Surface Expression of Collagen Receptor Fc Receptor-γ/Glycoprotein VI Is Enhanced on Platelets in Type 2 Diabetes and Mediates Release of CD40 Ligand and Activation of Endothelial Cells

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Diabetes is associated with an enhanced collagen-mediated platelet activation that contributes significantly to thromboischemic complications. In this study, the platelet collagen receptor glycoprotein VI (GPVI) was studied in 385 patients with type 2 diabetes. Surface expression of the platelet Fc receptor that forms a functional complex with GPVI was significantly increased in patients with diabetes compared with those without diabetes ($P = 0.02$). Fc receptor expression correlated with GPVI expression and was found to be independently associated with diabetes ($r = 0.529$, $P < 0.001$). Stimulation of GPVI through a specific anti-GPVI monoclonal antibody significantly enhanced surface expression of CD40L ($P = 0.006$). Because CD40L is a potent platelet-derived cytokine that is involved in thrombosis and atherosclerosis, we evaluated the effect of GPVI-mediated release of CD40L on activation of endothelial cells. Coincubation of GPVI-stimulated platelets resulted in substantial enhanced endothelial surface expression of CD62P, $\alpha_v\beta_3$, and intercellular adhesion molecule 1 ($P < 0.05$) and secretion of monocyte chemoattractant protein 1 of cultured human umbilical vein endothelial cells ($P < 0.01$). These results suggest that the function of collagen receptor GPVI is altered in type 2 diabetes and may play an important role in atherothrombotic complications. Inhibition of GPVI may be a promising pharmacological target in the treatment of high-risk diabetic patients. *Diabetes* 53:2117–2121, 2004

Type 2 diabetes is associated with a two- to threefold risk of death from coronary artery disease (1,2). Furthermore, accelerated atherosclerosis and diabetic microvascular disease make diabetes a leading cause of ischemic stroke, retinopathy, and chronic renal failure (3). Alteration of platelet function contributes to microthrombus formation and may play an important role in the pathogenesis of diabetic micro- and macroangiopathies (4–9). Diabetes has a number of effects on platelet function that may predispose to atherosclerosis and thrombosis. These include increased primary and secondary platelet aggregation (10–13); increased platelet activation (14,15) with release of the contents of α-granules, including β-thromboglobulin and platelet factor 4 (13); and enhanced surface expression and activation of platelet glycoprotein IIb-IIIa complex (14). Moreover, platelet nitric oxide synthase activity is reduced in diabetes (16). Furthermore, a hypersensitivity of platelets to collagen, the major extracellular matrix protein present in atherosclerotic tissue that induces platelet activation, has been described in diabetes (17). Elevated expression levels of the platelet Fc receptor that correlated with an increase in collagen-induced aggregation have been observed in diabetes (18,19). Only recently, the platelet glycoprotein VI (GPVI) has been identified as the major platelet collagen receptor (20). GPVI is a 60- to 65-kDa type I transmembrane glycoprotein that belongs to the Ig superfamily (20). GPVI forms a complex with the γ-chain of the Fc receptor at the platelet surface (20). Ligand binding to GPVI triggers tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif of the Fc receptor γ-chain, initiating downstream signaling via Syk kinases, linker for activation of T-cells, Src homology 2 domain–containing leukocyte phosphoprotein of 76 kDa, and phospholipase C, thus inducing platelet activation and secretion (20,21). We have recently shown that GPVI is critically involved in platelet-mediated arterial thrombosis (22), making the receptor a promising target for antiplatelet treatment in high-risk patients. The purpose of the present study was to evaluate the role of GPVI for platelet activation and platelet-mediated endothelial activation in patients with type 2 diabetes.

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Received for publication 11 August 2003 and accepted in revised form 19 April 2004.

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GPVI, glycoprotein VI; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; mAb, monoclonal antibody; MCP-1, monocyte chemoattractant protein 1; VCAM-1, vascular cell adhesion molecule-1.

RESEARCH DESIGN AND METHODS

A total of 385 patients who were referred to our hospital for evaluation of coronary artery disease were studied consecutively. We hypothesized that enhanced platelet surface expression of the collagen receptor GPVI is associated with type 2 diabetes. Type 2 diabetes was defined as possessing a fasting glucose >140 mg/dl or taking an oral hypoglycemic agent or insulin. Venous blood samples were taken from the cubital vein before coronary angiography. Using a multiple-syringe sampling technique, the first 2 ml of blood was discarded. Thereafter, 5 ml of blood was collected into a polypropylene syringe that contained citrate (23).

Platelet flow cytometry and monoclonal antibodies. Evaluation of surface expression of platelet membrane glycoproteins (CD32, GPVI, CD62P, CD40L, and CD61) with specific monoclonal antibodies (mAbs) and two-color whole-blood flow cytometry was conducted as described in detail elsewhere (24,25). Specific mAb binding was expressed as relative intensity of immunofluorescence and was used as a quantitative measurement of glycoprotein surface expression. Five thousand events were analyzed. The following mAbs were used: mAb anti-CD32 (clone AT10; Immunotech, Marseille, France), anti-CD62P (clone CLB Thromb/6; Biozol, Eching, Germany), anti-CD40L (clone 24–31; Calbiochem, Darmstadt, Germany), anti-CD61 (clone PM6/13; Biozol), anti-$\alpha_v\beta_3$ (LM609; provided by Dr. D. Cheresh, Scripps Clinic, La Jolla, CA), anti–intercellular adhesion molecule-1 (ICAM-1; clone 84H10; Beckman Coulter), and anti–vascular cell adhesion molecule-1 (VCAM-1; clone 1.G11B1; Serotec). mAb 4C9 was generated against soluble human GPVI in rat, and mAb 2D1 raised also in rat recognizes an irrelevant human antigen.

GPVI-dependent platelet secretion. Whole blood was drawn from normal individuals and collected in test tubes that contained 3.8% sodium citrate. Platelet-rich plasma was incubated with 0.1 μg/ml mAb 4C9 or 2D1, respectively, for 60 min. Thereafter, surface expression of CD62P and CD40L, as markers for platelet release, was determined by flow cytometry (26).

Incubation of endothelial monolayers with platelets. Primary human umbilical vein endothelial cells (HUVECs) were harvested by use of collagenase (Worthington) digestion and cultivated as described (26). Platelets were isolated from acid-citrate-dextrose–anticoagulated whole blood as described. Washed platelets were resuspended in Tyrode's solution–HEPES buffer (mmol/l: 2.5 HEPES, 150 NaCl, 12 $NaHCO_3$, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and 5.5 D-glucose, and 1 mg/ml BSA [pH 7.4]) to obtain a final platelet count of 2×10^8/ml. Thereafter, platelets were preincubated with mAb 4C9 or 2D1 (0.5 μg/ml each) for 30 min. The activated platelet suspension was added to wells of a 24-well culture plate covered with confluent monolayers of endothelial cells. Incubation was performed at 37°C without agitation in culture condition atmosphere for 2 h. Thereafter, platelets were removed through multiple gentle washing steps, and medium was added for another 10 h (27,28).

Determination of endothelial monocyte chemoattractant protein 1 secretion and surface expression of adhesion molecules. The supernatant of cultured endothelial cells that were treated with platelets was aspirated, centrifuged at 4,000 rpm for 10 min, and stored at −80°C. Concentration of monocyte chemoattractant protein 1 (MCP-1) was determined by enzyme-linked immunosorbent assay (Biermann) with a detection limit of 5 pg/ml. Surface expression of ICAM-1, VCAM-1, CD62P, $\alpha_v\beta_3$, and CD41 was determined by fluorescein isothiocyanate–conjugated specific mAbs and flow cytometry as described (29). After aspiration of the supernatant, endothelial monolayers were incubated with mAbs and the DNA-staining fluorochrome LDS 751 (Styry 18; Exciton) for 20 min. Thereafter, cells were mechanically detached through repetitive pipetting, and single-cell suspension was evaluated by flow cytometry for immunofluorescence in the forward scatter versus LDS 751 fluorescence scatter plot.

Statistics. Differences between the two study groups were evaluated by means of the appropriate nonparametric test (Mann-Whitney *U* test). A multiple logistic regression analysis that implemented an automatic stepwise selection algorithm for risk factor inclusion was performed to assess independent risk factors for diabetes.

RESULTS

Platelet surface expression of collagen receptor GPVI in patients with type 2 diabetes. We evaluated prospectively the surface expression of platelet Fc receptor (CD32) in a total of 385 consecutive patients. A total of 22.6% of these patients had type 2 diabetes, and 77.4% were nondiabetic. The group of nondiabetic patients compared with the diabetic group was not significantly different in regard to sex, smoking habits, hypercholesterolemia, or family history of coronary artery disease. Diabetic patients, however, had an increased BMI (diabetic vs. nondiabetic; 28.1 ± 4.7 vs. 26.7 ± 4.1 kg/m^2; $P = 0.015$), and hypertension was also significantly increased in this group (diabetic vs. nondiabetic: 80 [92.0%] vs. 201 [67.4%]; $P < 0.001$). Diabetic patients also had significantly higher creatinine levels (1.22 ± 0.48 vs. 1.07 ± 0.30 mg/dl; $P < 0.001$) mg/dl and enhanced serum levels of C-reactive protein (41.2 ± 81.3 vs. 20.4 ± 45.3 mg/l; $P = 0.002$) compared with nondiabetic patients. As shown in Fig. 1*A*, surface expression of the platelet Fc receptor (CD32) was significantly enhanced in diabetic patients compared with nondiabetic patients (42.4 ± 14.0 vs. 38.4 ± 12.3; $P = 0.02$). There was a significant correlation between platelet size as determined by the forward scatter and CD32 immunofluorescence ($r = 0.235$, $P < 0.001$; Fig. 1*B*). Moreover, forward scatter tended to be enhanced in diabetic compared with nondiabetic patients; however, this did not reach statistical significance (56.8 ± 12.9 vs. 54.6 ± 13.1; $P = 0.139$). To test whether CD32 is associated with diabetes independently of cardiovascular risk factors, we performed a multiple logistic regression analysis that included systemic hypertension, hypercholesterolemia, active smoker, BMI, and CD32. Among the variables tested, CD32 was associated independently with diabetes (coefficient 0.024; $P = 0.008$). In a subpopulation of patients ($n = 122$), we additionally analyzed surface expression of GPVI, which forms a complex with the γ-chain of the Fc receptor at the platelet plasma membrane (20). There was a strong correlation between expression of the Fc receptor and GPVI ($r = 0.529$, $P < 0.001$; Fig. 1*C*). CD32 and 4C9 were significantly correlated in both diabetic and nondiabetic patients (diabetes: $r = 0.381$, $P = 0.026$; no diabetes: $r = 0.529$, $P < 0.001$). 4C9 immunofluorescence tended to be enhanced in diabetic compared with nondiabetic patients, however, without reaching statistical significance (diabetes, 31.4 ± 8.1; no diabetes, 30.1 ± 10.1; $P = 0.202$). In patients with diabetes, we did not find a correlation between surface expression of the collagen receptor and HbA_{1c} values (data not shown). However, CD32 significantly correlated with actual blood glucose levels ($r = 0.121$, $P = 0.027$; Fig. 1*D*).

Effects of ligation of GPVI on platelet secretion of P-selectin and CD40 ligand. Interaction of collagen with platelets induces aggregation and secretion (20). Platelet surfaces express at least two distinct receptors for collagen, the integrin $\alpha_2\beta_1$ and GPVI (20). To evaluate the role of GPVI for platelet secretion, we stimulated GPVI with the specific mAb 4C9 or an irrespective control mAb (2D1). In preliminary experiments with platelets derived from healthy volunteers, we found that ligation of GPVI through mAb 4C9 resulted in substantial release of the α-granule protein P-selectin (CD62P) and the cytosolic cytokine CD40L ($P < 0.01$; Fig. 2). To test whether enhanced platelet expression of collagen receptor in diabetes is associated with an increase in GPVI-dependent platelet release reaction, we analyzed surface expression of CD40L upon 4C9 stimulation. Patients with type 2 diabetes showed a significantly enhanced surface expression of CD40L on GPVI-stimulated platelets compared with nondiabetic patients ($P = 0.003$; Fig. 3). The increments of CD40L upon stimulation were significantly greater in dia-

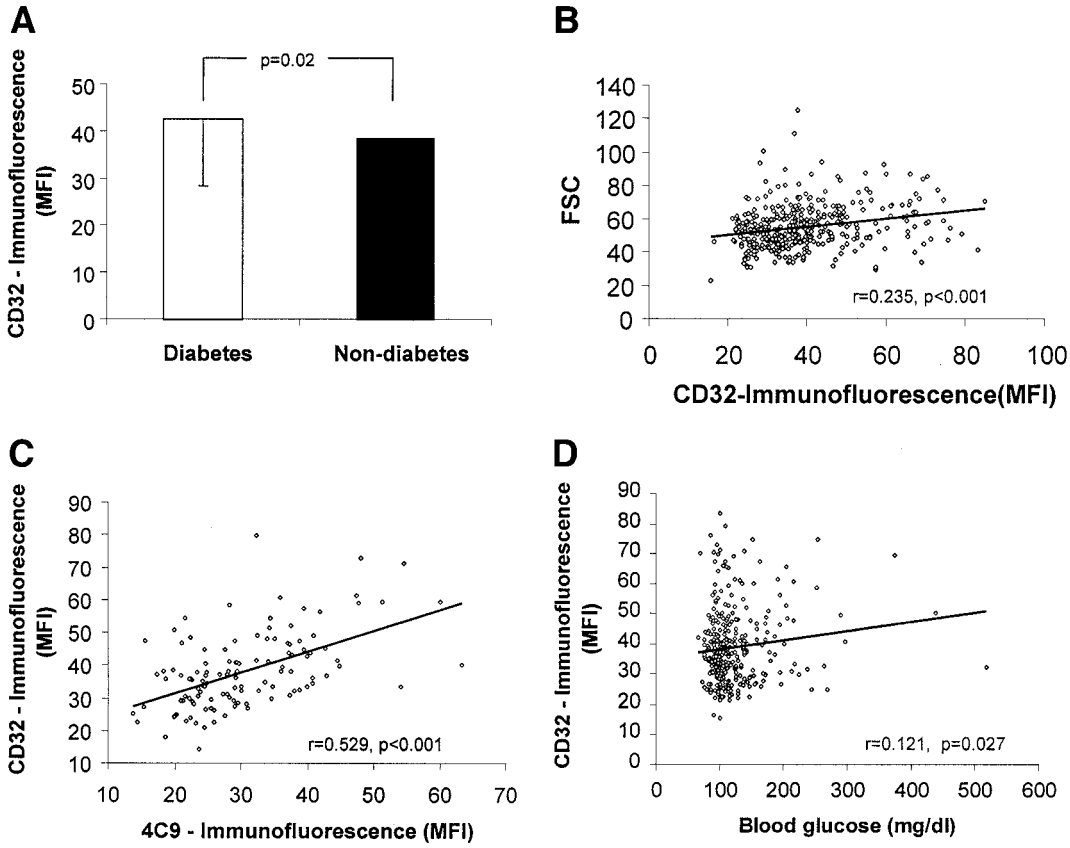

FIG. 1. Surface expression of platelet GPVI in diabetic patients. *A*: Surface expression of the platelet Fc receptor (CD32) in diabetic and nondiabetic patients. Data are presented as mean intensity of CD32 immunofluorescence (MFI). *B*: Correlation between platelet forward scatter and CD32 immunofluorescence. *C*: Correlation of surface expression of platelet Fc receptor (CD32) and of GPVI (mAb 4C9) in a consecutive subpopulation of diabetic and nondiabetic patients (n = 122). *D*: Correlation of CD32 immunofluorescence and blood glucose.

betic compared with nondiabetic patients (6.3 ± 5.1 vs. 3.5 ± 3.9, respectively; $P = 0.006$).

Effects of GPVI/ligation-stimulated platelets on activation of endothelial cells. Activated platelets release CD40L, a major platelet-derived proatherogenetic cytokine (30). To analyze the effect of GPVI-dependent platelet CD40L release on endothelial activation, monolayers of cultured HUVECs were coincubated with platelets that were pretreated with the specific mAb 4C9 or a control mAb 2D1. As shown in Fig. 4, coincubation of GPVI-stimulated platelets (4C9-treated) with HUVECs resulted in a significant increase in endothelial surface expression of CD62P, CD41, $\alpha_v\beta_3$, ($P < 0.05$), and ICAM-1 ($P = 0.09$) and secretion of MCP-1 compared with control (2D1; $P < 0.01$).

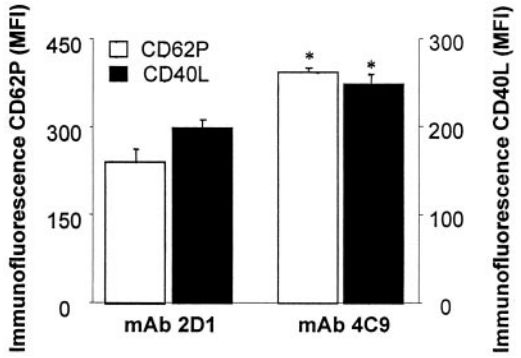

FIG. 2. Effects of ligation of GPVI on platelet secretion of platelets derived from healthy volunteers. Platelets obtained from healthy volunteers (n = 4) were incubated for 60 min with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter, surface expression of P-selectin and CD40L was analyzed by flow cytometry. Data are presented as MFI. *$P < 0.01$ between non- and 4C9-stimulated platelets.

DISCUSSION

The major findings of the present study are as follows: *1*) patients with type 2 diabetes have an enhanced platelet surface expression of the collagen receptor GPVI compared with individuals without diabetes, *2*) stimulation of GPVI results in substantial secretion of CD40L and GPVI-

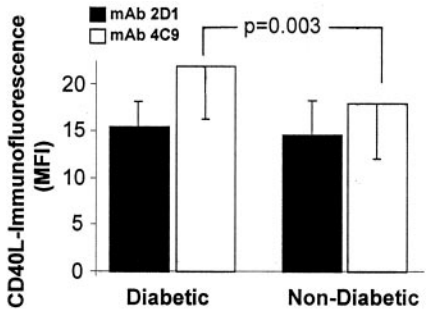

FIG. 3. Effects of ligation of GPVI on platelet secretion of CD40L in diabetic and nondiabetic patients. Platelets were incubated for 60 min with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter, surface expression of CD40L was analyzed by flow cytometry. Data are presented as CD40L MFI.

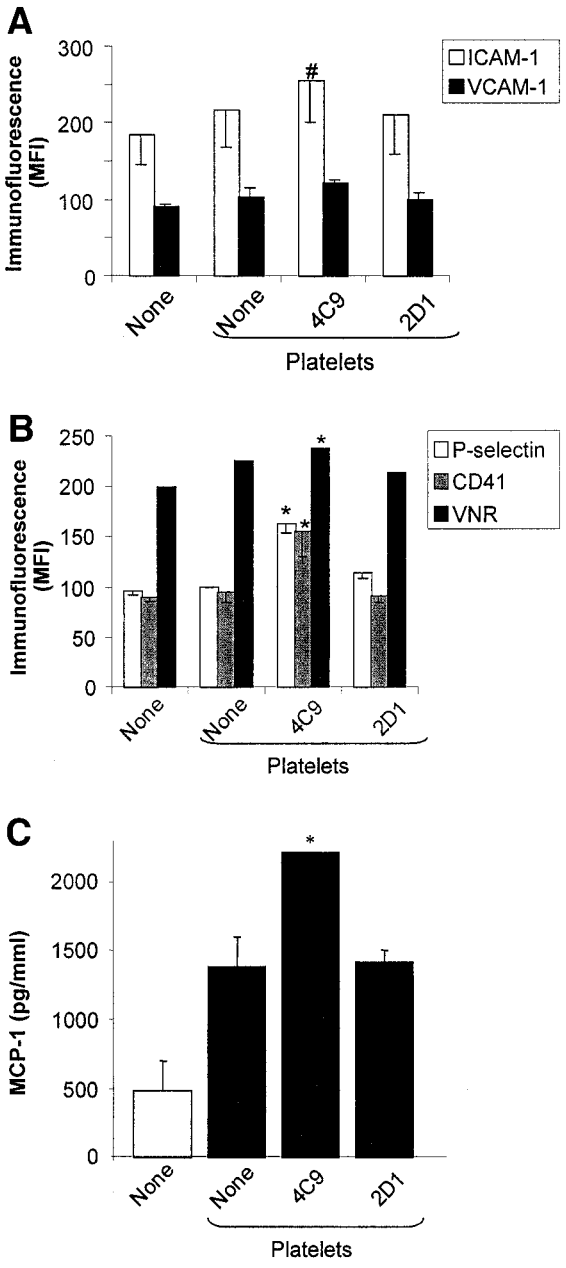

FIG. 4. Effects of coincubation of HUVECs with GPVI/ligation-stimulated platelets on endothelial cell activation. Monolayers of HUVECs were coincubated for 2 h with platelets in the presence of mAb 4C9 or mAb 2D1, respectively. Thereafter, platelets were removed and surface expression of VCAM-1, ICAM-1, CD62P, CD41, and $\alpha_v\beta_3$ on HUVECs (*A* and *B*) was determined after 10 h of further cultivation. *$P < 0.05$ between 4C9 and 2D1 values; #$P = 0.09$. *C*: Secretion of MCP-1 was determined as described (28). *$P < 0.01$ between 4C9 and 2D1 values.

dependent CD40L release is enhanced in type 2 diabetes, and *3*) coincubation of cultured endothelial cells with GPVI/ligation-stimulated platelets induces substantial endothelial activation. The present findings indicate that surface expression of the platelet collagen receptor is enhanced in diabetic patients, which may increase the risk for platelet-dependent thrombus formation and plaque progression.

Circulating activated platelets induce atherosclerosis and vascular complications by promoting microthrombus formation. We have recently shown that platelets are critical for the development of atherosclerosis (31). Chronic inhibition of platelet adhesion to the arterial wall attenuates substantial development of atherosclerotic lesions in mice (31). A variety of platelet functional changes have been described in diabetic patients, including abnormalities of signal transduction events (32–34), enhanced thromboxane A_2 formation (9), an increased surface expression of platelet membrane glycoproteins such as GPIIb-IIIa (14,35), and an enhanced sensitivity of diabetic platelets to collagen (32,33). Antiplatelet therapy with aspirin is recommended as a primary and secondary prevention strategy in patients with diabetes (36) and has been shown effectively to reduce morbidity and mortality in diabetes. In addition, chronic aspirin therapy reduces complications of diabetic retinopathy, possibly by reducing microthrombosis in retinal capillaries (37). Thus, effective antiplatelet therapy might be a promising therapeutic strategy in individuals with diabetes.

Only recently has the GPVI been recognized as the fundamental collagen receptor that mediates platelet adhesion to vascular lesions (20,22). Inhibition of GPVI abrogates arterial thrombus formation substantially, making GPVI an attractive pharmacological target. GPVI forms a complex with the Fc receptor γ-chain at the platelet surface (20). Ligand binding to GPVI triggers platelet activation, aggregation, and secretion (20). In the present study, we found that circulating platelets of patients with diabetes are characterized with enhanced surface expression of the Fc receptor that correlates with the expression of GPVI on the platelet plasma membrane. Enhanced surface expression of platelet Fc receptor has been shown to be associated with an increased aggregation response to collagen (18,19). Moreover, variation of GPVI surface density regulates thrombus formation on collagen (38). Thus, the enhanced surface expression of GPVI described herein may enhance the risk of thrombotic events and progression of diabetic vasculopathy. This conclusion is supported by the fact that GPVI ligation results in substantial release of the tumor necrosis factor–like cytokine CD40L. Moreover, we found that GPVI-dependent stimulation of platelets results in substantial alterations of the chemotactic and adhesive properties of endothelial cells. CD40L-mediated endothelial activation substantially contributes to atherogenesis, because inhibition of CD40L retarded the progression of atherosclerosis in mice (39) and led to a collagen-rich stable plaque phenotype very likely attributable to decreased matrix metalloproteinase-1 and -2 activity. We recently found that platelets can induce matrix-degrading activity in HUVECs via platelet-associated CD40L and that this mechanism is prominent in platelet-endothelial interaction (27). In the present study, we describe that inhibition of GPVI through soluble GPVI is effective in reducing platelet release of CD40L. Thus, inhibition of GPVI-mediated platelet function may offer a novel and promising therapeutic strategy for the prevention of vascular complications in patients with diabetes. Moreover, drug resistance to current antiplatelet drugs such as aspirin and clopidogrel has been described in high-risk patients (40,41)

In conclusion, our data indicate that GPVI is a major platelet receptor involved in the pathophysiology of thrombosis and atherosclerosis in diabetes. GPVI block-

ade may not only inhibit platelet adhesion to vascular lesions and thereby prevent physical vessel occlusion but also prevent platelet-CD40L–mediated inflammatory cascades, accelerating atherosclerosis and plaque progression in diabetes.

ACKNOWLEDGMENTS

This study was supported by grants from the Deutsche Forschungsgemeinschaft (Ga 481/4-2, Graduiertenkolleg "Vaskuläre Biologie" GRK 438) and Wilhelm Sander-Stiftung. Z.L. was supported by the Deutscher Akademischer Austauschdienst (DAAD, German Academic Exchange Service). The presented results are part of her doctoral thesis.

We thank Kirsten Langenbrink and Sandra Kerstan for assistance.

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