<sup>5</sup> in 100  $\mu$ l) were incubated with FITC-conjugated OKM5 (final concentration: 2.5  $\mu$ g/ml) or FITC-conjugated mouse IgG1 (final concentration: 2.5  $\mu$ g/ml) for 30 min at 4°C and assayed on a FACScan® (Becton Dickinson and Co., Mountain View, CA) as previously reported (24). Appropriate cell fractions for the analysis of monocytes were selected by a gating method with a two-dimensional display of forward scatter and side scatter of analyzed cells.

Isolation of human monocyte-derived macrophage. Mononuclear cells were isolated as described above. Mononuclear cells were plated at a density of  $2\times10^6$  cells in a 16-mm well (Primaria brand, Falcon Labware; Becton, Dickinson and Co.) in RPMI-1640 containing 10% (vol/vol) human type-AB serum. After 4 h of incubation at 37°C in 5% CO<sub>2</sub>, nonadherent cells were removed. Monocyte-derived macrophages cultured for 12–14 d were used for the assays.

Lipoproteins. Human LDL (density, 1.019-1.063 g/ml) was isolated by ultracentrifugation from the plasma of healthy volunteers as previously reported (28). LDL was acetylated with repeated additions of acetic anhydride (5, 29). Oxidized LDL was prepared by incubating LDL with 5  $\mu$ M CuSO<sub>4</sub> for 24 h. These lipoproteins were radioiodinated using the iodine monochloride method as previously reported (30, 31). OxLDL was also conjugated with FITC according to the method previously reported by Tomiyama et al. (32). In brief, OxLDL solution (1.7 mg/ml) was dialyzed against carbonate buffer (0.16 M Na<sub>2</sub>CO<sub>3</sub>, 0.33 M NaHCO<sub>3</sub>, pH 9.5). FITC (Sigma Chemical Co., St. Louis, MO) was dissolved with dimethylsulfoxide to give a concentration of 10 mg/ml. 200  $\mu$ g FITC per milligram OxLDL protein was added to the OxLDL solution. The mixture was incubated for 4 h with stirring in the dark at room temperature, and then dialyzed against PBS overnight.

Assays for binding, cell association, and degradation of OxLDL. Binding assays of 125I-OxLDL in macrophages from normal and CD36deficient subjects were performed at 4°C for 1 h. After washing with PBS three times, the cells were dissolved in 1.0 ml of 0.1 N NaOH and the radioactivity was measured by a gamma counter as binding of lipoprotein. The protein concentration of the dissolved aliquot of macrophages was determined by the method of Lowry et al. (33). Cell association and degradation of 125I-OxLDL in macrophages from normal and CD36-deficient subjects were also determined after 4 h of incubation at 37°C with 125I-OxLDL and 40-fold excess amounts of unlabeled lipoproteins. After washing with PBS three times, the cells were dissolved in 1.0 ml of 0.1 N NaOH and the radioactivity was measured as cell association of lipoproteins. Lipoprotein degradation was quantitated by measurement of trichloroacetic acid-soluble noniodide radioactivity in the medium after incubation (34). For degradation analysis, noncell degradation was subtracted from the data.

Assay of cellular cholesteryl ester (CE) content in macrophages. Macrophages were cultured for 12 d. On the 13th day AcLDL (50  $\mu$ g/ml) or OxLDL (50  $\mu$ g/ml) was added to the medium and the cells were

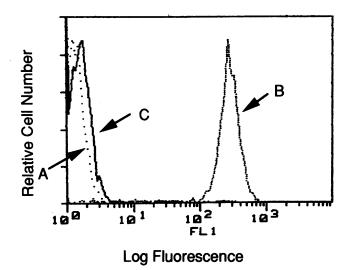


Figure 1. Flow cytometric analysis for surface expression of CD36 on monocytes from a CD36-deficient subject and a normal control. (A) Control monocytes incubated with control FITC-conjugated IgG1. (B) Control monocytes incubated with FITC-conjugated OKM5. (C) CD36-deficient monocytes incubated with FITC-conjugated OKM5.

incubated for 24 h. The incubation medium was then discarded and the cellular lipids were extracted by incubating the cells with hexane/ isopropanol (3:2, vol/vol) for 30 min at room temperature. The cholesterol content of the supernatant was determined by the enzymatic, fluorometric method by Heider and Boyett with minor modifications (35, 36). Briefly, the lipid extract was dried under nitrogen and dissolved in 180  $\mu$ l of isopropanol. 30  $\mu$ l of the supernatant was then added to 0.4 ml of an enzyme mixture containing 0.16 U/ml cholesterol oxidase and 60 U/ml cholesterol esterase. The reaction mixtures for measuring free cholesterol and those for total cholesterol were incubated at 37°C for 1 and 2 h, respectively, followed by the addition of 0.81 ml of 0.5 N NaOH to terminate the reaction. Fluorescence intensity was measured with excitation at 310 nm and emission at 407 nm. The mass of CE was calculated by subtracting free cholesterol from total cholesterol. After lipid extraction, the cellular protein was dissolved in 1.0 ml of 0.1 N NaOH and the protein concentration was determined by the method of Lowry et al.

#### **Results**

Fig. 1 shows a representative pattern of the flow cytometric analysis for surface expression of CD36 on monocytes. OKM5, an antibody against CD36, bound to the monocytes obtained from control subjects, while it did not bind to the monocytes from CD36-deficient subjects (Fig. 1). Expression of CD36 on monocyte-derived macrophages was investigated by an immunohistochemical analysis using FITC-conjugated OKM5. As shown in Fig. 2, expression of CD36 was demonstrated only in control monocyte-derived macrophages incubated with FITCconjugated OKM5. No immunofluorescence was detected on the macrophages from CD36-deficient subjects. Then, the binding of FITC-conjugated OxLDL to CD36 deficient and control monocytes was analyzed by FACScan®. The binding capacity of OxLDL to CD36-deficient monocytes was reduced and  $\sim 23\%$  of that to control monocytes when it was calculated as a mean fluorescence intensity.

By using macrophages, we studied the binding, cell associa-

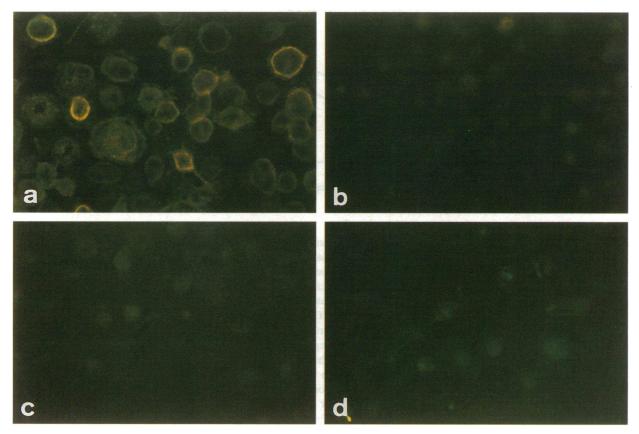


Figure 2. Fluorescent micrographs of expression of CD36 in normal and CD36-deficient monocyte-derived macrophages. Isolated monocytes were cultured in RPMI-1640 supplemented with 10% human type-AB serum on an 8-well polystyrene chamber slide (Lab-Tek; Nunc, Inc., Naperville, IL) for 12 d. Cultured monocyte-derived macrophages were washed in 0.01 M PBS containing 1% BSA for 5 min at 4°C, and incubated with FITC-conjugated mouse monoclonal antibody to CD36 (OKM5) or FITC-conjugated normal mouse IgG1 for the negative control for 30 min at 4°C. After fixation with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer for 5 min at 4°C, the specimens were mounted with aqueous mounting medium (Perma Fluor; IMMUNON, Pittsburgh, PA). (a) Control monocyte-derived macrophages incubated with FITC-conjugated CoKM5. (b) Control monocyte-derived macrophages incubated with FITC-conjugated control IgG1. (c) CD36 deficient monocyte-derived macrophages incubated with FITC-conjugated control IgG1.

tion, and degradation of Ox-LDL. Fig. 3 shows representative data of specific binding of  $^{125}$ I-OxLDL to macrophages. Scatchard analysis showed that the maximum binding of  $^{125}$ I-OxLDL to macrophages from CD36-deficient subjects was  $\sim 40\%$ 

lower than that from controls. The  $K_d$  was almost the same in both monocyte-derived macrophages (11.3  $\mu$ g/ml for CD36 deficiency and 7.4  $\mu$ g/ml for the control, respectively). As shown in Fig. 4, both cell association and degradation of

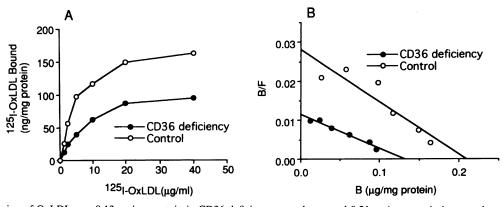
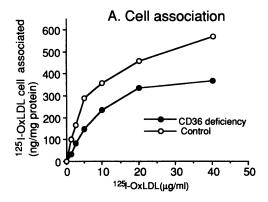


Figure 3. Binding of <sup>125</sup>I-OxLDL to normal and CD36-deficient macrophages (A) and Scatchard analysis of specific binding of <sup>125</sup>I-OxLDL to macrophages (B). Vertical bar in A shows the specific binding activities of <sup>125</sup>I-OxLDL to normal and CD36-deficient macrophages. CD36-deficient and normal control macrophages were incubated with indicated concentrations of <sup>125</sup>I-OxLDL for 1 h at 4°C in triplicate in the presence or absence of a 40-fold excess of unlabeled OxLDL. Maximum bind-

ing of OxLDL was  $0.13~\mu$ g/mg protein in CD36-deficient macrophages and  $0.21~\mu$ g/mg protein in control macrophages, respectively. The specific activities were calculated by subtracting the values obtained in the presence of an excess of unlabeled OxLDL from the values obtained in the absence of unlabeled OxLDL.



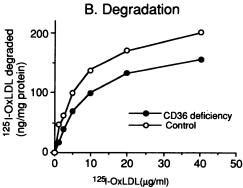
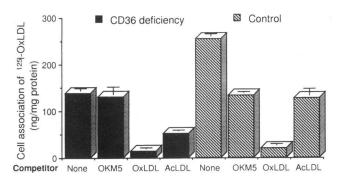


Figure 4. Cell association (A) and degradation (B) of <sup>125</sup>I-OxLDL to normal and CD36-deficient macrophages. Vertical bars in A and B showed specific cell association and degradation of OxLDL to normal and CD36-deficient macrophages. CD36-deficient and normal control macrophages were incubated with indicated concentrations of <sup>125</sup>I-OxLDL for 4 h at 37°C in triplicate in the presence or absence of a 40-fold excess of unlabeled OxLDL.

OxLDL in CD36-deficient macrophages were also lower than those in controls. These results demonstrated that monocytes and monocyte-derived macrophages from CD36-deficient subjects possessed a lesser capacity to bind or degrade OxLDL than those from control subjects.

Next, we studied the effects of two types of nonradiolabeled modified LDLs, OxLDL and AcLDL, on the cell association and degradation of OxLDL in CD36-deficient and control macrophages (Fig. 5). The effect of OKM5 was also studied. When



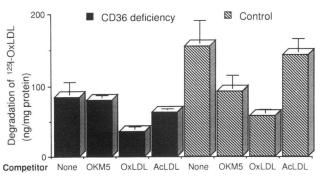


Figure 5. Effect of OKM5, unlabeled OxLDL, and unlabeled AcLDL on the cell association (top) and degradation (bottom) of  $^{125}\text{I-OxLDL}$  in macrophages from subjects with CD36 deficiency and normal subjects. OKM5 (4  $\mu$ g/ml), OxLDL (40-fold excess; 200  $\mu$ g/ml), and AcLDL (40-fold excess; 200  $\mu$ g/ml) were added to the incubation medium containing 5  $\mu$ g/ml of  $^{125}\text{I-OxLDL}$ . Cells were incubated for 4 h at 37°C and cell association and degradation were measured as described in Methods. 4  $\mu$ g/ml of OKM5 was ascertained to be adequate for the saturation of the antibody. Data are the average of four determinations and are a representative of three separate experiments in both case 1 and case 2. Data are shown as mean  $\pm$ SD.

percent inhibition is calculated as a percentage of specific association/degradation (total minus nonspecific values observed in the presence of an excess of unlabeled OxLDL), both specific activities of cell association and degradation of OxLDL were lower by 47 and 51%, respectively, in macrophages from the CD36-deficient subject than in macrophages from a normal control. The addition of OKM5 inhibited the cell association and degradation of OxLDL by 53 and 65%, respectively, in macrophages from control, while it did not significantly inhibit them in CD36 deficiency. These values (53 and 65%) nearly corresponded to the difference in specific activities of OxLDL uptake or degradation between CD36 deficiency and normal controls. These results suggest a major contribution of CD36 as an OxLDL receptor. The uptake and degradation of <sup>125</sup>I-OxLDL by macrophages were suppressed only partially by the addition of Ac-LDL in both normal controls and CD36-deficient subjects.

To determine the specificity of CD36 as an OxLDL receptor, the effects of competitors on the cell association of AcLDL to macrophages were also studied in CD36-deficient and normal control macrophages. As a competitor, OKM5 and excess amounts of OxLDL and AcLDL were used. As shown in Fig. 6, the cell association of AcLDL was not significantly inhibited by OKM5 in both CD36-deficient and normal macrophages.

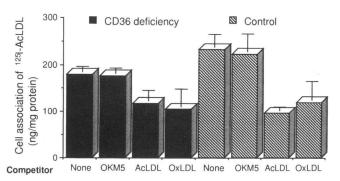


Figure 6. Cell association of AcLDL in macrophages from a subject with CD36 deficiency (case 2) and a normal subject. OKM5 (4  $\mu$ g/ml), OxLDL (40-fold excess; 200  $\mu$ g/ml), and AcLDL (40-fold excess; 200  $\mu$ g/ml) were added to the incubation medium containing 5  $\mu$ g/ml of <sup>125</sup>I-AcLDL. Cells were incubated for 4 h at 37°C and cell association was measured. Data are the average of quadruplicate determinations of cell association and are a representative of two separate experiments. Data are shown as mean $\pm$ SD.

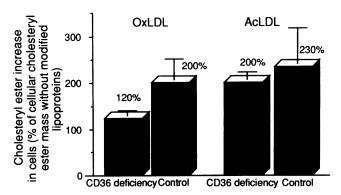


Figure 7. Cholesteryl ester accumulation in macrophages from a subject with CD36 deficiency and a normal subject. 50 μg/ml of OxLDL or AcLDL were incubated for 24 h at 37°C. Vertical bar shows percentage of cholesteryl ester mass in macrophages incubated with OxLDL or AcLDL against cholesteryl ester mass in macrophages incubated without modified lipoproteins. Control for CD36-deficient cells represents CD36 deficient cells incubated without OxLDL or AcLDL in RPMI-1640 containing 3% BSA and control for control cells represents control cells incubated without OxLDL or AcLDL in RPMI-1640 containing 3% BSA for 24 h. Data are the average of three separate determinations and are shown as mean±SD.

The cell association and degradation were suppressed to almost the same level by both unlabeled AcLDL and OxLDL. Specific AcLDL cell association to CD36-deficient macrophages was significantly lower by 54% than that of control macrophages.

We also examined the change in cholesterol content after incubation with OxLDL in CD36-deficient macrophages compared with that in control macrophages. Macrophages were incubated with 50  $\mu$ g/ml OxLDL or AcLDL for 24 h. When macrophages were incubated with OxLDL, the increase in CE accumulation was remarkably smaller in the CD36-deficient macrophages than in normal controls (120% increase vs 200% increase), whereas the difference in CE accumulation was rather small between both macrophages when incubated with AcLDL (Fig. 7).

## **Discussion**

The current study investigated, for the first time, the capacity of binding and uptake of OxLDL in CD36-deficient macrophages. We demonstrated that CD36 plays an important role as an OxLDL receptor in human monocyte-derived macrophages.

First, we demonstrated that the macrophages from CD36 deficiency have a low capacity to bind and internalize OxLDL. Furthermore, the macrophages from CD36 deficiency accumulated a smaller amount of CE than control macrophages. These results point to the notion that reduced binding capacity for OxLDL in human monocyte-derived macrophages due to CD36 deficiency may lead to a decreased accumulation of CE in macrophages. The number of OxLDL receptors in monocyte-derived macrophages may be one of the causes of variable levels of foam cell formation in atherosclerotic lesions of subjects with similar levels of common risk facers, such as hypercholesterolemia.

Secondly, we demonstrated a significant contribution of CD36 as an OxLDL receptor in vitro. Based on the competition

with OKM5 and the difference in the capacity of binding or uptake between CD36-deficient and control macrophages, CD36 appeared to account for at least 40% of the OxLDL uptake (Figs. 3-5). Although CD36 was found to contribute to a significant part of OxLDL metabolism, CD36-deficient macrophages retained an ~ 50% capacity for OxLDL binding and uptake, suggesting that receptors other than CD36 may also play a significant role in OxLDL metabolism. Arai et al. (13) and Sparrow et al. (14) suggested heterogeneity among OxLDL and AcLDL receptors. Other than CD36, the AcLDL receptor which is the so-called scavenger receptor identified by Kodama et al. (7), or SR-B1, which has recently been cloned as a receptor for OxLDL (37), or the 94-97-kD receptor described by Ottnad et al. (38) may be involved in the remaining OxLDL uptake in monocyte-derived macrophages (Fig. 5).

Thirdly, we have shown that OxLDL is degraded through CD36, since a difference in the degradation of OxLDL can be observed between the macrophages from CD36 deficiency and normal controls. OKM5 inhibited the degradation of OxLDL, supporting the hypothesis that OxLDL is degraded through CD36. In our unpublished experiments, we transfected CD36 cDNA to 293T cells and observed the cell association and degradation of  $^{125}$ I-OxLDL. We found that OxLDL was degraded through CD36 in these transfected cells. Furthermore, the degradation was partially, but significantly inhibited by  $100~\mu\mathrm{M}$  chloroquine. These findings confirm that OxLDL is degraded through CD36 and that some part of the degradation of OxLDL may occur in the lysosomes.

In CD36-deficient macrophages, AcLDL did not inhibit the cell association of OxLDL to the level of the inhibition by unlabeled OxLDL (Fig. 5). These data may suggest the presence of specific receptors which bind only OxLDL, but not AcLDL. In contrast with OxLDL cell association, AcLDL cell association was competed by OxLDL to the same extent as AcLDL (Fig. 6), suggesting that there may be no specific receptors for AcLDL in human monocyte macrophages, although Arai et al. (13) suggested the presence of a specific receptor for AcLDL which binds only to AcLDL in mouse peritoneal macrophages, but not to OxLDL.

We observed that the cell association of AcLDL was reduced by 54% in CD36 deficiency compared with that in control macrophages. These data might suggest that AcLDL binds to CD36. Although OKM5 did not compete with the cell association of AcLDL, OKM5 probably will not compete with the binding sites for AcLDL. Controversy exists on the ligand specificity for CD36. Endemann et al. (27) showed that CD36 does not bind to AcLDL, while Anton et al. (37) reported that CD36 actually binds to AcLDL. The difference in the results of these studies may be related to the character of the cells undergoing transfection. In our unpublished observations using transfected 293T cells, AcLDL did not bind to CD36. A possible explanation for these discrepancies is that the binding of AcLDL to CD36 may need the involvement of other factors, or the expressed CD36 may exist in different forms among the transfected cells.

In our experiments, both monocytes and macrophages expressed CD36. Is the role of CD36 the same between monocytes and macrophages in vivo? Previous studies have shown that CD36 may play a role in monocytes adhesion to collagen or thrombospondin. Therefore, as suggested by Endemann et al.

(27), CD36 could play a role in atherogenesis independent of and in addition to its potential role in foam cell formation.

Will a deficiency of CD36 increase or decrease susceptibility to atherogenesis? Our subjects with CD36 deficiency currently do not show any signs or symptoms of premature atherosclerosis, however the small number of patients available with this rare disorder precludes any firm conclusions. A significant decrease in the rate of uptake of OxLDL by arterial macrophages should slow the development of macrophage-derived foam cells and thus the rate of progression of fatty streaks. From this point of view, a deficiency in CD36 might be antiatherogenic. On the other hand, as has been pointed out (39), failure to take up OxLDL, which is cytotoxic and a growth factor for smooth muscle cells and prothrombotic, might have quite opposite effects. Studies in transgenic mice overexpressing CD36 or deficient in CD36 may be necessary to resolve this complex issue.

In summary, we have presented data suggesting that CD36 on human monocyte-derived macrophages can account for up to 40% of the binding and uptake of OxLDL under the in vitro conditions used. We therefore suggest that CD36 may contribute to foam cell formation under in vivo conditions. However, when we predict the quantitative role of CD36 in the uptake of OxLDL by arterial macrophages on the basis of these in vitro studies, a careful interpretation must be made. For example, the absolute rate of degradation observed here was low compared, for example, to that in mouse peritoneal macrophages (13), suggesting that the rate of degradation may be much greater even in human macrophages in the vessel wall than in vitro because of the phenotypic shifts. Therefore, the relative contribution of CD36 in vivo may be difficult to predict and will depend upon the extent to which conditions in the artery wall induce the expression of several receptors that are known to bind OxLDL (15, 27, 37, 38, 40). Studies of the expression of these receptors in developing lesions (e.g., in situ hybridization) will be needed to evaluate this issue.

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