A Novel Role for CD36 in VLDL-Enhanced Platelet Activation

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Type 2 diabetes is characterized by increased plasma triglyceride levels and a fourfold increase in ischemic heart disease, but the mechanism is unclear. CD36 is a receptor/transporter that binds fatty acids of lipoproteins. CD36 deficiency has been linked with insulin resistance. There is strong evidence of in vivo interaction between platelets and atherogenic lipoproteins suggesting that atherogenic triglyceride-rich lipoproteins, such as VLDL, that are increased in diabetic dyslipidemia are important in this process. This study demonstrates that VLDL binds to the platelet receptor CD36, enhances platelet thromboxane A2 production, and causes increased collagen-mediated platelet aggregation. VLDL enhanced collagen-induced platelet aggregation by 1) shortening the time taken for aggregation to begin (lag time) to 70% of control (P < 0.001); 2) increasing maximum aggregation to 170% of control (P = 0.008); and 3) increasing thromboxane production to 3.318% of control (P = 0.004), where control represents platelets stimulated with collagen (100%). A monoclonal antibody against CD36 attenuated VLDL-enhanced collagen-induced platelet aggregation by 1) inhibiting binding of VLDL to platelets by 75% (P = 0.041); 2) lengthening lag time to 190% (P < 0.001); and 3) decreasing thromboxane production to 8% of control (P < 0.001). In support of this finding, platelets from Cd36-deficient rats showed no increase in aggregation, thromboxane production, and VLDL binding in contrast to platelets from rats expressing CD36. These data suggest that platelet Cd36 has a key role in VLDL-induced collagen-mediated platelet aggregation, possibly contributing to atherothrombosis associated with increased VLDL levels. Diabetes 52:1248–1255, 2003

Individuals with type 2 diabetes have at least four times greater risk of developing ischemic heart disease (IHD) than the general population. Type 2 diabetes and the metabolic syndrome are also associated with the atherogenic lipoprotein phenotype (1), characterized by elevated plasma triglyceride, increased VLDL production, increased small dense LDL levels, as well as decreased HDL. Recent data provide convincing evidence that increased plasma triglyceride levels are an independent risk factor for IHD (2), but the mechanism linking increased plasma triglyceride-rich lipoproteins, such as VLDL, to IHD remains uncertain.

Type 2 diabetes is associated with increased plasma markers of platelet activation (3–5), and pharmacological intervention to reduce plasma triglycerides results in reduction in markers of platelet activation (6). Platelet activation plays an integral role in atherothrombosis (7), since platelet aggregation forms the scaffolding for the initiation of a platelet plug after damage to the vascular wall or rupture of atherosclerotic plaque. It is increasingly apparent that atherogenic lipoproteins and platelets interact both in vivo and in vitro, producing a procoagulant phenotype (8–11), but the molecular interaction between triglycerides and platelets has not been elucidated, despite the increased risk of atherothrombosis and hypertriglyceridemia in type 2 diabetes.

CD36 is a fatty acid transporter (8) that binds native and oxidized lipoproteins in transfected cells (9–13) and facilitates supply of fatty acids for platelet thromboxane production (14), suggesting that CD36 may be capable of mediating interaction between triglyceride-rich VLDL and platelets. Recent data strengthen this suggestion since decreased expression of CD36 on macrophages was associated with lower uptake of hypertriglyceridemic VLDL remnants (15). In addition, elegant studies demonstrate the importance of CD36 in the development of insulin resistance and features of the metabolic syndrome (16–20). The contribution of CD36 to atherogenesis in the context of increased concentration of triglyceride-rich lipoproteins is further supported by transgenic mice data, showing that mice with a deletion of both Cd36 and apoE develop 77% less lesion area than control apoE knockout mice (21). Pharmacological reduction of Cd36 expression was associated with less atherosclerosis in vivo (22). In addition, Cd36 mediated oxidized phospholipid/LDL and macrophage interactions are important for atherosclerosis (23,24). The incidence of atherothrombotic events in

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CD36-deficient humans has not been studied directly, but macrophages from CD36-deficient humans bind and internalize 40% less oxidized LDL than macrophages that express CD36, and antibodies against CD36 inhibit up to 80% of oxidized LDL-induced foam cell production (25), suggesting that CD36 may play an important role in atherosclerosis. It is essential that further knowledge about the role of CD36 in atherothrombosis and insulin resistance in humans is obtained, since the incidence of CD36 deficiency is as high as 4% in African, Japanese, Thai, and Korean subjects, yet is almost undetectable in Caucasians (26).

Increasing evidence implicates triglyceride-rich lipoproteins in atherothrombosis. VLDL is present in the atherosclerotic plaque (27), and both humans and mice with type III hyperlipidemia apoE2/E2 homozygosity have increased levels of VLDL and remnant triglyceride-rich lipoproteins (28). Mice expressing human apoE2 (22) have virtually all of the characteristics of type III hyperlipoproteinemia, and their plasma cholesterol and triglyceride levels are two to three times those in normolipidemic mice that express human apoE3 (3/3). The 2/2 mice are markedly defective in clearing migrating VLDL particles and spontaneously develop atherosclerotic plaques, even on a regular diet.

The aim of this study was to investigate the interaction between VLDL and platelets, with a specific focus on the role of CD36 in VLDL-enhanced platelet aggregation.

RESEARCH DESIGN AND METHODS

Lipoprotein isolation. The protocol was approved by the local ethics committees (Cambridge and Southampton), and volunteers gave informed consent. Blood was obtained in EDTA from normal healthy volunteers 2 h after eating a light meal. By choosing the 2-h time point and by advising low-fat content of the meal preceding venepuncture, the production of chylomicrons and their subsequent remnants would be minimal. Plasma was diluted in a ratio of 4:1 with Optiprep (Nycoderm Pharma, Oslo, Norway), and VLDL was isolated by ultracentrifugation at 350,000g for 3 h at 17°C using a Beckman vertical 50Ti rotor. The resultant VLDL- and LDL-enriched fractions were purified further by gel filtration using Sepharose CL-6B (29) and PBS (pH 7.4), and purity was assessed using agarose gel electrophoresis and Sudan Black (Sigma, Poole, U.K.) staining (29). Copper oxidized LDL was prepared (29), and total protein, Apo B48 (Sigma), and cholesterol (Sigma) were quantified. Lipid peroxides (Peroxidase from Pierce, through Perbio) and electro- phoresis mobility (29) increased significantly after oxidation of LDL (from 5 to 1,000 μmol/l peroxides for 100 μg/ml LDL) and VLDL (from 5 to 60 μmol/l peroxides for 100 μg/ml VLDL). Lipoproteins did not contain any more endotoxin than PBS (E-Toxate; Sigma), suggesting that lipoprotein effects on platelets were not due to endotoxin contamination.

Platelet preparation. Blood from healthy young volunteers was obtained by venepuncture without a tourniquet after obtaining consent. Blood was collected gently into 0.109 mol/l sodium citrate (9:1 vol/vol). By using young venepuncture without a tourniquet after obtaining consent. Blood was collected gently into 0.109 mol/l sodium citrate (9:1 vol/vol). By using young venepuncture without a tourniquet. Blood was collected gently into 0.109 mol/l sodium citrate (9:1 vol/vol).

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Platelet aggregation. Platelet aggregation studies were performed on an APACT-4 aggregometer. When platelets aggregate, increased light is transmitted to allow both light absorption and inhibitory effects of VLDL to be observed. The effect of aspirin (acetylsalicylic acid; Sigma) was examined in certain experiments (40 μmol/l aspirin incubated with PRP for 20 min at room temperature). Typically, each run consisted of PRP + PBS + collagen, PRP + VLDL + collagen, PRP preincubated with aspirin + PBS + collagen, and PBS preincubated with aspirin + VLDL + aspirin. Experiments were also repeated with a maximum of oxidized VLDL. Maximum light transmission percentage (representing percent maximum aggregation) and lag time (i.e., the time for aggregation to begin after addition of collagen) were assessed.

Quantification of thrombomycin. The stable metabolite of thrombomycin A2 thrombomycin B2 (TXB2), was measured after aggregation using collagen ± aspirin ± VLDL. Immediately after the control sample (PRP + PBS) reached maximum aggregation, 80 μmol/l aspirin (in excess of the pharmacological dose used earlier) and 40 μmol/l EDTA were added before promptly freezing at −80°C. The samples were extracted using sequential acetone, hexane, and chloroform extraction steps and assayed at three dilutions for TXB2 by nonradioactive enzyme immunoassay as described by the manufacturer (Amersham Pharma Bioch T.I. Limited, Buckinghamshire, U.K.). Standard TXB2 was used to validate the extraction procedure in the presence of high lipoprotein levels.

Inhibition of collagen-induced aggregation by anti-CD36. The monoclonal antibody against CD36,18S-1G2 was chosen because it binds to a site on CD36 that inhibits CD63 function, including binding of oxidized LDL (13,22). This antibody was a kind gift from the VIth Human Leukocyte Differentiation Antigen Workshop via Dr. M. de Haas, CLB, Amsterdam, The Netherlands. The concentration of anti-CD36, preincubated for 5 min with PRP, was adjusted for each donor (0.2–200 μg/ml) to allow aggregation to occur within 10 min of the subsequent experiment. In the subsequent experiments the time-dependent concentration of anti-CD36, or an equal volume of PBS, were incubated with PRP for 5 min before adding VLDL or PBS for a further 5 min. A control isotype-matched antibody (against CD4 [ox35]) was used at an equal concentration to anti-CD36 and was a kind gift from Professor M. Glennie, Tenovus Research Laboratory, Southampton, U.K. A further control antibody, which was demonstrated by flow cytometry to bind specifically to platelets, was also used at the same concentration as anti-CD36 (anti-glucorticoid receptor receptor [GR]; AutogenBioCell, Wiltshire, U.K.). Anti-GR was selected since it is a monoclonal antibody that binds to platelets but has no effect on platelet activation, unlike many antibodies against other integrins on platelets.

Inhibition of VLDL binding to platelets by anti-CD36. To assess the inhibitory effect of anti-CD36 on VLDL binding to platelets, gel-filtered platelets ± anti-CD36/C4 ± VLDL were incubated with collagen in the aggregometer to allow binding of VLDL. To separate the bound VLDL so that the only VLDL detected was platelet-bound VLDL, the activated platelets were then washed three times to remove unbound VLDL, resuspended in a smaller volume of PBS to concentrate bound VLDL, and the platelet samples frozen. The platelet sample for analysis by enzyme-linked immunosorbent assay was 20 μl of either PBS or VLDL, preincubated with collagen, PRP preincubated with aspirin + VLDL + aspirin. Experiments were also repeated with a maximum of oxidized VLDL. Maximum light transmission percentage (representing percent maximum aggregation) and lag time (i.e., the time for aggregation to begin after addition of collagen) were assessed.

Inhibition of platelet thrombomycin production by anti-CD36. A time course of TXB2 production during platelet aggregation was carried out. From the time course experiments, aggregation was halted as the control reached maximum aggregation in subsequent experiments with anti-CD36. This time point was selected to study the dynamics of interaction of VLDL and CD36 rather than to assess total TXB2 production after platelet aggregation was complete.

The effects of plasma lipoproteins were eliminated by removal of plasma after centrifugation of PRP at 409g. Washed platelets were used in this experiment because gel-filtration resulted in too low a concentration of platelets. Activation by washing was markedly and significantly lower than activation by collagen as assessed by flow cytometry. Activation was compared between the same donor’s platelets with different treatment, and therefore suboptimal activation after washing was controlled for. The suspended platelets were used to repeat the aggregation experiments (± anti- CD36/C4 ± VLDL) before washing. The platelets were incubated with anti-CD36 (50 μl) for 30 min before being used in subsequent experiments with anti-CD36.

The antibody against CD36 was characterized further, in addition to the information provided by the VIth Human Leukocyte Differentiation Antigen
CD36, VLDL, AND PLATELET ACTIVATION

Workshop (29). Control experiments were run with an equal concentration of anti-CD4. Binding of anti-CD36 to platelets was confirmed by incubating anti-CD36 (0.4 mg/ml) with PRP and detecting platelet-bound antibody with rabbit-anti-mouse-horseradish peroxidase (RAM-HRP, 1:500; Dako). Lack of reactivity against collagen was assessed by binding collagen (0.2 mg/ml) to a microtiter plate, incubating with anti-CD36 (0.4 mg/ml) then detecting any bound antibody with RAM-HRP. Lack of reactivity of anti-CD36 directly against VLDL was assessed by binding anti-CD36 to a microtiter plate then incubating with VLDL (150 µg/ml) before detecting any bound VLDL using anti-ApoB-HRP (29).

**Platelet aggregation and lipoprotein binding in Cd36-deficient rats.** Six-month-old Spontaneously Hypertensive Rats (SHR/NCr1BR, designated as SHR throughout, known to be deficient in Cd36) and Wistar Kyoto Rats (WKY/NCr1BR, designated as WKY throughout) were killed by CO₂ inhalation, and blood was collected into citrate after cardiac puncture of the right ventricle, in accordance with Home Office animal guidelines. PRP was prepared, platelet aggregation ≥ VLDL initiated with collagen, and VLDL-binding experiments carried out as above. TXB₂ was measured after aggregation of platelets from 10-month-old SHR and WKY.

**Statistics.** Because the aim of the study was to examine the effect of normal VLDL, VLDL was isolated from different individuals with no apparent health problems, and the data were pooled for analysis. VLDL from different donors and PRP from different donors gave individual responses, but the overall direction of response was similar. Therefore, because of donor variability, data were normalized relative to control (platelets + collagen) as 100%. Within each run, the same donor’s VLDL was used; therefore, paired Student’s t tests were used to compare means of normally distributed data and data normalized by log transformation. By comparing the effects of the same donor’s VLDL in the presence of aspirin or anti-CD36, the inter-individual variations (e.g., differences in meals and physiological VLDL production and clearance) were minimized.

**RESULTS**

**The role of VLDL in platelet aggregation.** VLDL enhanced maximum collagen-stimulated platelet aggregation (represented by % light transmission) when compared with control (175% compared with control normalized to 100%; P = 0.008; n = 8 different platelet donors, and 8 different VLDL donors, Fig. 1A). VLDL shortened lag time, i.e., aggregation occurred more quickly (70% of control, P = 0.001; Fig. 1B). A representative platelet aggregation trace is shown in Fig. 2, labeled to define lag time and maximum aggregation. The effects of VLDL were dose dependent (Fig. 3). VLDL did not significantly alter aggregation in the presence of ADP or protease-activated receptor 1 (PAR-1) activating peptide or in the absence of agonist (data not shown). VLDL had no effect on platelet aggregation in the absence of collagen. Oxidized LDL had very similar enhancing effects to VLDL on collagen-induced platelet aggregation (data not shown).

**The effect of aspirin on VLDL-enhanced platelet aggregation.** Since aspirin is known to be effective in decreasing platelet activation in vitro and in both primary and secondary prevention of coronary heart disease (33,34), PRP was incubated with a pharmacological dose of aspirin or buffer before adding VLDL or PBS to the aggregometer and stimulating with collagen. While VLDL increased aggregation compared with collagen alone, pre-incubation of platelets with aspirin decreased aggregation in the presence of VLDL (175% of control decreased to 64% of control with aspirin; P = 0.006; Figs. 1A and 2). While VLDL shortened lag time, aspirin lengthened lag time (154% of control; P < 0.001; Figs. 1B and 2). Similar effects were observed with oxidized LDL.

**The role of thromboxane in VLDL-enhanced collagen-induced platelet aggregation.** Since we had shown that aspirin markedly attenuated the effect of VLDL-enhanced collagen-induced platelet aggregation, we measured TXB₂ production (n = 8 different platelet donors). Platelet aggregation with collagen was performed ± VLDL ± aspirin. VLDL dramatically increased TXB₂ when compared with collagen alone (3.318% compared with 100% control; P = 0.004; Fig. 1C). This increase was abolished with aspirin (decreased to 27% of control; P < 0.001). TXB₂ production by ADP-stimulated platelets pretreated with aspirin or buffer was not different, and there was no effect of VLDL in the absence of collagen (data not shown). Oxidized LDL produced similar results to VLDL (data not shown).

**The role of CD36 in VLDL-enhanced collagen-stimulated platelet aggregation.** CD36 binds both lipoproteins and collagen; therefore, we examined the effect of a monoclonal antibody against a site on CD36 that inhibits

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**FIG. 1. The effects of VLDL on collagen-stimulated platelet aggregation in the presence or absence of aspirin (n = 8 platelet donors, and 8 VLDL donors).** Results are expressed as percentage of control (platelets stimulated with collagen). Raw data were collected as light transmission (A) (percent representing maximum aggregation), lag time (B) (in seconds), and picogram of thromboxane produced (C). A: **control vs. control + VLDL, P = 0.008; control vs. control + aspirin, P = 0.025; **control + VLDL vs. control + VLDL + aspirin, P = 0.006; control + VLDL + aspirin vs. control + aspirin, P = 0.234. B: **control vs. control + VLDL, P = 0.001: control vs. control + aspirin, P = 0.002: ***control + VLDL vs. control + VLDL + aspirin, P < 0.001: control + VLDL + aspirin vs. control + aspirin, P = 0.911. C: **control vs. control + VLDL, P = 0.004: control vs. control + aspirin, P < 0.001: ***control + VLDL vs. control + VLDL + aspirin, P < 0.001: control + VLDL + aspirin vs. control + aspirin, P = 0.076. Control + aspirin = 13% of control, control + aspirin + VLDL = 27% of control.
CD36 function including oxidized LDL binding to CD36 on VLDL-enhanced platelet aggregation. PRP was preincubated with anti-CD36, anti-CD4, or PBS before incubating with VLDL or PBS and stimulating aggregation with collagen \( (n = 9 \) different platelet donors). Lag time was measured since anti-CD36 did not alter maximum aggregation. VLDL decreased lag time for collagen-stimulated platelet aggregation compared with control (70% of control; \( P = 0.013 \); Fig. 4A), while anti-CD36 increased the lag time in both the presence and absence of lipoproteins (to 190% of control; \( P < 0.001 \)). Therefore, anti-CD36 attenuated the lag-shortening effect of VLDL. The inhibitory effects of anti-CD36 were dose-dependent. Oxidized LDL produced similar results as VLDL (data not shown).

Anti-CD4 was used to control for the effects of nonspecific binding of an isotype IgG (Fig. 4A and C), and anti-GR was used as a control anti-platelet antibody (Fig. 4B; \( n = 6 \) platelet donors). Anti-CD4, anti-GR, and PBS all gave similar results. These data suggest that anti-CD36 increases lag time independently of nonspecific antibody effects on platelet function.

Aggregation was repeated after removal of plasma lipoproteins, and activation of platelets was assessed by flow cytometry using expression of CD62 (\( n = 7 \) different platelet donors). There was a trend for increased expression of CD62 in the presence of VLDL compared with collagen alone (219 ± 40.5 vs. 163 ± 37.2 mean fluorescence, respectively, \( P = 0.15 \)). Anti-CD36 significantly reduced activation in the presence of VLDL compared with

![FIG. 2. Representative platelet aggregation trace demonstrating VLDL-enhanced collagen-stimulated platelet aggregation and the effects of aspirin. The x-axis represents time (trace represents 7.5 min), while the y-axis represents light transmission (or aggregation). Lag time and maximum aggregation are defined on the traces.](image1)

![FIG. 3. Effect of various concentrations of VLDL on maximum platelet aggregation (A) and lag time (B). VLDL concentrations are given in micrograms of total (lipo)protein per milliliter. This is a representative dose-response curve since different donors of platelets and different donors of VLDL give various responses.](image2)

![FIG. 4. A: The effects of VLDL and anti-CD36 or anti-CD4 on lag time of collagen-stimulated platelet aggregation (\( n = 9 \) different platelet donors). Control represents platelets stimulated with collagen (100%). There were no significant differences between \( \alpha \)CD4 and control experiments throughout or between \( \alpha \)CD36 ± VLDL. Control vs. control + VLDL, \( P = 0.013 \); control vs. control + \( \alpha \)CD36, \( P < 0.001 \); control + VLDL vs. control + \( \alpha \)CD36 + VLDL, \( P < 0.001 \). B: The effects of anti-CD36 and the control antibody, anti-GR, on VLDL-inhibited lag time (\( n = 6 \) different platelet donors). Results are expressed relative to anti-GR (100%). Anti-GR vs. anti-GR + VLDL, \( P = 0.036 \); anti-GR + VLDL vs. anti-CD36 + VLDL, \( P = 0.009 \). The anti-GR data are in agreement with data generated with PBS and anti-CD4. C: Inhibition of VLDL binding to platelets with anti-CD36 (\( n = 5 \) different platelet donors). Results are expressed relative to control (100%). Control vs. \( \alpha \)CD36, \( P = 0.041 \); control vs. \( \alpha \)CD4, \( P = 0.772 \).](image3)
Collagen + VLDL (102.4 ± 18.1 vs. 219.4 ± 40.5 mean fluorescence, P = 0.004) but not when lipoproteins were absent. Similar effects were shown with the control antibody (anti-GR) as with the PBS control. **Inhibition of VLDL binding to platelets by anti-CD36.** Since CD36 binds lipoproteins, we determined whether anti-CD36 inhibited VLDL binding to platelets. Anti-CD36 inhibited binding of VLDL (detected as ApoB) compared with control (25% of control; P = 0.041; n = 5 different platelet donors; Fig. 4C). Collagen-stimulated platelets incubated with VLDL bound 0.39 μg/ml ApoB compared with 0.25 μg/ml in the absence of exogenous VLDL. No inhibition of VLDL binding was observed with the control antibody. Similar inhibition by anti-CD36 was also observed with oxidized LDL but not with native LDL (data not shown). **The role of thromboxane in CD36-mediated platelet aggregation.** To determine appropriate time points for comparisons of TXB₂ production, simultaneous time courses of aggregation and TXB₂ production were produced with anti-CD36 (a representative graph is shown in Fig. 5). Anti-CD36 delayed lag time but did not affect maximum aggregation. TXB₂ increased just before platelet aggregation became detectable. The production of TXB₂ was delayed with anti-CD36. In subsequent anti-CD36 experiments for TXB₂ measurement, aggregation was terminated at the control reached maximum aggregation (marked with an arrow on Fig. 5). During the early stages of aggregation, VLDL enhanced TXB₂ production (140% of control; P = 0.01; n = 6 different platelet donors; Fig. 6), while anti-CD36 attenuated VLDL-enhanced platelet TXB₂ production (to 8% of control; P < 0.001). In comparison, anti-CD4 did not decrease TXB₂ production. **Characterization of antibody against CD36.** The actions of the antibodies against CD36 and CD4 were characterized. We confirmed that anti-CD36, but not anti-CD4, bound to platelets. The effects of anti-CD36 on aggregation and VLDL binding were dose dependent. The antibodies did not cross-react with VLDL or collagen. It was demonstrated that both VLDL and oxidized LDL bound to platelets and that anti-CD36 inhibited binding of VLDL and oxidized LDL. Anti-CD36 did not activate platelets in the absence of an agonist. Throughout the study, an antibody against CD4 (not expressed on platelets) of the same isotype subclass as anti-CD36 was used. In addition, these results were reproduced in the presence of an antibody shown to bind to platelets (anti-GR). **Aggregation and VLDL binding in platelets from Cd36-deficient rats.** SHR from a colony known to be Cd36-deficient due to a specific deletion mutation (16) and WKY that express Cd36 were used to verify that Cd36 mediates the effects of VLDL on platelets. Maximum platelet aggregation was enhanced in platelets from WKY incubated with human VLDL compared with control buffer (P = 0.041; n = 6 rats from each strain; Fig. 7A), but there was no enhancement of aggregation in platelets from SHR incubated with human VLDL. Lag time was decreased in platelets from WKY with human VLDL (P < 0.001, Fig. 7B), but there was no decrease in lag time in platelets from SHR + VLDL. In addition, there was a trend toward decreased VLDL binding to platelets from SHR compared with WKY (P = 0.090; data not shown). TXB₂ production was measured after collagen-induced aggregation ≥ VLDL (n = 7). TXB₂ was increased in WKY in the presence of VLDL compared with the PBS control (P = 0.044, Fig. 7C), but this effect was abolished in SHR (P = 0.407). **DISCUSSION** The metabolic syndrome and type 2 diabetes are associated with elevated plasma triglycerides and an increased risk of IHD. The relation between increased plasma triglycerides and increased risk of atherothrombosis is currently poorly understood, but our study provides a potential molecular explanation for this association. This study is the first to demonstrate that CD36 mediates VLDL-enhanced collagen-induced platelet aggregation. CD36 increases collagen-induced platelet aggregation by shortening the lag time, increasing thromboxane production, and increasing maximum aggregation. Antibodies to CD36 remove the enhancing effect of VLDL by inhibiting binding of VLDL to platelets, attenuating thromboxane production, and prolonging the lag time. VLDL also enhanced platelet activation, and anti-CD36 inhibited the effects of VLDL to enhance platelet activation when as-

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**FIG. 5.** A representative comparison of thromboxane production with aggregation in the presence of VLDL and anti-CD36 to determine when to halt aggregation for TXB₂ measurements (Fig. 6). —, control; ...,control + VLDL; —, control + α-CD36; and —, control + α-CD36 + VLDL. Control represents platelets stimulated with collagen. * represents the point at which later experiments were terminated (Fig. 6).**

**FIG. 6.** The effects of VLDL and anti-CD36 on collagen-stimulated thromboxane production (n = 6 different platelet donors). Aggregation was halted when control reached maximum (Fig. 5). Control represents platelets stimulated with collagen (100%). Control vs. control + VLDL, P = 0.010; control vs. control + αCD36, P = 0.002; control + VLDL vs. control + αCD36 + VLDL, P < 0.001. αCD4 gave similar results to control.
FIG. 7. Maximum platelet aggregation (A) lag time (B), and thromboxane (C) production in platelets from Cd36-deficient SHR and Cd36-expressing WKY rats (n = 6 rats of each strain). Results are expressed relative to platelets from WKY rats incubated with buffer and stimulated with collagen. A: WKY vs. WKY + VLDL, P = 0.041; SHR vs. SHR + VLDL, P = 0.453; WKY + VLDL vs. SHR + VLDL, P = 0.012. B: WKY vs. WKY + VLDL, P < 0.001; SHR vs. SHR + VLDL, P = 0.953; WKY + VLDL vs. SHR + VLDL, P < 0.001. C: WKY vs. WKY + VLDL, P = 0.044; SHR vs. SHR + VLDL (expressed relative to SHR-VLDL), P = 0.407.

The changes in platelet aggregation, thromboxane production, and lipid binding demonstrated in this study could be due to a variety of Cd36 activities. We have clearly shown that Cd36 acts as a lipoprotein receptor. There is currently limited published evidence that VLDL binds to Cd36 (30); however, in addition to the evidence presented in this study, it is plausible that VLDL binds to Cd36 in a manner similar to oxidized LDL, long-chain fatty acids, and phospholipids from apoptotic cells. We have shown that Cd36 is unlikely to act as a collagen receptor, since VLDL does not alter the amount of collagen V binding to platelets from SHR or WKY rats and Cd36-deficient rats do not bind less collagen V than WKY rats expressing Cd36 (data not shown). It has previously been demonstrated that Cd36 facilitates supply of fatty acids necessary for platelet metabolism and thromboxane production (14), and it is therefore possible that Cd36 is acting as a lipid-internalizing receptor in this study. Finally, Cd36 could be acting as a transmembrane signaling receptor, although little is known about Cd36 signal transduction in platelets. VLDL binding to Cd36 may affect the collagen receptors, GPVI and α2-integrin, but this role warrants further investigation at a later stage. It is interesting to speculate that the activities of Cd36 demonstrated in our study make Cd36 a novel therapeutic target in the future. It would be desirable to downregulate platelet Cd36 without altering expression of Cd36 in the adipocyte, since this may exacerbate diabetes. One possible therapeutic target would be to differentially regulate...

We focused on Cd36 because Cd36 deficiency has been linked with insulin resistance (16–20) via defective fatty acid transport into muscle and adipose. Cd36 is a scavenger receptor that binds lipoproteins (9–13) and mediates uptake of arachidonic acid and thromboxane production by human platelets (35). In addition, the importance of Cd36 in atherogenesis has been demonstrated, since transgenic mice with a deletion of both Cd36 and apoE develop 77% less lesion area than control apoE knockout mice (21). Given that Cd36 expression has been reported to be similar in platelets from humans with type 2 diabetes and controls (36), it is plausible that the high levels of markers of platelet activation in type 2 diabetes (3–5) result from triglyceride-enhanced platelet activation via Cd36 rather than changes in platelet Cd36 expression per se.

We have demonstrated that a monoclonal antibody, raised against amino acids 155–183, which are important for oxidized LDL binding to Cd36 (13,32), significantly prolongs lag time, overcoming the lag-shortening effect of VLDL. Anti-Cd36 decreases binding of VLDL to platelets, suggesting that Cd36 is an important receptor for VLDL binding on platelets. Anti-Cd36 also negates the enhancing effects of VLDL on thromboxane production by platelets during aggregation, suggesting that VLDL binding to Cd36 is important for the thromboxane amplification loop. Control experiments carried out with an isotype IgG and an antibody that we have shown binds specifically to platelets were no different from PBS control experiments (Fig. 4) and were significantly different from results obtained with anti-Cd36, suggesting that anti-Cd36 has specific effects on VLDL binding to Cd36 rather than by virtue of antibody-related properties. In addition, to overcome potential problems of using antibodies in platelet function studies outlined below, we demonstrated that platelets from rats deficient in Cd36 showed no change in aggregation, VLDL binding, and thromboxane production in the presence of human VLDL compared with the control buffer. In contrast, platelets from rats expressing Cd36 showed enhanced aggregation and decreased lag time with human VLDL. Platelets from rats deficient in Cd36 did not exhibit increased VLDL-binding capacity or increased thromboxane production, unlike platelets from rats expressing Cd36. This strain of SHR has been well characterized with respect to Cd36 deficiency (16), including Cd36 deficiency in platelets (37), using cDNA microarrays, Western blots, and flow cytometry to demonstrate lack of Cd36 expression in adipose, heart muscle, and platelets from SHR (16,37).

It is important to consider the role of Cd36 in the present study, since VLDL does not alter the amount of collagen V binding to platelets from SHR or WKY rats and Cd36-deficient rats do not bind less collagen V than WKY rats expressing Cd36 (data not shown). It has previously been demonstrated that Cd36 facilitates supply of fatty acids necessary for platelet metabolism and thromboxane production (14), and it is therefore possible that Cd36 is acting as a lipid-internalizing receptor in this study. Finally, Cd36 could be acting as a transmembrane signaling receptor, although little is known about Cd36 signal transduction in platelets. VLDL binding to Cd36 may affect the collagen receptors, GPVI and α2-integrin, but this role warrants further investigation at a later stage. It is interesting to speculate that the activities of Cd36 demonstrated in our study make Cd36 a novel therapeutic target in the future. It would be desirable to downregulate platelet Cd36 without altering expression of Cd36 in the adipocyte, since this may exacerbate diabetes. One possible therapeutic target would be to differentially regulate Cd36 expression in adipose, heart muscle, and platelets from SHR (16,37)
transcription of CD36 via modification of tissue-specific transcription factor activity.

The use of platelets from rats deficient in Cd36 overcomes a number of problems associated with using antibodies in platelet function tests. The antibody against CD36 was selected specifically because it binds to a region of CD36 important for oxidized LDL binding (although the main binding site is likely to be 28–93 [13]), and it also does not activate platelets (13,32). It is known that platelets are activated by certain antibodies through the FcγRII receptor or complement. However, in our studies, use of this antibody was not associated with platelet activation. Moreover, FcRIIA receptors are found at low density on platelets and only bind aggregated or complexes of IgG. The results using platelets from SHR/WKY (Fig. 7) entirely support our observations obtained with anti-Cd36, suggesting that anti-Cd36 did not operate via the FcγRII receptor or complement. The SHR/WKY experiments also limit the probability that the effects of anti-Cd36 are due to interaction of anti-Cd36 with other known collagen receptors. In addition to decreasing the effects of VLDL on platelet function, anti-Cd36 also decreased collagen-induced aggregation in the absence of added VLDL (Fig. 4A). The inhibitory effect of anti-Cd36 on samples without exogenous VLDL is likely to be due to the inhibition of plasma lipoprotein binding to Cd36 (these experiments were carried out in platelet rich plasma containing endogenous lipoproteins). We verified that the inhibitory effect was due to plasma lipoproteins by examining purified platelets by flow cytometry. Anti-Cd36 significantly reduced platelet activation in the presence of VLDL but had no effect when lipoproteins were absent. The SHR/WKY experiments (Fig. 7) verify that VLDL binds to Cd36, altering aggregation, and that the results using anti-Cd36 are unlikely to be artifact.

We have compared the Cd36-deficient SHR strain with the Cd36-expressing WKY strain in this study. While these strains differ in more than just expression of Cd36, the data obtained with these models complement and confirm the data obtained with human platelets and the antibody against Cd36, to suggest that Cd36 mediates the triglyceride-induced changes in platelet aggregation. Collagen-induced platelet aggregation depends highly on the thromboxane amplification loop, and our study clearly demonstrates that VLDL enhances thromboxane production with collagen. The decrease in VLDL-enhanced platelet aggregation with aspirin treatment also strengthens the suggestion that thromboxane is important in VLDL-enhanced platelet aggregation. The aggregation/thromboxane results of our study agree with some earlier studies of LDL and VLDL (38–40) but disagree with others (41). Differences may be due to the collagen used (maybe the proportion of type V collagen [42]) or the experimental system used (e.g., adhesion versus aggregation, anticoagulant, and divalent cations). However, our data are the first to implicate VLDL in a thromboxane-mediated platelet aggregation pathway linked to Cd36.

The concentrations of lipoproteins used in this study range from physiological to pathological as assessed by cholesterol and apoB concentration (31) and are relevant to the concentrations found in individuals with the metabolic syndrome and type 2 diabetes. It is unclear just how high a concentration of lipoprotein may be attained in local microenvironments, but conceivably this could be very high in the vicinity of damaged endothelium or a partially ruptured atherosclerotic plaque.

In conclusion, we have shown that Cd36 mediates binding of VLDL to platelets and that this increases collagen-induced platelet aggregation and thromboxane production. Given that individuals with the metabolic syndrome and type 2 diabetes have increased concentrations of triglyceride-rich lipoproteins, such as VLDL, interaction between VLDL and platelet Cd36 may provide a novel cause of atherothrombosis in the metabolic syndrome and type 2 diabetes.

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CD36, VLDL, AND PLATELET ACTIVATION

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