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Reduced Adhesion of Monocyte-Derived Macrophages from CD36-Deficient Patients to Type I Collagen

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CD36 is an 88-kDa glycoprotein expressed on platelets and monocyte/macrophages (Mϕ). CD36 is a multifunctional receptor for collagen, thrombospondin, oxidized low density lipoproteins (LDL), and long-chain fatty acids. The present study was performed to investigate whether CD36 can function as an adhesion molecule which is involved in mediating human macrophages (Mϕ) adhesion to type I collagen *in vitro*. The Mϕ of human CD36-deficient as well as normal control subjects were isolated and cultured on the multi-well plates coated with type I collagen, a natural ligand for CD36. Up to 2 h of incubation, the Mϕ from CD36-deficient patients showed almost a ~55% decrease in adhesion to type I collagen in comparison to those from controls ($P < 0.01$). However, there was no significant difference in the adhesion thereafter. Furthermore, the addition of antibody against CD36 into the media of control Mϕ significantly inhibited the adhesion by ~50% ($P < 0.05$). The addition of oxidized LDL (OxLDL) did not alter adhesion of Mϕ from both CD36-deficient and controls. These data suggest that CD36 is involved in the adhesion of Mϕ to type I collagen, especially in the early stage of adhesion.

Key Words: **monocyte-derived macrophages; CD36 deficiency; type I collagen; cell adhesion; receptor.**

CD36 is an 88-kDa transmembrane glycoprotein expressed in a variety of cell types such as platelets, monocytes, monocyte-derived macrophages, and adipocytes. It functions as an endothelial cell adhesion receptor for *Plasmodium falciparum* (malaria)-parasitized erythrocytes. This adhesion characteristic of CD36 is of clinical importance as it has been demonstrated on the microvasculature of brain, liver, kidney, and lung tissues from patients who died from severe falciparum malaria (1). It also functions as a cellular receptor for thrombospondin-1 and may participate in platelet-monocyte adhesion, platelet-tumor cell adhesion, cell substratum adhesion, and angiogenesis (2). The interaction of platelets with collagen is an important step in their adhesion to the vessel wall (3). Genetic CD36 deficiency was first described in 1990 (4) and we have reported the molecular basis of this disorder by identifying three mutations in subjects with CD36 deficiency (5–7). Endemann *et al.* demonstrated that a mouse homolog of human CD36 binds oxidized low density lipoproteins (LDL) (8). Using the macrophages (Mϕ) from the CD36-deficient patients, we have clarified that one of its major physiological functions is a receptor for oxidized LDL (OxLDL) by demonstrating that CD36 is responsible for ~50% of uptake for OxLDL in human monocyte-derived-macrophages (9). Mϕ are believed to play a central role in the development and progression of atherosclerosis, which is characterized as a chronic inflammatory disorder where the persistent presence of Mϕ is evident at all stages (10). Hence, the phenotype of patients with CD36 deficiency suggest that CD36 may play an important part in the pathogenesis of atherosclerosis and hemostasis process, at least in populations in which the prevalence of CD36 deficiency is rather high.

To obtain a more precise definition of the role of CD36, we have examined the adhesion of control and CD36-deficient Mϕ to type I collagen. We also studied the effects of control Mϕ adhesion to collagen in the presence of human monoclonal antibody against CD36. Our data demonstrated that the adhesion of Mϕ to type I collagen is reduced in the absence of functional CD36 and that this reduction occurs at the earliest stage of the adhesion process.

MATERIALS AND METHODS

Study subjects. Blood was obtained with an informed consent from 7 healthy, unrelated, CD36-positive control subjects and 5 unrelated, CD36-deficient patients identified by screening with spe-

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cific antibodies and immunofluorescent flow cytometry, who were matched for age. Their monocytes lacked CD36 on cell surface (Fig. 1).

Lipoprotein preparation. Low density lipoproteins (d = 1.019–1.063 g/ml) were isolated by sequential ultracentrifugation as described previously (11) from serum of 2 normolipidemic volunteers who had fasted overnight for 12–14 h. The protein content of LDL was determined by the method of Lowry *et al.* with bovine serum albumin (BSA) as a standard. Oxidation of LDL was performed in a cell free system. Briefly, to prepare OxLDL, native LDL was diluted to 1 mg of protein/ml with phosphate-buffered saline (PBS) and dialyzed at 4°C for 24 h against PBS in the absence of EDTA. The LDL were sterilized by ultrafiltration using 0.45 mm Millipore filter, diluted to a concentration of 400 μg of protein/ml with PBS and incubated at 37°C for 8 h in the presence of 5 mM $CuSO_4$.

Isolation and culture of macrophages. Mixed mononuclear cells from blood samples of fasting healthy CD36-positive volunteers and CD36-deficient subjects were isolated by a density gradient centrifugation method using lymphocyte separation solution (Nacalai Tesque, Kyoto, Japan). Twenty milliliters of blood (anticoagulated with 10 units/ml sodium heparin) was layered over 15 ml of Ficoll-Paque and centrifuged at $1 \times 10^4 g$ for 30 min. 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 1×10^5 in collagen type I-coated culture dishes (Primaria Labware; Becton Dickinson and Co., U.S.A.) in RPMI-1640 containing 10% (vol/vol) human type AB serum with antibiotics. Monocytes were incubated at 37°C in 5% CO_2, and then the adherent cells were washed 3 times to remove loosely adherent cells. These cells represent monocytes in an early stage of differentiation and therefore are referred to as monocyte/macrophages (Mϕ) in the text. Cell viability determined by trypan blue exclusion was >98% in all studies.

Adhesion assays. Measurement of adhesion activity of Mϕ to type I collagen coated culture dishes (Iwaki Glass Ware, Tokyo, Japan) using a crystal violet method was performed as previously reported (13). The inherent sensitivity of the assay procedure for determination of cell number using crystal violet staining permits the application of 96-well plates. As reported previously (14), internal standardization of the procedure includes a fixation step before cell staining which minimizes loss of cells due to manipulation associated with the experimental procedures. Briefly, 1×10^5 cells with or without OxLDL preincubation were applied to 96-microtiter plate wells precoated with type I collagen. After gently washing the wells 3 times with PBS, adherent cells were fixed with 1% glutaraldehyde in PBS for 15 min and stained with 0.1% crystal violet solution for 30 min. After destaining with distilled water for 15 min, the dyes were solubilized with 200 μl distilled water containing 0.3% Triton X-100, and then the absorbance was measured at 620 nm. In order to compare the number of cells inoculated with that of cells adherent at the time of fixation, cells stained with crystal violet were counted before the dye was solubilized. This counting was repeated in at least three separate experiments.

Statistical analysis. Values were given as the means ± SD. Statistical significance between the groups was evaluated by unpaired *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Since the Mϕ which reside in areas rich in extracellular matrices (ECM) constituents, are constantly exposed to collagens, fibronectin, and a variety of proteoglycans, the specific interaction of Mϕ with collagen is a vital yet elusive topic of investigation. CD36 has been proposed to play a major role in monocyte-collagen

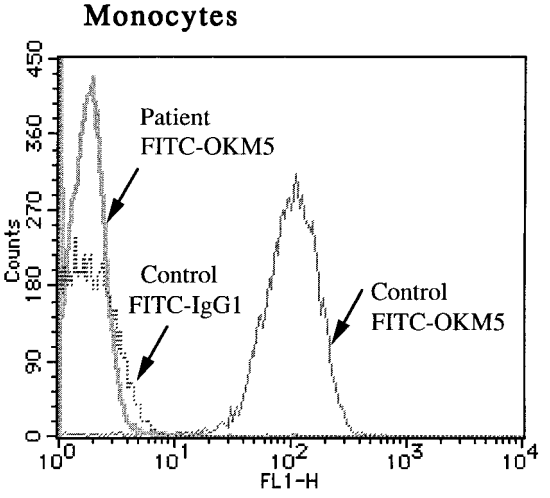

FIG. 1. Immunofluorescent flow cytometric analysis of CD36-deficient and control monocyte-macrophages. Expression of CD36 was determined by flow cytometry using an anti-CD36 antibody fluorescence distribution of the counted cell population. Monocytes from CD36-deficient and control subjects were incubated with either FITC-conjugated IgG1 or FITC-conjugated monoclonal antibody OKM5. Typical illustration of the left shift of the curve in a CD36-deficient subject is shown.

interaction (15). Therefore, CD36 has been shown to meet several criteria for a collagen receptor and has also been proposed as a thrombospondin receptor on platelets (1).

To assess the adhesion of Mϕ to type I collagen and confirm the acceleratory role of CD36, we performed adhesion assays on Mϕ from 7 healthy unrelated, CD36-positive control subjects and 5 unrelated CD36-deficient patients, identified by screening with specific antibodies and immunofluorescent flow cytometry (Fig. 1). The Mϕ from CD36-deficient patients showed a ~50% reduction in adhesion to type I collagen ($P < 0.01$). This difference between control and CD36-deficient Mϕ was significant up to 2 h time point but not thereafter (Fig. 2). These results strongly suggest that CD36 is one of the major receptors responsible for adhesion of Mϕ to type I collagen at least in the initial steps.

Human anti-CD36 antibodies have been shown to partly inhibit platelet adhesion to fibrillar collagen under both static and flow conditions. In our preliminary experiments, we determined the dose-dependent effect of anti-CD36 monoclonal antibody, OKM5. Maximum inhibition was obtained at a concentration of 1 μg/ml. To further investigate the reduced adhesion of CD36-deficient Mϕ to type I collagen compared with control the Mϕ, we examined the adhesion patterns of control Mϕ incubated with human anti-CD36 antibodies. It was apparent that the Mϕ preincubated with specific anti-CD36 antibody markedly showed a reduced adhesion to type I collagen, while non-specific antibody IgG1 had no effect (Fig. 3). These two sets of

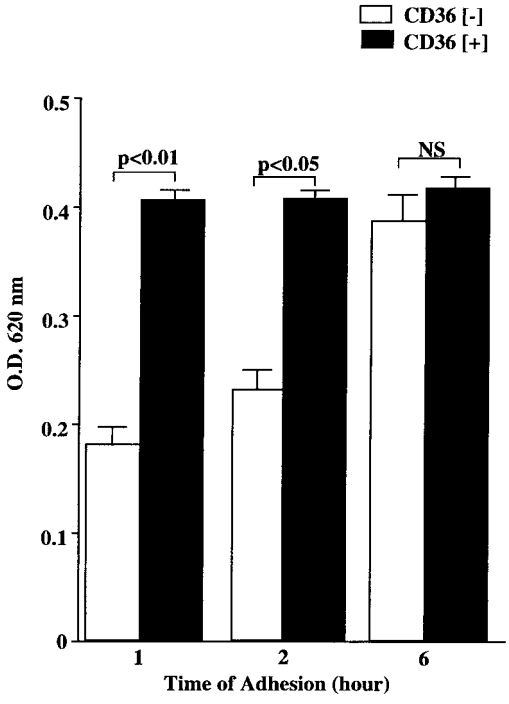

FIG. 2. Time-dependent adhesion of monocyte-macrophages to type I collagen coated culture plates. Freshly isolated human monocyte-derived macrophages were added to 96-well plates, which had been precoated with type I collagen and incubated for various times. The number of adherent cells was determined by a crystal violet staining method as described under Materials and Methods. Values shown are the mean ± SD of experiments using monocyte-macrophages from unrelated subjects each run in triplicate. Similar results were obtained in repeated independent experiments.

results demonstrate that the presence of functional CD36 accelerates the initial interaction of platelets with collagen, but that CD36 is not obligatory because, ultimately, the adhesion in all cases reaches the similar levels as in controls.

As mentioned earlier, platelets of Nak^a-negative phenotype constitutively lack CD36. The Nak^a-negative phenotype occurs with a high incidence in the Japanese blood donor population but with an incidence of less than 0.2% in the US blood donor population (16, 17).

On the other hand, we reported that CD36 functions as a physiological receptor for OxLDL, demonstrating that CD36 is responsible for ~50% of uptake for OxLDL in human monocyte-derived Mϕ (9). This led us to simultaneously determine whether the presence of OxLDL affects the adhesion of Mϕ to type I collagen *in vitro.* To test this we cultured Mϕ from the control and CD36-deficient subjects in the presence of 40 μg/ml OxLDL. There was no significant difference in the adhesion between the two groups (Fig. 4).

DISCUSSION

CD36 is a multifunctional protein involved in adhesion, scavenging, transport of long-chain fatty acids and cell signaling. Characterization of its structural organization is critical to understand its complex biological functions. The evidence that CD36 plays a vital role in vascular medicine has been strengthened recently by reports from our own group and others (18, 19). CD36 plays a central role in the accumulation of lipids and formation of foam cells, which is a characteristic feature of atherosclerosis. In atherosclerotic plaques, there is an abundance of type I collagen. As shown by Rechter *et al.*, the amount of type I collagen increases with the progression of atherosclerotic lesions. Increased collagenase activity has been shown at these sites where various degrees of matrix repair and remodeling are continuously under way (21). The abundance of Mϕ at the sites of atherosclerotic plaques may result from their ability to adhere to type I collagen. Therefore, the ability of Mϕ to adhere to type I collagen in atherosclerotic lesions via CD36 may play some role in the pathogenesis of atherosclerosis. This engagement may be involved in signaling the release of proinflammatory cytokines and growth factors, possibly exacerbating the progression of the disease (22–24). Recently, Gowen *et al.* have shown that Mϕ adhere selectively to denatured forms of type I collagen via their scavenger receptors, but did not specify which among the many scavenger receptors is involved (25).

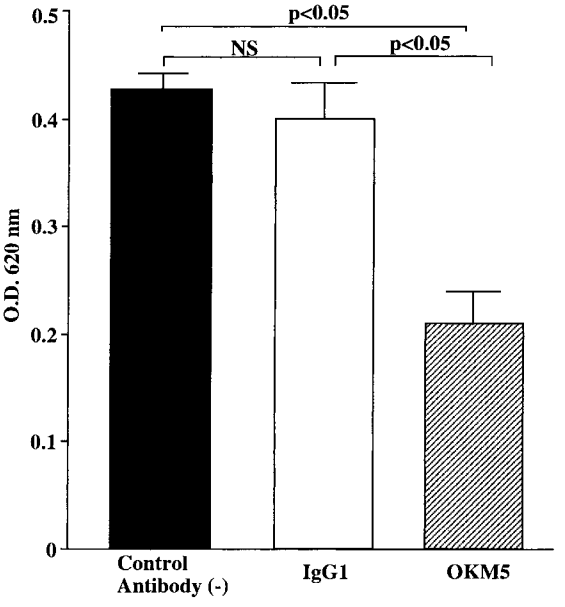

FIG. 3. Effect of antibodies directed against CD36 (OKM5) on adhesion of monocyte-derived macrophages to surfaces coated with type I collagen. To determine if CD36 is involved in acceleration of the initial interaction of monocyte-derived macrophages with type I collagen coated plates, freshly prepared monocyte-macrophages from the control subjects were incubated at 37°C with specific anti-human CD36 monoclonal antibody, FITC-OKM5, or nonspecific mouse antibody FITC-IgG1 and cells without addition of antibody served as control. Values shown are the mean ± SD of experiments in triplicate using monocyte-macrophages from unrelated subjects. Similar results were obtained in repeated experiments.

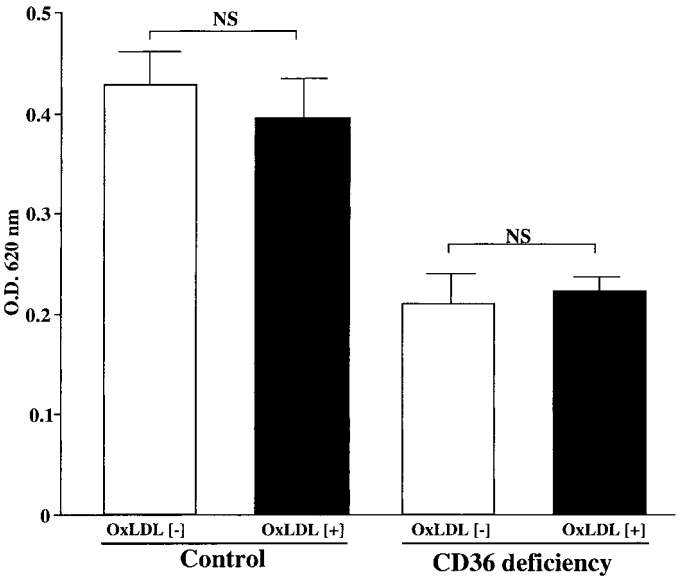

FIG. 4. Effect of OxLDL on adhesion of control monocyte-macrophages to type I collagen coated microtiter plates. To test whether the presence of OxLDL affects the adhesion of Mϕ on microtiter plates coated with type I collagen. We cultured Mϕ from the controls and CD36-deficient subjects in the presence or absence of 40 μg/ml OxLDL. Values shown are the mean $\pm$ SD of experiments in triplicate using monocyte-macrophages from unrelated subjects. Similar results were obtained in repeated independent experiments.

Our data support their results, and specify the involvement of class B scavenger receptor, CD36. Despite previous findings, which suggested the involvement of integrins, Gowen *et al.* could not significantly inhibit the adhesion of Mϕ to type I collagen with anti-β1 or β2 integrin antibodies. These results suggest that Mϕ scavenger receptors, CD36, other than these integrins previously reported to function as receptors for type I collagen, may be responsible for mediating adhesion to type I collagen.

Our data clearly showed that the Mϕ from CD36-deficient patients had significantly reduced adhesion to type I collagen in comparison to control CD36-positive subjects at the earliest stages of adhesion. It is noteworthy that the differences in adhesion were not significant after 2 h (Fig. 2). Furthermore, our assumption that CD36 plays a vital role in the early stage of adhesion was reinforced by the fact that the addition of antibody against human CD36 attenuated the adhesion of nonstimulated Mϕ from the controls. These sets of results indicate that the presence of functional CD36 accelerates the initial interaction of Mϕ with type I collagen, but that CD36 is not obligatory because, ultimately, the adhesion in all cases reaches the same level after 2 h of seeding (Fig. 2). Thus, acceleration of Mϕ interaction with collagen may arise through signal transduction mechanisms mediated by CD36-coupled tyrosine protein kinases and phosphorylation processes as suggested previously (26). The mechanism of interaction between Mϕ and collagen that is a major component of subendothelium is still not fully understood because collagen has a macromolecular structure and would therefore be expected to interact with many other proteins. In the current study we observed some degree of adhesion despite the complete lack of CD36. These observations suggest that other receptors may participate in adhesive interaction of Mϕ with type I collagen, although CD36 remains to play a vital role at least in experimental conditions. The biological relevance of the decreased adhesion of Mϕ to type I collagen is yet to be established. However, diversity of adhesive phenotypes expressed on the surface of infected erythrocytes has led to a considerable interest in the identification of those particular phenotypes associated with severity of the disease. Sequestration of malaria-infected erythrocytes in the peripheral circulation has been associated with the virulence of *Plasmodium falciparum*. Pain *et al.* showed that adhesion of *P. falciparum* is mediated by platelets and the formation of clumps of infected erythrocytes and platelets requires expression of platelet surface glycoprotein CD36. Pain *et al.* also reported that platelet-mediated adhesion is strongly associated with severe malaria (27). However, a long-term follow-up of our patients is in progress to understand the exact mechanism involved and clinical outcome of this reduced adhesion phenomenon and the reason for the diversity of clinical presentation of individuals with malaria.

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