The cytoadherence of erythrocytes (red blood cells) infected with *Plasmodium falciparum* (pRBCs) to endothelial cells and the uptake of oxidized low-density lipoprotein (oxLDL) by macrophages are both mediated, in part, by the glycoprotein receptor CD36. The interaction of lipoproteins and pRBCs competing for the human CD36 receptor was examined by use of Chinese hamster ovary cells expressing human CD36. OxLDL competitively inhibits the adherence of pRBCs to CD36, but native LDL and high-density lipoprotein do not. Modification of Lys residues in CD36 inhibits both oxLDL and pRBC binding; however, only oxLDL binding is inhibited by receptor iodination, and only pRBC binding is influenced by pH variations and receptor reduction. Furthermore, peptide inhibitors of the pRBC/CD36 interaction do not influence oxLDL binding. These results suggest that, although oxLDL competitively inhibits the adherence of pRBCs, these ligands interact with distinct domains on the CD36 receptor.

The majority of the morbidity and mortality associated with malaria is due to a single species, *Plasmodium falciparum* [1]. *P. falciparum*–parasitized erythrocytes can adhere to endothelial cell receptors, including chondroitin sulphate [2], vascular cell adhesion molecule–1 [3], intercellular adhesion molecule–1 [4], and CD36 [5]. These receptor-ligand interactions contribute to the process of sequestration that allows the parasite to avoid splenic clearance by residing in the microvascular beds, where the adhesive erythrocytes contribute to the hypoxia, metabolic disturbances, and organ dysfunction that characterize severe falciparum malaria [6]. Although several receptors are capable of contributing to rolling interactions under flow conditions, CD36 appears to be necessary for the static adhesion of infected cells to the endothelium [7].

CD36, an 88-kDa membrane glycoprotein that belongs to the scavenger receptor family, is found on several cell types, including platelets, monocytes, macrophages, and endothelial cells [8]. CD36, reported to function as a receptor for erythrocytes (red blood cells) infected with *P. falciparum* (pRBCs), is also a receptor for apoptotic neutrophils [9], thrombospondin (TSP) [10], collagen [11], and oxidized low-density lipoprotein (oxLDL) [12]. Recent evidence [13, 14] indicates that CD36 is also a high-affinity receptor for the native lipoproteins LDL, high-density lipoprotein (HDL), and very LDL (VLDL). CD36 may play a role in cardiovascular disease, since excessive uptake of oxLDL by macrophages contributes to atherogenesis [15]. Lipid-loaded macrophages constitute the foam cells seen in the “fatty streak” that characterizes atherosclerotic plaques [16], and CD36 is upregulated in cells that take up and degrade oxLDL [17]. The binding domains of some CD36 ligands have been characterized to a low degree of resolution by using monoclonal antibodies [18–20]. However, it is unknown whether multiple ligands are interacting with a single domain or whether each ligand interacts with a unique domain. This question has potential significance in the design of therapeutic agents, since it may be possible to block deleterious CD36-ligand interactions, such as sequestration, without disrupting physiological receptor functions.

The objectives of the present study were to determine whether native and oxidized lipoproteins competitively inhibit the adherence of pRBCs to CD36 and to determine whether two competing ligands, pRBCs and oxLDL, bind to the same or to unique domains in human CD36. We demonstrate that native lipoproteins do not influence pRBC binding to CD36 and that, although *P. falciparum*–infected erythrocytes and oxLDL competitively inhibit each other’s binding, this appears to result from steric inhibition rather than from competition for a single binding domain.
Materials and Methods

P. falciparum cultures. The I1G line of P. falciparum was cultured in A- blood obtained by venipuncture of volunteers. Cultures were maintained by the method of Trager and Jensen [21], by use of RPMI 1640 supplemented with 10% human serum and 50 μM hypoxanthine. Prior to their use in an adhesion assay, erythrocytes were pelleted and resuspended in Bis Tris saline (BTS; 25 mM BTS, 135 mM NaCl, pH 6.8) [22].

pRBC adhesion assays. Adhesion assays were carried out as described elsewhere [23, 24] by use of Chinese hamster ovary (CHO) cells stably transfected with human CD36 [25] that had been grown to 10% confluence in 12 well plates (Corning, Corning, NY) in the presence of the selective agent G418 (GIBCO, Burlington, Ontario, Canada). Immediately prior to use, the cells were fixed with 4% formalin in phosphate-buffered saline for 10 min and then rinsed three times with BTS. Adhesion assays using a total volume of 300 μL of infected blood (5% hematocrit and 5% parasitemia) were maintained by the method of Trager and Jensen [21], by use of SigmaPlot (Jandel Scientific, Chicago), assuming the mean values, with standard error of the mean indicated by bars. Where appropriate, binding parameters were determined by use of SigmaPlot (Jandel Scientific, Chicago), assuming a noncooperative ligand/receptor interaction.

LDL, HDL, and oxLDL. Human LDL and HDL were isolated from serum by ultracentrifugation at densities of 1.019–1.063 g/mL (LDL) and 1.063–1.21 g/mL (HDL) at 45,000 rpm for 18 h (LDL) or 48 h (HDL) at 10°C, using a model 50.3 Ti rotor in a L8-80 centrifuge (Beckman Instruments, Fullerton, CA) [26]. Isolated lipoprotein was dialyzed against 0.15 M NaCl and 0.24 mM Na2EDTA, pH 7.4, and was then diluted with 1 M glycine/NaOH buffer, pH 10.0, and iodinated with Na125I [27]. Unbound iodine was removed by use of QAE-Sephadex chromatography (Pharmacia, Baie d’Urfé, Quebec, Canada). The lipoprotein was extensively dialyzed against phosphate-buffered saline, pH 7.4. For oxidation, LDL was diluted to 1–1.5 mg protein/mL and mixed with 4 μM CuSO4 to a final concentration of 20 nmol CuSO4/mg LDL [28]. The oxidized LDL was dialyzed against RPMI-1640 containing 25 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, pH 7.4. Specific activity was typically 300–600 cpm/ng LDL protein, with >90% of the radioactivity being precipitated by trichloroacetic acid. “Cold” oxidized LDL was produced by the same method, except that NaI was used in place of radiiodine.

OxLDL binding assay. OxLDL binding to CHO-CD36 was carried out using confluent CHO-CD36 cells in 12 well plates (Corning). Twenty-four hours prior to the assay, 3 × 10^5 CHO cells were seeded into wells. Cells were incubated on a rotator in the presence of 5 μg of [125I]oxLDL for 2 h at 4°C. Alternatively, CHO-CD36 cells were fixed in formalin, and the assay was performed at room temperature for 2 h. Specific binding of oxLDL was determined by subtracting counts obtained in the presence of a 40-fold excess of unlabeled oxLDL from those obtained in the absence of unlabeled oxLDL, as described elsewhere [29]. Mock-transfected (vector alone) CHO cells were assayed for oxLDL binding activity on a routine basis, and binding to CHO-CD36 binding was at least twice that observed with binding to CHO-mock.

Chemical modification and pH. For pRBC and oxLDL adhesion assays involving iodination of the CD36 receptor, CD36-expressing CHO cells were subjected to one of the following: (1) no additions, (2) the addition of 10 mM NaI in phosphate-buffered saline with Mg2+ and Ca2+ (PBS; GIBCO), (3) the addition of an Iodobead (Pierce, Rockford, IL), or (4) the addition of both 10 mM NaI and an Iodobead. After a 5-min incubation at room temperature with gentle agitation, the bead was removed, and the wells were thoroughly washed three times with BTS prior to use in an adhesion assay. For dithiothreitol (DTT; Sigma, St. Louis) assays, the CHO-CD36 cells were treated with DTT at varying concentrations in PBS for 30 min at room temperature prior to the cells’ being washed with BTS and used in an assay. For Lys residue modification experiments, acetic acid N-hydroxysuccinimide ester (NHS, Sigma) was dissolved in 1 volume of DMSO prior to the addition of 33 volumes of PBS [30]. This solution was immediately diluted and added to wells containing fixed CHO-CD36 cells. After a 30-min incubation at room temperature, the wells were washed three times with PBS and then used for an assay. Peptide inhibition assays of pRBC and oxLDL binding were performed using serial dilutions of commercially synthesized peptides (BSC-HSC, Toronto, Ontario, Canada).

Results

Oxidized and native lipoproteins [13, 14] and pRBCs [31] have all been demonstrated to bind to CD36. However, it is unknown whether these ligands bind to the same or to unique domains of CD36. We undertook competitive inhibition assays to determine whether oxLDL, LDL, and HDL inhibit the adhesion of pRBCs to CD36-expressing cells. Significant inhibition of cytoadherence was observed for the oxidized form of LDL but not for native LDL or HDL (figure 1). Addition of increasing amounts of oxLDL resulted in ≈80% inhibition of pRBC adherence with an IC50 value of 5 μg/mL, the equivalent of a concentration of ~10^-8 M. In these competition experiments, native LDL resulted in only slight inhibition (<10%) at the highest value assayed, 1.3 mg/mL, and HDL appeared to have no effect on the adherence of pRBCs. The range of “control adherence” values for these assays was 20–32 pRBCs/CHO-CD36 cell, whereas adherence of pRBCs to mock-transfected cells was observed to be in the range of 1–2 pRBCs/CHO cell (data not shown). To exclude the possibility that the inhibition by oxLDL was caused by the presence of copper or another reactive product [32], bovine serum albumin (BSA) was subjected to the same treatment and assayed for inhibitory potential. The addition of native or oxidized BSA had no effect on the adherent ability of infected erythrocytes (data not shown).
JID 1999;180 (August) pRBC and oxLDL Binding Domains of CD36

Figure 1. Effect of lipoproteins on the adherence of erythrocytes infected with Plasmodium falciparum (pRBCs) to Chinese hamster ovary (CHO) CD36 cells. The ability of pRBCs to adhere to CHO CD36 cells was determined in the presence of varying amounts of high-density lipoprotein (HDL), low-density lipoprotein (LDL), and oxidized LDL (oxLDL). Mean observed values of 20 representative CHO cells per well are shown, with standard errors in the sample indicated by error bars. Best-fit lines assuming competitive inhibition, produced by the computer program SigmaPlot (Jandel Scientific, Corte Madera, CA), are indicated. OxLDL was found to inhibit pRBC adherence to a maximum of 80% with an IC50 of 5.5 ± 1.1 μg/mL. The range of control adherence values for these assays was 20–32 pRBCs/CHO-CD36 cell. Adherence of pRBCs to mock transfected cells was observed to be in the range of 1–2 pRBCs/CHO cell (data not shown).

Figure 2. Sensitivity of binding of erythrocytes infected with Plasmodium falciparum (pRBCs) and binding of oxidized low-density lipoprotein (oxLDL) to iodination of CD36. The effect of prior iodination of CD36 on the ability of pRBCs and oxLDL to bind was determined. Chinese hamster ovary cells transfected with CD36 were left untreated (−) or were iodinated (+), then were assayed for binding of oxLDL or pRBCs. Prior experiments (not shown) determined that addition of NaI or an Iodobead (Pierce) alone did not affect the adherence of either ligand. The results are expressed as the average of triplicate determinations ± SEM.

indicating that the inhibitory effect was not caused by the oxidation method or the presence of oxidation reagents alone.

The ability of oxLDL to inhibit the adherence of pRBCs to CD36-bearing cells could be explained by the two ligands competing for a common binding domain, or it might result from steric hindrance resulting from the two large ligands’ binding to separate but adjacent domains. Previous work on the binding of pRBCs to CD36 has demonstrated that the interaction is strongly influenced by protein modification methods and that alteration of particular amino acid residues influences the characteristics of pRBC/CD36 binding [33]. We therefore assayed pRBC binding and oxLDL binding to determine whether modifying residues in CD36 affected both interactions equally. The adherence of pRBCs to human CD36 is pH dependent in the range 6.0–7.4 because of a His residue at position 242 of human CD36 [33]. Varying the pH of the buffer in the oxLDL binding assay from 6.0 to 7.4 had no significant effect on oxLDL binding (data not shown). Adherence of pRBC to rodent, but not human, CD36 is sensitive to iodination [33]. We observed that treatment of CHO-CD36 cells with iodide in combination with an Iodobead prior to an adhesion assay reduced the capacity of CD36 to bind to oxLDL (P < .01, Student’s t test) but not to pRBCs (P > .10, Student’s t test; figure 2). Treatment of CHO-CD36 cells with NHS under conditions that would favor modification of Lys residues affected both pRBC and oxLDL adhesion (figure 3A). Finally, CD36 was subjected to treatment with DTT prior to adhesion assays. DTT significantly reduced pRBC binding (P < .01, Student’s t test) but not oxLDL binding (P > .10, Student’s t test; figure 3B), suggesting that pRBC binding requires an intact Cys-rich domain [34], whereas oxLDL binding does not. We concluded that the amino acid residues on CD36 that influenced or comprised the pRBC and oxLDL binding domains were dissimilar.

Adherence of pRBCs has been demonstrated to be inhibited by peptides with an H/KxxxKxY motif [35, 36]. Apo B, the only protein present in an LDL particle, contains the sequence FKHLRKYTY (residues 20–28) [37], which is similar—but not an exact match—to motifs that have been reported to inhibit pRBC adhesion to CD36 [35, 36, 38]. To examine the possibility that a common adhesion motif was responsible for oxLDL and pRBC interactions with CD36, a peptide based on the apo B sequence (FKHLRKYTY) and a positive control peptide (HPLQKTY) were assayed for their ability to inhibit the adherence of pRBCs and oxLDL. Both sequences inhibited pRBC adherence with an IC50 value of ~10−7 M (figure 4A), but these peptides had no effect on the binding of oxLDL to CHO-CD36 cells (figure 4B). This indicates that, although apo B and pRBCs both contain a potential H/KxxxKxY adhesion motif, these sequences only disrupt the pRBC-CD36 interaction.

Discussion

CD36 belongs to a family of receptor proteins that interact with a diverse array of ligands [39]. Although it may be useful...
Figure 3. Effect of Lys modification or reduction of disulfide bonds of human CD36 on binding of erythrocytes infected with Plasmodium falciparum (pRBCs) and binding of oxidized low-density lipoprotein (oxLDL). The effect of prior Lys modification of Chinese hamster ovary (CHO) CD36 cells with N-hydroxysuccinimide ester (NHS; panel A) or reduction of CD36 with dithiothreitol (DTT; panel B) was determined. CHO CD36 cells were grown to 10% confluence prior to fixation and rinsing with Bis Tris saline (BTS; see Methods). The cells were then exposed to varying concentrations of NHS and DTT (see x-axis) prior to three rinses with BTS and the addition of pRBCs or labeled oxLDL. Binding assays were completed as described in Methods, and the results were expressed as the mean of triplicate determinations ± SEM in the case of oxLDL binding (left axis) or as the mean of observed values of 30 representative CHO cells per well, with SEM indicated by error bars (right axis).

to consider the interaction of a single ligand with CD36 in isolation, in vivo many of the ligands may be present simultaneously and may influence the outcome of a P. falciparum infection if they compete for the same receptor. Of particular interest is the potential competition between serum lipoproteins and pRBCs, because lipoproteins will be present during a P. falciparum infection and may modify the pRBC-CD36 interaction and thus the outcome of the infection.

When individual lipoproteins were assayed for their ability to inhibit pRBC binding to CD36, it was observed that binding of oxLDL to CD36 prevented the binding of pRBCs; however, HDL and native LDL had little or no effect (figure 1). LDL and HDL serum levels [40] are 1000-fold higher than the µg/mL IC₅₀ values reported for their interaction with CD36 [13, 14], and therefore we would expect the LDL and HDL binding domains of CD36 to be saturated under physiological conditions. Since the highest concentrations of native lipoproteins used in our in vitro pRBC binding assay approach normal serum levels of LDL and HDL, we conclude that the presence of native lipoproteins does not likely result in the displacement of pRBCs from CD36 in vivo. This conclusion is in agreement with the observed role of CD36-mediated pRBC adherence in the pathology of falciparum malaria.

Although it is not immediately obvious how two large ligands, a pRBC and a lipoprotein, can bind to an 88-kDa receptor, several explanations are possible. If LDL, HDL, and pRBCs are binding to a common population of CD36 receptors, ≥1 of the ligands may be using a bridging molecule, such as TSP [41], that does not sterically hinder the binding of other ligands. A second explanation may be that the preference of CD36 for either pRBCs or native lipoproteins may be modulated by a covalent modification in CD36, as is observed with the TSP/collagen binding domain [11]. Such modulation may produce two distinct CD36 populations, one that binds native lipoproteins and one that interacts with pRBCs. A third explanation may be that the surface distribution or aggregation state of CD36 may influence its ligand preference. It has been suggested [42] that multimerization of CD36 may lead to its targeting to cell surface caveolae [43] instead of a diffuse surface arrangement. This explanation would suggest that the cell type in which CD36 is expressed—such as COS [13], CHO [25], or SF9 [44] cells—may influence the ligand-binding preferences. Finally, pRBCs may produce nonreciprocal inhibition of native lipoproteins [45].

The presence of an H/KxxxKxY-like motif in the apo B sequence suggested that these ligands might share a common binding domain in CD36. The results of the pH studies, residue modification experiments, and peptide inhibition assays suggest
Figure 4. Inhibition, by peptides, of adherence of erythrocytes infected with *Plasmodium falciparum* (pRBCs) and of binding of oxidized low-density lipoprotein (oxLDL). The ability of three peptides to inhibit pRBC adherence and oxLDL binding was determined. The adherence assays were performed, as described in figure 3, with a serial dilution of the peptides FKHLRKYTY, HPLQKTY, and LYPQHKT (a scrambled control of HPLQKTY). Data points represent mean values of bound pRBCs (panel A), determined by counting >19 representative Chinese hamster ovary–CD36 cells, with SEMs indicated by error bars, or oxLDL binding assay (panel B) performed in triplicate, with SEMs indicated by error bars. Solid lines indicate best fit of the data, assuming competitive inhibition of adherence by the peptides as determined by the program SigmaPlot (Jandel Scientific, Corte Madera, CA). Control adherence is the value observed for the lowest dilution of peptide that was observed to be at least 3 log units below the IC₅₀s of the peptides.

that this hypothesis is incorrect. Comparisons of pRBC binding to human, rat, and mouse CD36 suggest that the His residue at position 242 in human CD36 strongly influences the pRBC/CD36 interaction [33]. In contrast, oxLDL binding is not influenced by pH, indicating that a His residue (at position 242 or elsewhere) does not influence the behavior of the oxLDL binding domain. Iodination of the available Tyr residues in CD36 decreases oxLDL but not pRBC binding, suggesting that the oxLDL domain alone is influenced by iodination. Treatment of CD36 with the reducing agent DTT was expected to reduce the disulfide bridges to thiol present in CD36 [46] and thereby have a more global effect on protein structure, particularly on the Cys-rich segment of CD36 (residues 243–333) that is adjacent to His 242. The modification of Lys residues did not produce a differential effect on the two ligand interactions; therefore, we conclude either that Lys modification produces a global change in the structure of CD36 or that a Lys residue is part of, or important to, the conformation of both binding domains.

The peptide inhibition results support the conclusion that oxLDL and pRBCs compete for CD36 in an infected individual, since supporting epidemiological evidence may be difficult to obtain—atherosclerosis and malaria are major health problems in geographically distinct populations. It is of interest that the presence of pRBCs in the microvasculature leads to high concentrations of reactive oxygen intermediates (ROIs) [47], and these may lead to increased levels of the oxidized form of LDL. Since oxLDL and pRBCs compete for CD36, it is possible that ROIs may have both antimicrobial and antisequestration effects and that their presence may select for pRBCs, which express different ligands and can use other receptors to bind to endothelial cells. On a practical note, the presence of LDL in malaria cultures and its tendency to oxidize over time may introduce another variable into comparisons of the cytoadherent ability of different parasite lines and should be taken into account when comparisons of results are made. The observation that adhesion of pRBCs and oxLDL involves two distinct binding domains is important, since it suggests that antisequestration therapies can be tailored to interact with discrete regions of the CD36 protein and have
little, or no, effect on other physiological functions of this receptor.

References


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