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der Technischen Universität München
Deutsches Herzzentrum München des Freistaates Bayern
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Characteristics of Platelet Surface Expression of Glycoprotein VI in Type 2 Diabetes

Zhongyan Li

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2. Univ.-Prof. A. Kastrati

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Contents

Abbreviations

AA, arachidonic acid

ACE inhibitors, angiotensin-converting enzyme inhibitors

ACS, acute coronary syndrome

ADP, adenosine diphosphate

CAD, coronary artery disease

CD, cluster of determinants

CD40L, CD40 ligand

CHOS, cholesterol

Col, collagen

CRP, C-reactive protein

ECM, extracellular matrix

ELISA, enzyme linked immuno-sorbent assay

FACS, fluorescence-activated cell sorter

Fb, fibrinogen

FcR, Fc receptor

FcγR, Fc receptor γ-chain

FITC, fluorescein isothiocyanate

Fn, fibronectin

GP, glycoprotein

HbA1c, hemoglobin A1c

HUVEC, human umbilical vein endothelial cell

ICAM-1, intercellular adhesion molecule-1

Ig, immunoglobulin

ITAM, immunoreceptor tyrosine-based activation motif

Lam, laminin

LDL, low density lipoprotein

mAb(s), monoclonal antibody(ies)

MCP-1, monocyte chemotactic protein-1

MI, myocardial infarction

MMP, matrix metalloproteinase

NF-κB, nuclear factor-κB

NO, nitric oxide

NOS, nitric oxide synthase

PBS, phosphate buffer saline

PDGF, platelet-derived growth factor

PE, phycoerythrin

PFA, paraformaldehyde

PF4, platelet factor 4

PRP, platelet-rich plasma

SAP, stable angina pectoris

TNF, tumor necrosis factor

tPA, tissue-type plasminogen activator

TxA_2, thromboxane A_2

UAP, unstable angina pectoris

uPA, urokinase-type plasminogen activator

Vn, vitronectin

vWF, von Willebrand factor

1 Introduction

1.1 Blood platelets in primary and secondary hemostasis

The normal function of platelets is to arrest hemorrhage from wounds after tissue trauma, which requires adhesion to altered vascular surfaces and rapid cellular activation with the ensuing accumulation of additional platelets and fibrin into a growing thrombus. The main trigger for the formation of a hemostatic thrombus after traumatic vascular injury is the loss of the endothelial cell barrier between extracellular matrix (ECM) components and flowing blood (Figure 1-1 B). The response of platelets to this event develops in three successive but closely integrated phases that involve adhesion, activation and aggregation.

Blood platelets play a central role in the physiology of primary hemostasis. Adhesion of still resting platelets to the damaged vessel wall is the first step of primary hemostasis and is known as "primary adhesion" (4). Attachment of already activated platelets to structures of the subendothelium is known as "secondary adhesion".

The adhesion process is regulated by glycoproteins (GPs) of the platelet membrane. The first contact between circulating blood platelets and the vessel wall lesion (platelet tethering) is established by an interaction of the platelet glycoprotein Ib-V-IX with collagen-immobilized von Willebrand factor (vWF) (103, 119). The vWF-GPIb interaction is "fast-on" and relatively "fast-off", and results in a rolling of platelets along the exposed subendothelium (122, 123). This slowing of the platelets allows binding of the activating collagen-receptor, GPVI, to its ligand resulting in activation of platelet integrins and subsequent firm adhesion, where the reactions between receptor and ligand are relatively "slow-on" but irreversible (99) (Figure 1-1 B). Direct GPVI-collagen interactions are crucial for initial platelet tethering and subsequent stable platelet adhesion and aggregation at sites of arterial injury (88). Ligation of GPVI during platelet-collagen interactions can shift $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins from a low to a high affinity state (99). The bindings of integrin $\alpha_2\beta_1$ to collagen and $\alpha_{IIb}\beta_3$ to vWF are the principal interactions underlying firm adhesion (123) (Figure 1-1 C).

The binding of the platelet collagen receptor to collagen, in particular, leads to activation and to shape changes of the adherent platelets (activation and spreading). A primary hemostatic clot can form completely after activation of the platelets. Starting from released arachidonic acid (AA) the adherent and activated platelets form thromboxane A_2 (TxA_2) that reinforces the activation process after the release into the extracellular space and binding to a specific thromboxane receptor (Figure 1-1 D).

During adhesion and shape change the platelet begins to release stored substances into its surroundings. This process is known as secretion, release or degranulation.

The thrombocytic release of adenosine diphosphate (ADP) that is contained in the dense bodies is of central importance in the activation and recruitment of resting platelets to the platelet aggregate (platelet recruitment). ADP can activate the glycoprotein IIb-IIIa complex (GPIIb-IIIa) through binding to a specific membrane receptor (45) (Figure 1-1 D).

In addition to hemostasis, the platelet interacts with many physiological mechanisms via released factors. Released growth factors such as platelet-derived growth factor (PDGF) have mitogenic effects for fibroblasts in the vicinity of a platelet thrombus and participate in proliferative processes in the region of a vessel wall lesion and the formation of intima. Furthermore, pro-inflammatory factor CD40 ligand (CD40L) is released from activated platelets. CD40L causes decisive changes in the chemotactic and adhesive properties of vessel wall cells (54) (Figure 1-1 D).

The interaction of circulating platelets with adherent platelets proceeds through activated $\alpha_{IIb}\beta_3$ integrin receptors. This stimulates further platelets to undergo aggregation. Two phases of aggregation are distinguished: primary and secondary aggregation. During the primary phase the platelets are loosely linked to each other by "fibrinogen bridges" (Figure 1-1 E). This process is reversible. Secondary aggregation sets in after a time lag and begins when the platelets have released granule components. Secretion of the granules reinforces the activation process and initiates the secondary, irreversible phase of aggregation (45). Shear forces (that can increase the probability of contact between two platelets), Ca^{2+} and fibrinogen are decisive for a normal aggregation process (45). The glycoprotein IIb-IIIa complex plays a central role in aggregation (Figure 1-1 E). In the resting state, soluble plasma fibrinogen cannot bind to the platelet surface as binding sites for fibrinogen in the

region of the glycoprotein IIb-IIIa complex only become accessible after activation. The binding of GPIIb-IIIa is strongly dependent on Ca^{2+} and leads to the formation of platelet aggregates (Figure 1-1 E).

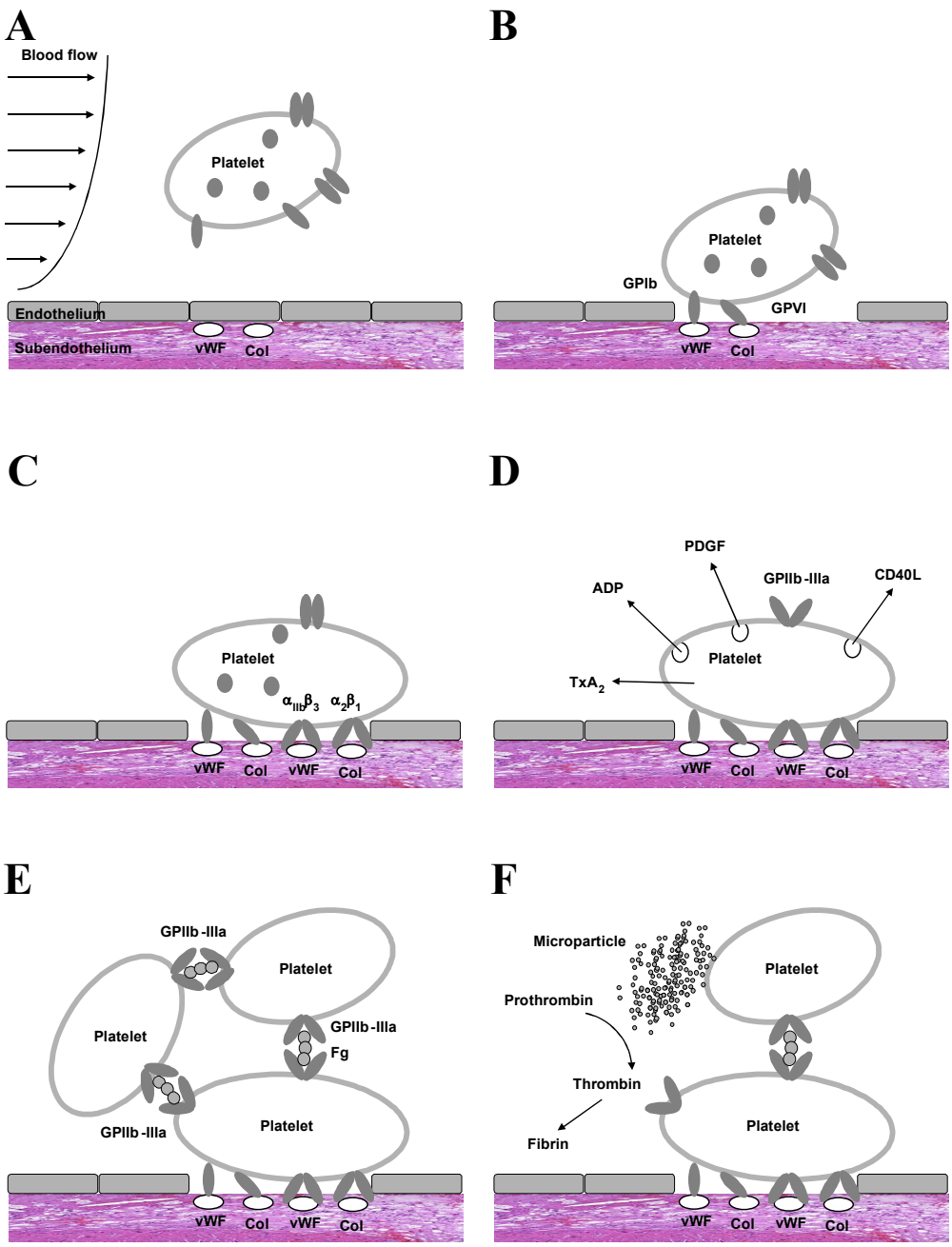

Figure 1-1. Blood platelets in primary and secondary hemostasis. *vWF: von Willebrand factor; Col: collagen; TxA_2: thromboxane A_2; ADP: adenosine diphosphate; PDGF: platelet-derived growth factor; CD40L: CD40 ligand; Fg: fibrinogen; GPIb: glycoprotein Ib; GPVI: glycoprotein VI; GPIIb-IIIa: glycoprotein IIb-IIIa. (Adapted from reference 47)*

The primary platelet aggregation is relatively unstable and an efficient hemostasis requires the consolidation of the platelet-rich thrombus (secondary hemostasis). Secondary hemostasis begins with the activation of the coagulation cascade and the formation of thrombin and fibrin (Figure 1-1 F). The activated platelet surface plays a decisive role in activating the coagulation cascade (procoagulant activity) (33). Deposition of fibrin on the platelet aggregate leads to a consolidation of the thrombus via cross-linking. The platelet-fibrin conglomerate contracts (clot retraction) and thus further strengthens the hemostatic blood clot.

During the activation process, platelets extrude and expel small membrane vesicles (microparticles) from their plasma membranes (Figure 1-1 F); these particles exert a strong procoagulant activity in the vicinity of the platelet activity by formation of the prothrombinase complex on their surfaces (45). The GPIIb-IIIa receptor participates in the platelet-dependent formation of thrombin and in the generation of microparticles. Formation of microparticles around the platelet aggregates catalyses thrombin generation and thus fibrin formation that stabilizes the platelet thrombus (Figure 1-1 F).

1.2 Platelet membrane glycoproteins

Platelets express glycoproteins on their membranes that mediate the interactions of the platelets among themselves as well as with the subendothelial matrix, with plasmic coagulation factors, and with endothelial cells or leukocytes.

Platelet membrane glycoproteins are classified into different groups according to their characteristic molecular structures: integrins, leucine-rich glycoproteins, selectins, immunoglobulin-like adhesion receptors and lysosomal integral membrane proteins (103) (Table 1-1).

Integrins are adhesion receptors that link structures of the cytoskeleton with the extracellular matrix. Integrins consist of α- and β- subunits and are subdivided on the basis of the β-chain which pairs with a specific α-chain and together the two proteins form a functional receptor. Integrins interact with numerous glycoproteins (e.g. collagen, fibronectin, fibrinogen, laminin, thrombospondin, vitronectin, von Willebrand factor) (58). To date, five different integrins have been described on platelets, three

of the β_1 class ($\alpha_2\beta_1$ = collagen receptor, $\alpha_5\beta_1$ = fibronectin receptor, $\alpha_6\beta_1$ = laminin receptor) and two of the β_3 class ($\alpha_{IIb}\beta_3$ = fibrinogen receptor, $\alpha_v\beta_3$ = vitronectin receptor) (103) (Table 1-1).

Table 1-1. Platelet membrane glycoproteins

Classification	Electrophoretic classification	Cluster of determinants	Number of receptor copies	Ligand specificity
Integrins				
$\alpha_2\beta_1$	GPIa-IIa	CD49b	1000	Col
$\alpha_5\beta_1$	GPIc-IIa	CD49c	1000	Fn
$\alpha_6\beta_1$	GPIc´-IIa	CD49f	1000	Laminin
$\alpha_{IIb}\beta_3$	GPIIb-IIIa	CD41-CD61	60,000-100,000	Fb, Fn, Vn, vWF
$\alpha_V\beta_3$	GPαv-IIIa	CD51-CD61	100	Vn, Fb, Fn
Leucine-rich glycoproteins				
--	GPIb-V-IX	CD42a-b-c	25,000	vWF, Thrombin
--	GPIV(GPIIIb)	CD36	15,000-25,000	Col, Thrombospondin
Selectins				
--	P-selectin	CD62P	12,000	αMb2, PSGL-1
Immunoglobulin-like adhesion receptors				
--	ICAM-2	CD102	5000	LFA-1
--	PECAM-1	CD31	3000	?
--	GPVI	?	3700	Col
Lysosomal integral membrane proteins				
--	GP53	CD63	3000	?

Col: collagen; Fb: fibrinogen; Fn: fibronectin; Vn: vitronectin; vWF: von Willebrand factor. (Adapted from references 43, 45)

Platelets contain two membrane glycoprotein complexes, GPIb-V-IX and GPIV, which are characterized by their richness in the amino acid leucine. The GPIb-V-IX complex forms adhesion receptors for von Willebrand factor and plays a central role

in primary hemostasis. The main task of GPIb-V-IX is the adhesion of circulating platelets to vWF immobilized in collagen fibrils in spite of the high shear forces that exist in regions of arterial flow. The GPIb-V-IX complex consists of four subunits. GPIbα (150kDa) and GPIbβ (27kDa) are covalently linked to each other by disulfide bridges. The GPIbα subunit is of decisive significance for the receptor function. In the region of the extracellular domain GPIbα possesses binding sites for von Willebrand factor and thrombin (118).

Selectins are vascular adhesion receptors that mediate the heterotypical interactions of cells. P-selectin in platelets is stored in thrombocytic α-granules. P-selectin is not expressed on resting platelets. However, activation leads to the rapid release and surface expression of P-selectin on platelets. So it can be used as a marker of platelet activation.

1.3 Platelet collagen receptors and their signaling pathways

The first step in the hemostatic cascade is platelet interaction with the exposed ECM at sites of injury. Among the macromolecular constituents of the ECM, collagen is considered to play a major role in this process. Platelet adhesion and aggregation on collagen is an integrated process that involves several platelet agonists which act through a variety of surface receptors, including integrins, immunoglobulin (Ig) -like receptors and G-protein-coupled receptors.

1.3.1 GPVI and its signaling pathway

GPVI was first identified as a 60-65 kDa platelet glycoprotein by 2-D gel electrophoresis over twenty years ago (20). GPVI is a type I transmembrane glycoprotein, which belongs to the immunoglobulin receptor superfamily (21, 101) . Human GPVI is composed of 339 amino acids and contains two Ig-C2-like extracellular domains formed by disulfide bonds, a mucin-like stalk, a transmembrane region, and a short 51 amino acid cytoplasmic tail (Figure 1-2).

GPVI has a positively charged arginine in its transmembrane region which is essential for association with the Fc receptor γ-chain (FcR γ-chain, FcγR) (10, 154). The first six juxtamembrane amino acids are essential for the interaction with the FcR γ-chain (Figure 1-2). The GPVI cytosolic tail contains a proline rich motif that binds selectively to the SH2 domain of the Src family tyrosine kinases, Fyn and Lyn (133). The cytoplasmic part of GPVI contains a calmodulin binding domain (5). Calmodulin is constitutively associated with GPVI in platelets and undergoes delayed dissociation upon activation although the functional significance of this is not known.

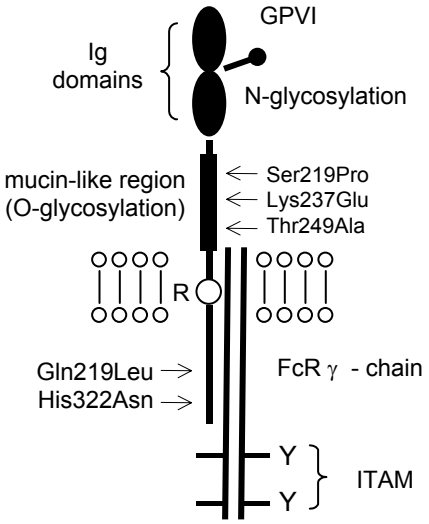

Figure 1-2. The GPVI-Fc receptor γ-chain complex. *GPVI consists of two Ig domains linked to a mucin-rich region that has a number of sites for O-linked glycosylation. The transmembrane domain has an arginine group that is required for the association with the Fc receptor γ-chain (FcR γ-chain) through a salt bridge. The FcR γ-chain is present as a disulfide-linked homodimer and has two tyrosines in a conserved sequence known as an immunoreceptor tyrosine-based activation motif (ITAM). (Adapted from reference 101)*

Crosslinking of GPVI leads to tyrosine phosphorylation of the FcR γ-chain on its immunoreceptor tyrosine-based activation motif (ITAM) by the Src kinases Fyn and Lyn (14, 30, 108). This leads to binding and subsequent activation of the tandem SH2-domain-containing tyrosine kinase, Syk, which initiates a downstream signaling cascade that culminates in activation of a number of effector enzymes including

PLCγ2, small G proteins and PI 3-kinase (134). The adapter LAT and SLP-76 play critical roles in this signaling cascade. So in this process the GPVI-FcγR complex transduces outside-in signals by an immunoreceptor-like mechanism that involves $p72^{SYK}$ activation, results in PLCγ2 activation, and leads to release of granule contents and platelet aggregation (6).

In general, ligand binding to GPVI triggers tyrosine phosphorylation of the ITAM of the Fc receptor γ-chain initiating downstream signaling via Syk kinase, LAT, SLP-76, and phospholipase C, thus, induces platelet activation and secretion (1, 101).

1.3.2 GPVI is the major signaling receptor for collagen on platelets

Platelet surface expresses at least two distinct receptors for collagen, the integrin $\alpha_2\beta_1$ and the platelet-specific receptor GPVI (101). A third receptor that figures prominently at the very onset of adhesion, the GPIb-V-IX complex, does not bind directly to collagen, but rather to von Willebrand factor that has become immobilized onto collagen. More recently, it has been shown that the GPVI/ FcR γ-chain complex is a key receptor for all types of collagen (59).

Jung and Moroi demonstrated that the affinity of the integrin $\alpha_2\beta_1$ for collagen is regulated by intracellular signals mediated by GPVI. They showed that several platelet agonists, including ADP, thromboxanes and GPVI-specific stimuli, increase the affinity of $\alpha_2\beta_1$ for monomeric or soluble collagen from a low to an intermediate or high affinity state (62, 63, 95). This work led to revision of the original so called "two-site, two-step" model (121) (which proposed $\alpha_2\beta_1$ as the major collagen receptor in hemostasis and thrombosis) and the proposal that the initial interaction of collagen through GPVI leads to activation of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, and that this in turn mediates stable adhesion to collagen and thereby reinforces the signaling through GPVI (99, 148).

Platelet adhesion to collagen at high shear rates ($>$600 s^{-1}) requires vWF immobilized on collagen. This interaction is essential for the initial capture or tethering of platelets by vWF and is critically dependent on the fast-on rate of association between vWF and GPIbα (123). This interaction, however, also has a fast-off rate of association that leads to rolling of platelets on a vWF surface for

several minutes until $\alpha_{IIb}\beta_3$ –mediated stable adhesion (via vWF) is seen (122). In contrast, stable adhesion occurs rapidly on a collagen-coated surface through integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ (99, 123). Nieswandt et al. speculated that the GPVI/FcR γ-chain complex may be involved in the process of platelet activation, leading to firm adhesion to vWF through activated $\alpha_{IIb}\beta_3$ (Figure 1-3) (101). Together, these observations show an important role for the interaction of vWF with GPIb and $\alpha_{IIb}\beta_3$ in platelet adhesion to collagen that is largely dependent on functional GPVI.

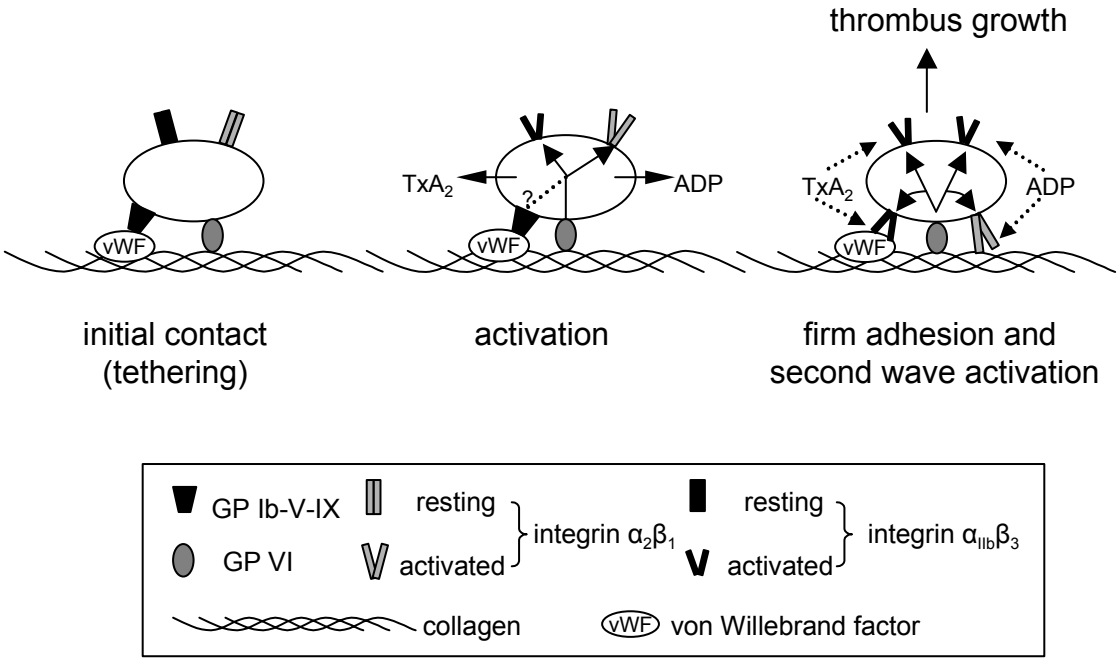

Figure 1-3. Revised model for platelet adhesion to collagen. *The initial contact (tethering) to the extracellular matrix is mediated predominantly by GPIbα-vWF and GPVI-collagen interactions. In a second step, GPVI-collagen interactions initiate cellular activation followed by shifting of integrins to high affinity state and the release of second wave agonists, most importantly ADP and TxA_2. GPIb-mediated signaling may amplify GPVI-induced activation pathways. Cellular activation and upregulation of integrin affinity is proposed to be a strict pre-requisite for adhesion. Finally, firm adhesion of platelets to collagen through activated $\alpha_2\beta_1$ (directly) and $\alpha_{IIb}\beta_3$ (indirectly via vWF or other ligands) results in sustained GPVI-signaling, enhanced release, and procoagulant activity. In this process, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ have partially redundant roles. Released ADP and TxA_2 amplify integrin activation on adherent platelets and mediate thrombus growth by activating additional platelets. (adapted from reference 101)*

It is now established that the initial platelet contact with collagen and the subsequent initiation of integrin activation, i.e. adhesion and thrombus growth is strictly dependent on functional GPVI. These developments identify a new sequence of events in the initial phase of hemostasis and thrombosis and place GPVI in a central position in this complex process (Figure 1-3) (101). It is now proposed that under high shear flow conditions, GPIbα and GPVI act in concern to tether platelets to the ECM through their respective ligands, vWF and collagen. The fast-off rate of these interactions prevents the rapid onset of stable adhesion. The generation of intracellular signals from GPVI, and possibly GPIb converts β_1– and β_3– integrins ($\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$) from a low to a high affinity state and induces the release of soluble agonists, most importantly ADP and TxA_2 (which also induce integrin activation). Activated $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ integrins now initiate firm adhesion by binding to collagen and vWF, respectively, and this process is reinforced by the autocrine action of the released mediators. In turn, integrin-mediated adhesion strengthens GPVI-collagen interactions leading to enhanced signaling and further upregulation of integrin activity, enhanced release, and the development of procoagulant activity. Integrin-mediated signaling events are also likely to contribute to these processes. Finally, the accumulation of released ADP and TxA_2 results in the activation of further platelets, i.e. thrombus growth (Figure 1-3).

The aforementioned revised model of platelet attachment to the subendothelium highlights a central role of GPVI-collagen interactions in all major phases of thrombus formation, i.e. platelet tethering, firm adhesion and aggregation at sites of arterial injury (e.g. during acute coronary syndrome).

1.4 Platelet CD40 ligand

CD40 ligand (CD40L, CD154, gp39) is a 39 kDa transmembrane pro-inflammatory glycoprotein belonging to the tumor necrosis factor (TNF) family. CD40L was originally identified in T lymphocyte, where it has a role in the immune response by binding to its receptor on B cells, CD40 (125). Both CD40L and CD40 have also been identified on other cells within the vasculature, including endothelial cells, smooth muscle cells, monocytes, and macrophages, where they have been

implicated as mediators of inflammation (80). The pioneering work of Henn and coworkers (54) established that CD40L and CD40 also exist in platelets and that platelets can also mediate functions via CD40L. They showed that CD40L is cryptic in unstimulated platelets but rapidly becomes exposed on the platelet surface after platelets are activated (54). They further showed that surface-expressed CD40L is proinflammatory and capable of inducing the expression of chemokines (e.g. Interleukin-8 and monocyte chemotactic protein-1), adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin) (54), and tissue factor by ligating CD40 on endothelial cells and monocytes (128).

CD40L has now been demonstrated to have an important role in inflammation. Platelets express CD40L on activation, which induces proinflammatory changes in endothelial cells via endothelial CD40 (54, 128). Ligation of CD40 on endothelial cells (69) results in activation with adhesion molecule and tissue factor expression and production of proinflammatory cytokines and chemokines. Platelet CD40L can also mediate the inflammatory cascades, leading to matrix degradation and plaque rupture. In addition to its role in inflammation and atherosclerosis, CD40L is involved in thrombosis: at high shear stress, CD40L binds directly to platelet $\alpha_{IIb}\beta_3$ via the integrin binding sequence KGD, enhancing thrombus formation and inducing platelet spreading via outside-in integrin signaling (3). Platelet CD40L may be a pivotal link between the processes of thrombosis, inflammation and atherosclerosis (2).

1.5 Platelets and inflammation

Besides their fundamental role in hemostasis and thrombosis, platelets have been recognized to be involved in inflammatory mechanisms (116). Platelets contain a variety of proinflammatory compounds such as eicosanoids, cytokines, and growth factors that are stored in substantial amounts in their granules and that are released within seconds upon platelet activation (52). Therefore, accumulation of activated platelets at sites of vascular lesions might result in high concentrations of platelet-derived substances that alter chemotactic and adhesive properties of vascular cells (52). Thus, platelets might support chemotaxis and recruitment of monocytes into the subendothelium at an early stage in atherogenesis (116).

Endothelium dysfunction and injury are the basis of the onset of the atherosclerotic process (117). Platelet-derived substances have been shown to induce a variety of genes within endothelial cells involved in molecular mechanisms of early inflammation (52, 116). Among the early inflammatory response genes, monocyte chemotactic protein-1 (MCP-1) is expressed in activated endothelium (106). MCP-1 belongs to the c-c chemokine family and attracts blood monocytes at subnanomolar concentrations to inflammatory sites (142). MCP-1 gene expression is regulated on a transcriptional level involving transcription factor nuclear factor-κB (NF-κB) (140) (Figure1-4). Increased levels of MCP-1 mRNA were found in atherosclerotic lesions (75). Chemotaxis and transmigration of circulating monocytes through the endothelial surface is a prerequisite for monocyte-macrophage transformation-a mechanism involved in early steps of atherosclerosis (117).

Intercellular adhesion molecule-1 (ICAM-1, also referred to as CD54) is a major adhesion receptor of the immunoglobulin-type family and is expressed in an activation-dependent manner on endothelium (56, 149). ICAM-1 can mediate the adhesion of neutrophils, monocytes and, later, lymphocytes to the inflamed vessel wall (131).

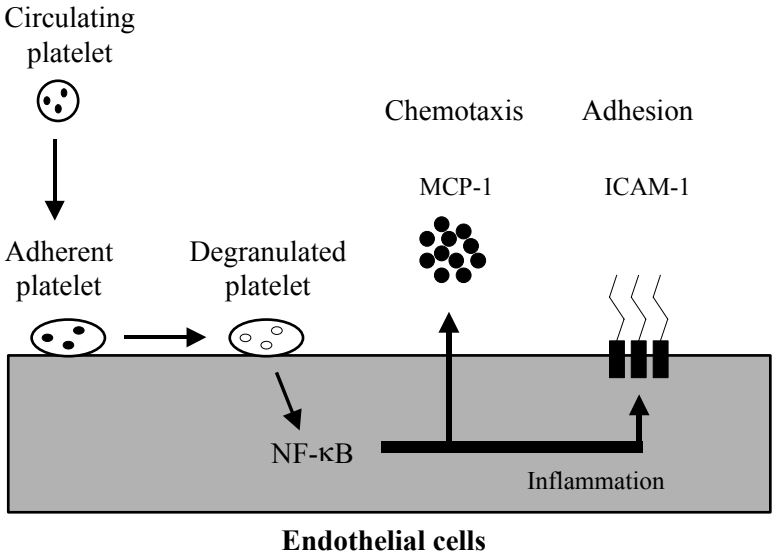

Figure 1-4. Platelet-endothelium interaction. *Activated platelets can alter the chemotactic and adhesive properties of endothelial cells. NF-κB is decisive for the platelet-mediated endothelial formation of MCP-1 and ICAM-1. NF-κB: transcription factor nuclear factor-κB; MCP-1: monocyte chemotactic protein-1; ICAM-1: intercellular adhesion molecule-1. (adapted from reference 45)*

The expression of early inflammatory response genes such as MCP-1 or adhesion molecules like ICAM-1 is regulated by transcription factor NF-κB in endothelium (23)(Figure 1-4). Our group has shown that activated platelets induce activation of the transcription factor NF-κB (41, 44).

It has recently been shown that activated platelets can decisively alter the chemotactic (MCP-1) and adhesive (ICAM-1) properties of endothelial cells (41, 44, 54), via an NF-κB-dependent mechanism (41), which is the early step in atherogenesis. Platelet-induced activation of the NF-κB system might contribute to early inflammatory events in atherogenesis.

As discussed in the preceding text, the secretion of chemotactic substances such as MCP-1 and the surface expression of ICAM-1, which represents a major receptor for monocyte adhesion to endothelial cells (71), are induced through the release of potent, cytokine-like substances (interleukin-1, CD40 ligand) by the activated platelets (27, 44, 54). These experimental results support the hypothesis that inflammatory changes in the vessel wall occur either in the vicinity of a platelet-rich thrombus or arise after contact of activated platelets with the intact vessel wall and that these changes favor the insertion of monocytes (formation of foam cells) and the migration of smooth muscle cells (intima proliferation) and thus promote the atherogenetic process. In this way blood platelets may play a central role in the occurrence of atherosclerotic reconstruction process in the vicinity of the vessel wall.

1.6 Historical background of diabetes mellitus and coronary artery disease

Diabetes mellitus magnifies the risk of cardiovascular morbidity and mortality (112). Besides the well-recognized microvascular complications of diabetes, such as nephropathy and retinopathy, there is a growing epidemic of macrovascular complications, e.g. coronary artery disease (CAD), particularly in the burgeoning type 2 diabetic population. The role of diabetes as a major independent risk factor for CAD has been well established. We focus on type 2 diabetes, characterized by insulin resistance and inadequate beta cell insulin secretion, because the patients represent more than 90% of those with diabetes and atherosclerosis.

CAD causes much of the serious morbidity and mortality in patients with diabetes, who have a two- to fourfold increase in the risk of CAD (31). In one population-based study (49), the 7-year incidence of first myocardial infarction (MI) or death for patients with diabetes was 20%, but was only 3.5% for non-diabetic patients. Patients with diabetes but without previous MI carry the same level of risk for subsequent acute coronary events as non-diabetic patients with previous MI.

Diabetes also worsens early and late outcomes in acute coronary syndrome (ACS). In unstable angina pectoris (UAP) or non-Q-wave MI compared with control, the presence of diabetes increases the risk of in-hospital MI, complications of MI, and mortality (66, 82). Patients with diabetes also have an adverse long-term prognosis after MI, including increased rates of reinfarction, congestive heart failure, and death (82). In fact, the 5-year mortality rate following MI may be as high as 50% for diabetic patients-more than double that of non-diabetic patients (55). Thus, diabetes belongs to a special category of risk factors for vascular diseases.

The abnormal metabolic state that accompanies diabetes causes arterial dysfunction. Relevant abnormalities include chronic hyperglycemia, dyslipidemia, and insulin resistance. These factors render arteries susceptible to atherosclerosis. Atherogenesis is a complex process involving platelet-endothelium adhesion as early trigger for atherosclerotic lesion formation. Diabetes alters function of multiple cell types, including endothelium, smooth muscle cells, and platelets, indicating the extent of vascular disarray in this disease. Here, platelet dysfunction in diabetes will be emphasized.

1.7 Platelets and type 2 diabetes

These patients with type 2 diabetes mellitus show not only accelerated atherosclerosis but also increased morbidity and mortality due to thrombotic complications of atherosclerosis (141), so atherosclerosis and vascular thrombosis are major contributors, and it is generally accepted that platelets are contributory.

Diabetes has a number of effects on platelet function that may predispose to atherosclerosis and thrombosis. These include increased adhesiveness, an exaggerated primary and secondary platelet aggregation both spontaneous and in

response to stimulating agents (83, 120, 130, 153), increased platelet activation (137, 138) with release of chemical substances and proteins from their dense and α-granules, including thromboxane B_2 (38, 129), β-thromboglobulin (13, 129), platelet factor 4 (13, 130), and fibronectin (38).

Platelets from diabetic subjects are hypersensitive to stimulating agents and show a reduced threshold for aggregation when stimulated with agonists under *ex vivo* conditions (67). Platelets obtained from type 2 diabetic patients showed higher aggregation in response to ADP than platelets from healthy controls (74), which was especially apparent in diabetic patients with macrovascular disease (25). Recently, a hypersensitivity of platelets to collagen, the major extracellular matrix protein present in atherosclerotic tissue that induces platelet activation, has been described in diabetes (104), which has been proposed as a contributing factor to the increased incidence of vascular disease seen in diabetes.

It was reported that platelets from diabetic subjects had decreased membrane fluidity and changes in intraplatelet signaling pathways (145). In platelets, as in endothelial cells, elevated glucose levels lead to activation of protein kinase C, decreased production of platelet-derived nitric oxide (NO), and increased formation of O^{2-} (7). In diabetes, platelets also show disordered calcium homeostasis (77). Disordered calcium regulation may contribute significantly to abnormal activity, since intraplatelet calcium regulates platelet shape change, secretion, aggregation and thromboxane formation. Moreover, patients with diabetes have increased platelet surface expression and activation of glycoprotein Ib (GPIb), which mediates binding to von Willebrand factor, and GPIIb-IIIa, which mediates platelet-fibrin interaction (135, 137, 145). Recently, an elevated expression level of the platelet Fc receptor (FcγRIIA) has been observed in diabetes that correlated with an increase in collagen-induced aggregation (15, 16).

These abnormalities may result from decreased endothelial production of the antiaggregants nitric oxide and prostacyclin, increased production of fibrinogen, and increased production of platelet activators, such as thrombin and von Willebrand factor. Moreover, platelet nitric oxide synthase (NOS) activity is reduced in diabetes (84). Loss of sensitivity to the normal restraints exercised by prostacyclin and nitric oxide generated by the vascular endothelium presents as the major defect in platelet function in diabetes (145).

In experimentally-induced diabetes reduced fibrinolytic activity has been demonstrated which may result from platelet release of fibrinolysis inhibitors and may lead to a more thrombogenic state. A higher concentration and enhanced release of plasminogen activator inhibitor (PAI-1) exists in patients with type 2 diabetes (61, 109). It was postulated that PAI-1 synthesis by megakaryocytes may be under the control of insulin (61).

These results about platelet dysfunction in diabetes are summarized in Table 1-2.

Table 1-2. Assessment of platelet function in diabetes mellitus

Assay	Result	Reference
Membrane fluidity	Decreased	Vinik et al., 2001 (145)
<u>Platelet aggregation</u>		
ADP-induced	Increased	Sobol et al., 2000 (130) Yazbek et al., 2003 (153)
Arachidonic acid-induced	Increased	Yazbek et al., 2003 (153)
Collagen-induced	Increased	Osende et al., 2001 (104)
<u>Markers of platelet activation</u>		
Thromboxane B_2	Increased	Garcia Frade et al., 1987 (38) Small et al., 1986 (129)
β-Thromboglobulin (βTG)	Increased	Small et al., 1986 (129)
Platelet factor 4 (PF4)	Increased	Sobol et al., 2000 (130)
Fibronectin	Increased	Garcia Frade et al., 1987 (38)
<u>Glycoprotein expression</u>		
GPIb	Increased	Tschoepe et al., 1990 (135) Vinik et al., 2001 (145)
GPIIb-IIIa	Increased	Tschoepe et al., 1992 (137) Vinik et al., 2001 (145)
Fc receptor (FcγRIIA)	Increased	Calverley et al., 2002 (15) Calverley et al., 2003 (16)
NOS activity	Decreased	Martina et al., 1998 (84)
Release of PAI-1	Increased	Jokl et al., 1995 (61) Rabini et al., 1999 (109)

Taken together, diabetic abnormalities increase intrinsic platelet activation and decrease endogenous inhibitors of platelet activity. The causes for this activation are multifold: altered exposure and/or abundance of glycoprotein receptors for agonists and adhesive proteins on the platelet surface, increased binding of fibrinogen, decreased membrane fluidity, altered platelet metabolism and changes in intraplatelet signaling pathways (130). These mechanisms may explain the enhanced thrombotic potential characteristic of diabetes.

2 Background and objectives of the present study

Type 2 diabetes mellitus is associated with a two- to threefold risk of death from coronary artery disease (39, 64). Alteration of platelet function contributes to microthrombus formation and may play an important role in the pathogenesis of diabetic micro- and macroangiopathies (17, 24, 50, 152). Diabetes has a number of effects on platelet function that may predispose to atherosclerosis and thrombosis. These include increased primary and secondary platelet aggregation (83, 120, 130, 153), increased platelet activation (137, 138), and enhanced surface expression and activation of platelet glycoprotein IIb-IIIa complex (137). 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 (104), which has been proposed as a contributing factor to the increased incidence of vascular disease seen in diabetes. An elevated expression level of the platelet Fc receptor (FcγRIIA) has been observed in diabetes that correlated with an increase in collagen-induced aggregation (15, 16). Recently the platelet glycoprotein VI (GPVI) has been identified as the major platelet collagen receptor (101) . GPVI and the Fc receptor γ chain signaling subunit with which GPVI forms a complex at the platelet surface are both required for collagen-mediated platelet adhesion and activation (101). Our group has recently shown that GPVI is critically involved in platelet-mediated arterial thrombosis (88) making the receptor a promising target for antiplatelet treatment in high-risk patients. The GPVI expression in diabetes mellitus remains unclear. In this study, we quantitated platelet FcR γ-chain and GPVI expression in patients with diabetes after hypothesizing that this cohort may express

higher levels than non-diabetic patients. Activated platelets alter endothelial chemotactic and adhesive properties, which is a key event for early atherogenesis, plaque formation and development of vulnerable lesions. Whether GPVI-mediated platelets can activate endothelial cells remains poorly understood.

Accordingly, the current study was undertaken

1) to document the expression levels of GPVI/FcR γ-chain in diabetes population;
2) to evaluate the effect of ligation of GPVI on platelet secretion;
3) to characterize the effects of GPVI/ligation-stimulated platelets on activation of endothelial cells.

3 Materials and methods

3.1 Study and patients

3.1.1 Monoclonal antibodies for flow cytometry

In this present study, the following monoclonal antibodies (mAbs) were used as fluorescein isothiocyanate (FITC, green) or phycoerythrin (PE, red) conjugates as indicated.

Anti-CD61 is a monoclonal antibody representing the surface expression of the β_3 subunit (GPIIIa) of the platelet surface antigen GPIIb-IIIa and the vitronectin receptor $\alpha_v\beta_3$. We identified platelets by size and CD61-PE immunofluorescence. (Clone PM6/13, purchased as PE-conjugate from Biozol, Eching, Germany).

Anti-CD62P binds to the α-granule membrane glycoprotein P-selectin that is exclusively surface exposed on the activated platelet surface and is used as a marker for α-degranulation. (Clone CLB-Thromb/6, commercially obtained as FITC-conjugate from Immunotech, Marseille, France).

Anti-CD32 is a monoclonal antibody directed against platelet FcR γ-chain (FcγRIIA). (Clone AT10, FITC labeled anti-CD32 was purchased from Biozol, Eching, Germany).

Anti-CD40L is specific for platelet CD40-ligand. (Clone 24-31, purchased as FITC-conjugate from Calbiochem, Darmstadt, Germany).

mAb 4C9 was generated against soluble human GPVI in rat.

mAb 2D1 that was also raised in rat recognizes an irrelevant human antigen (β3-endonexin). In the study, 2D1 was used as a control antibody.

Human GPVI (hGPVI) was cloned from cultured megakaryocytes as described elsewhere (89). Purified GPVI was shown to inhibit collagen-induced platelet aggregation.

Fluorescein isothiocyanate: Sigma, Deisenhofen, Germany.

4C9-FITC: 4C9 was conjugated to FITC according to standard protocols in our laboratory and used to characterize platelet surface expression of GPVI.

Phosphate buffer saline (PBS): Sigma, Deisenhofen, Germany.

Paraformaldehyde (PFA): Sigma, Deisenhofen, Germany.

We used a fluorescence-activated cell sorting-Calibur (FACS Calibur) flow cytometer (Becton-Dickinson, Heidelberg, Germany).

3.1.2 Study population

A total of 385 patients that were admitted to German Heart Center Munich, Germany, with a diagnosis of cardiovascular diseases were entered randomly and consecutively onto the study. These cardiovascular diseases include coronary artery disease (stable angina pectoris ⟨SAP⟩, unstable angina pectoris ⟨UAP⟩, or myocardial infarction ⟨MI⟩) or other cardiovascular diseases (valvular heart disease, arrhythmia, cardiomyopathy, etc). CAD patients have been proven by angiography. Diabetes mellitus (one of the cardiovascular risk factors) was the major consideration. Prospectively, 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 blood glucose greater than 140 mg/dl, or taking oral hypoglycemic agents or insulin. In addition to diabetes, information was also obtained

regarding the presence of other cardiovascular risk factors (hypertension, abnormal lipid profile, current cigarette smoker, obesity, family history of premature coronary disease).

Venous blood samples were taken from the cubital vein prior to coronary angiography. Using a multiple-syringe sampling technique the first two milliliters of blood were discarded. Thereafter, five milliliters of blood were collected into a polypropylene syringe that contained citrate (42). Informed consents were obtained from all the patients enrolled in the study before blood sampling took place.

3.2 Platelet function analysis

3.2.1 Platelet preparation

We evaluated the surface expression of platelet membrane glycoproteins (CD32, GPVI, CD62P, CD40L, CD61) with specific monoclonal antibodies and two-color whole blood flow cytometry. Preparation and immunolabeling of platelets with mAbs for flow cytometric analysis were performed. Immediately after blood was collected from patients into 3.8% trisodium citrate, fresh blood was first diluted with PBS (in a ratio of 1:50) in order to minimize *in vitro* aggregate formation. The platelets in the whole blood sample were tagged with a fluorochrome-labeled, platelet-specific monoclonal antibody (CD61-PE = red). At the same time another fluorochrome-labeled, activation-specific antibody (CD32-FITC, 4C9-FITC, CD62P-FITC or CD40L-FITC = green) was added to the whole blood. After incubation in the dark at room temperature for 60 minutes, the incubation mixture was quenched with 300 μL 0.5% paraformaldehyde / PBS solution (PH 7.4) and then used for the flow cytometric analysis. Table 3-1 illustrated the protocol of the experiment.

Table 3-1. Platelet flow cytometry

35μl citrated (3.8%), anticoagulated whole blood diluted 1:50 with PBS
+ 5μl PE-labeled anti-CD61 antibody (red fluorescence)
+ 5μl FITC-labeled CD32, 4C9, CD62P or CD40L antibody (green fluorescence)
+ 5μl PBS, 4C9 or 2D1
Incubation for 60 minutes at room temperature in the dark
Quenching and dilution with 300μl 0.5% paraformaldehyde / PBS solution

Final concentration : 4C9: 0.1 μg/ml
2D1: 0.1 μg/ml

Samples were analyzed in a FACS Calibur flow cytometer (Becton-Dickinson, Heidelberg, Germany) at a low flow rate. Before the flow cytometric measurement the mixture was stored at 4°C for less than 24 hours. Five thousand events falling within the platelet gate were counted per test. Data were recorded and analyzed using CELLQuest cell analysis software (Becton-Dickinson, Heidelberg, Germany). The light scatter and the fluorescent channels were set at logarithmic gain (forward scatter was E00 with a threshold of 52 and side scatter was 366).

The platelet population in the whole blood sample was identified on the basis of a size parameter (forward scatter) and the red, platelet-specific immunofluorescence (CD61-PE profile). Logarithmic amplification was used for the fluorescence and light scatter signals. Specific monoclonal antibody binding was expressed as mean intensity of immunofluorescence and was used as a quantitative measurement for glycoprotein surface expression. (Figures 3-1, 3-2, 3-3)

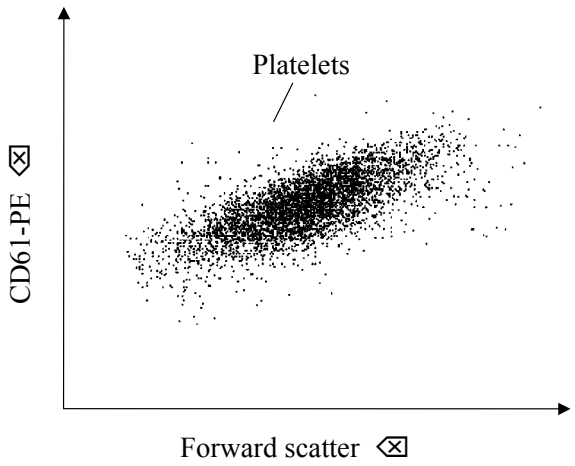

Figure 3-1. Flow cytometric analysis of platelet membrane glycoproteins (two-color, whole blood method). *Platelets are identified in whole blood by size and platelet-specific CD61 antigen in the forward scatter versus CD61 immunofluorescence (phycoerythrin fluorescence) plot.*

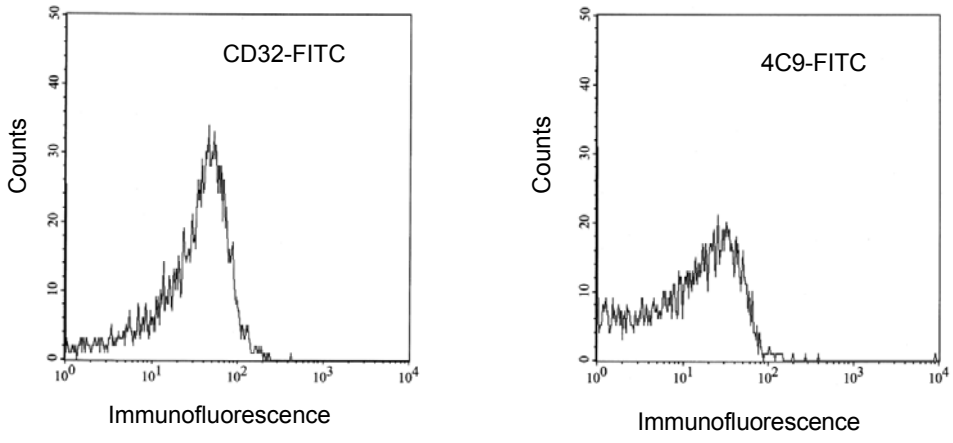

Figure 3-2. Flow cytometric analysis of platelet surface expression of FcγRIIA and GPVI. *FcγRIIA was evaluated by use of specific mAb CD32-FITC. GPVI expression was analyzed using mAb 4C9-FITC. Representative immunofluorescence histograms were depicted.*

3.2.2 GPVI-dependent platelet secretion

Whole blood was drawn from four normal individuals and collected in test tubes containing 3.8% sodium citrate. Platelet-rich plasma (PRP) was harvested from this anticoagulated whole blood after centrifugation at 1,000 rpm (Megafuge 1.0 R, Heraeus, Germany) for 15 min at room temperature. 5μl activation-specific antibody (CD62P-FITC or CD40L-FITC) and 5μl PRP were added to 35μl PBS. The mixture was incubated for 30 min with 5μl 0.1 μg/ml of mAb 4C9 or 2D1, respectively. Thereafter, the incubation mixture was quenched with 300 μL 0.5% paraformaldehyde / PBS solution (PH 7.4) and surface expression of CD62P and CD40L, as marker for platelet release, was determined by flow cytometry. Data from 10,000 events per test were obtained and analyzed.

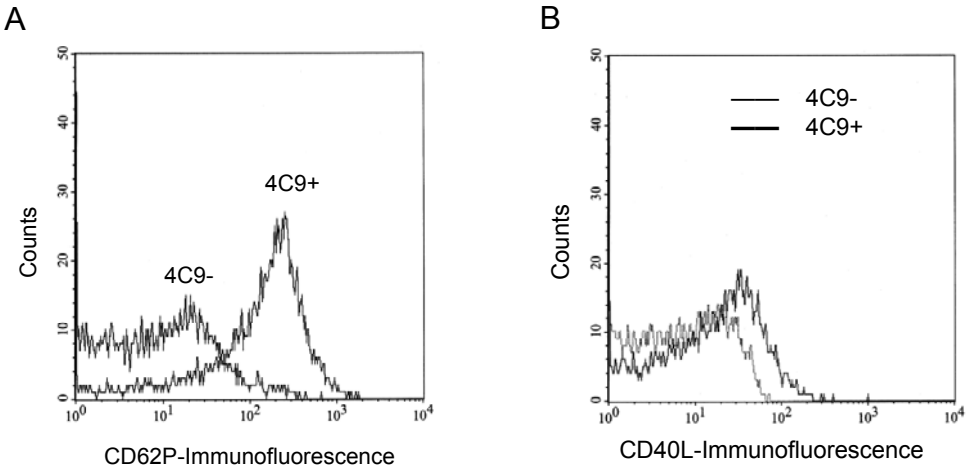

Figure 3-3. Flow cytometric analysis of platelet surface expression of P-selectin (A) and CD40L (B). A: *P-selectin was detected via mAb CD62P-FITC. The left curve showed non-stimulated platelets. The right curve indicated 4C9-stimulated platelets.* ***B:*** *mAb CD40L-FITC was used. The light line indicated non-stimulated and the bold one indicated 4C9-stimulated platelets. Representative immunofluorescence histograms were depicted.*

3.2.3 Effect of soluble GPVI on GPVI-dependent platelet secretion

To test the effect of soluble GPVI on GPVI-dependent release of CD40L, platelets were incubated with anti-GPVI mAb 4C9 (0.1 μg/ml) in the presence or absence of 2 μg/ml human GPVI for 60 min. Thereafter, CD40L surface expression was evaluated by flow cytometry.

3.3 Statistical analysis

For categorical variables, the data were summarized as counts or percentages, and Pearson chi square test was used to assess group differences. Descriptive statistics were reported as the mean value ± SD for continuous variables and differences between groups were tested by using Student *t* test for unpaired values. When the Kolmogorov-Smirnov test showed that the data were not normally distributed, we chose the Mann-Whitney *U* test for comparison of two different groups.

Cytometric data that were not normally distributed were reported as the value of the mean intensity of immunofluorescence obtained after specific staining. Differences between the two study groups were evaluated by means of appropriate unpaired nonparametric test (Mann-Whitney *U* test). A Pearson correlation was employed to test the association between platelet FcγRIIA expression and GPVI surface expression, and r is the correlation coefficient.

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. All the statistical analyses were performed with the use of software SPSS 11.0 for windows. Differences were regarded as statistically significant if the two-tailed p value was < 0.05.

3.4 Platelet interaction with endothelium

3.4.1 Incubation of endothelial monolayers with platelets

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (St. Katharinen, Germany). Cells were grown in 24-well culture plates (Nunc) in complete medium composed of EGM medium (Clonetics, St. Katharinen, Germany), 10% FCS, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 mg/L streptomycin and were used as confluent monolayers after 1 to 2 passages. Platelets were isolated from acid-citrate-dextrose (ACD)-anticoagulated whole blood as described below: Platelet-rich plasma (PRP) was harvested from this ACD-anticoagulated whole blood after centrifugation at 1,000 rpm (Megafuge 1.0 R, Heraeus, Germany) for 20 minutes at room temperature. 10ml PRP was diluted with 25ml Tyrode's solution pH 6.5 + 0.1% BSA / Glucose. After centrifugation at 2,100 rpm (Megafuge 1.0 R, Heraeus, Germany) for 10 minutes at room temperature, the resulting pellet was resuspended in 500µl Tyrode's solution pH 6.5 + 0.1% BSA / Glucose, thereafter, was mixed with 500µl Tyrode's solution pH 7.4 + 0.1% BSA / Glucose. Washed platelets were resuspended in Tyrode's solution–HEPES buffer (mmol/L: HEPES 2.5, NaCl 150, $NaHCO_3$ 12, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, D-glucose 5.5, and 1 mg/ml BSA, pH 7.4) to obtain a final platelet count of $2x10^8$ platelets/ml. Thereafter, platelets were pre-incubated with mAb 4C9 or 2D1 (0.5 µg/ml each) for 30 min. The activated platelet suspension was added to the wells of the 24-well culture plate covered with confluent monolayers of endothelial cells. Incubation was performed at 37°C without agitation in culture condition atmosphere for 1 hour. Thereafter, platelets were removed through multiple gentle washing steps, and EGM medium was added for another 10 hours (adapted from references 46, 90).

3.4.2 Determination of endothelial MCP-1 secretion

The supernatant of cultured endothelial cells treated with platelets was aspirated, centrifuged at 4000 rpm (Megafuge 1.0 R, Heraeus, Germany) for 10 minutes, and stored at -80°C. Concentrations of MCP-1 protein in the supernatant were determined by use of specific enzyme linked immuno-sorbent assay (ELISA) reagents (Quantikine R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer´s instruction.

3.4.3 Endothelial surface expression of ICAM-1

Surface expression of ICAM-1 was determined by FITC-conjugated anti-CD54 monoclonal antibody (which binds to ICAM-1) and flow cytometry. After aspiration of the supernatant, endothelial monolayers were incubated with anti-CD54 (50 µg/mL, Clone 84H10, was purchased as FITC conjugate from Immunotech, Marseille, France) and the DNA-staining fluorochrome LDS-751 (Styry 18, Exciton Inc) for 20 minutes. Thereafter, endothelial cells were mechanically detached and separated into single-cell suspension through repetitive pipetting, and single-cell suspension was evaluated by flow cytometry for ICAM-1 immunofluorescence in the forward scatter versus LDS-751 fluorescence scatter plot. 5000 events per test were evaluated, and the mean intensity of CD54-FITC immunofluorescence was used as the parameter of ICAM-1 expression.

4 Results

4.1 Baseline characteristics of the study population

385 patients were randomized to the study group. All the patients enrolled in the experiment were divided into two groups. 22.6 % (n=87) of these patients suffered from type 2 diabetes. The demographic and clinical characteristics of the study subjects are given in Table 4-1. There were no significant differences between the diabetes group (n=87) and the non-diabetes group (n=298) with respect to female gender, abnormal lipid profile, current smoker and family history of CAD. In diabetes group, a significantly higher proportion of patients suffered from hypertension, obesity, coronary artery disease. There was a significant trend that diabetic patients were likely to be older compared with non-diabetic patients (Table 4-1).

The basic laboratory parameters of the study subjects are summarized in Table 4-2. Glucose was significantly increased in diabetic patients over the non-diabetic group (170.1 $\pm$ 76.1 vs 106.7 $\pm$ 22.4, $p<0.001$). Diabetic and non-diabetic patients did not differ significantly in terms of blood platelet count, plasma low-density lipoprotein, cholesterol, whereas diabetic patients had significantly higher level of creatinine and C-reactive protein compared with non-diabetic patients as shown in Table 4-2.

Table 4-1. Demographic and clinical characteristic of studied patients

	All (n=385)	Diabetes (n=87, 22.6%)	Non-diabetes (n=298, 77.4%)	p* value
Age (yr), mean ± SD	64.6 ± 11.6	69.8 ± 9.7	63.1 ± 11.7	<0.001
Female, n (%)	116 (30.1%)	30 (34.5%)	86 (28.9%)	0.315
Hypertension, n (%)	281 (73.0%)	80 (92.0%)	201 (67.4%)	<0.001
Hypercholesterolemia, n (%)	227 (59.0%)	48 (55.2%)	179 (60.1%)	0.414
Current smoker, n (%)	55 (14.3%)	11 (12.6%)	44 (14.8%)	0.619
BMI, mean ± SD	27.0 ± 4.3	28.1 ± 4.7	26.7 ± 4.1	0.015
CAD, n (%)	296 (76.9%)	76 (87.4%)	220 (73.8%)	0.008
Family history of CAD, n (%)	97 (25.2%)	20 (23.0%)	77 (25.8%)	0.590
Medications				
Aspirin, n (%)	342 (88.8%)	82 (94.3%)	260 (87.2%)	0.068
Clopidogrel, n (%)	313 (81.3%)	77 (88.5%)	236 (79.2%)	0.05
β-blockers, n (%)	327 (84.9%)	79 (90.8%)	248 (83.2%)	0.082
ACE inhibitors, n (%)	252 (65.5%)	68 (78.2%)	184 (61.7%)	0.005
Statins, n (%)	236 (61.3%)	57 (65.5%)	179 (60.1%)	0.358

* indicates significant differences between diabetic and non-diabetic patients if p is < 0.05.

Data presented are the absolute number and percent (%) of patients or mean value ± SD.

BMI = body mass index

CAD = coronary artery disease

ACE inhibitors = angiotensin-converting enzyme inhibitors

Table 4-2. Basic laboratory parameters of studied patients

	All (n = 385)	Diabetes (n=87, 22.6%)	Non-diabetes (n=298, 77.4%)	p* value
Platelets (10^9/l)	226 ± 63	218 ± 56	228 ± 65	0.188
Creatinine (mg/dl)	1.10 ± 0.35	1.22 ± 0.48	1.07 ± 0.30	<0.001
LDL (mg/dl)	116.9 ± 42.5	112.2 ± 44.6	118.2 ± 41.9	0.272
CHOS (mg/dl)	197.6 ± 49.3	193.5 ± 55.4	198.8 ± 47.4	0.392
Glucose (mg/dl)	121.0 ± 48.9	170.1 ± 76.1	106.7 ± 22.4	<0.001
CRP (mg/l)	25.1 ± 56.1	41.2 ± 81.3	20.4 ± 45.3	0.002
HbA1c (%)		7.7 ± 1.4		

* indicates significant differences between diabetic and non-diabetic patients if p is < 0.05.

Data presented are mean value ± SD.

LDL = Low-density lipoprotein

CHOS = Cholesterol

CRP = C-reactive protein

HbA1c = Hemoglobin A1c

4.2 Platelet surface expression of collagen receptor in diabetic patients

4.2.1 Surface expression of platelet FcγRIIA

We evaluated prospectively surface expression of platelet FcγRIIA (CD32) in a total of 385 consecutive patients. 87 patients were randomized to the diabetic group and 298 patients to the non-diabetic group. Anti-CD32 is directed against FcγRIIA on platelets and was studied by flow cytometry. As shown in Fig.4-1, surface expression of the platelet FcγRIIA was significantly enhanced in diabetic patients compared with non-diabetic patients (42.4 ± 14.0 vs. 38.4 ± 12.3, p=0.02) (Fig. 4-1).

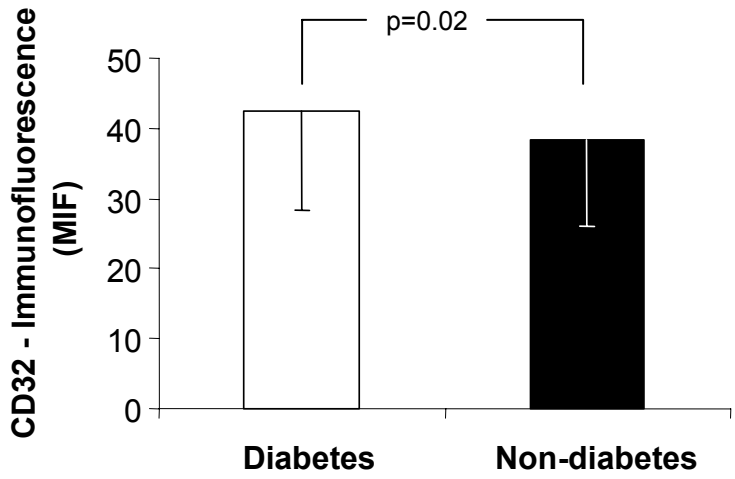

Figure 4-1. Bar graphs showing the surface expression of the platelet FcγRIIA (CD32) in diabetic (open bar) and non-diabetic (closed bar) patients. *Expression levels of the marker were analyzed by flow cytometry as described in the method section. Data are presented as mean intensity of CD32-Immunofluorescence (MIF). n=87 for diabetic group and n=298 for non-diabetic group.*

We divided all the enrolled patients into CAD and non-CAD groups. The patients with CAD were further divided into diabetic and non-diabetic groups. In the patients with CAD, the situation is the same, that is, diabetic subjects were more likely to show a significantly increased platelet surface expression of CD32 as compared with non-diabetic patients (42.4 ± 14.2 vs. 38.3 ± 12.0, p=0.034) (Fig. 4-2).

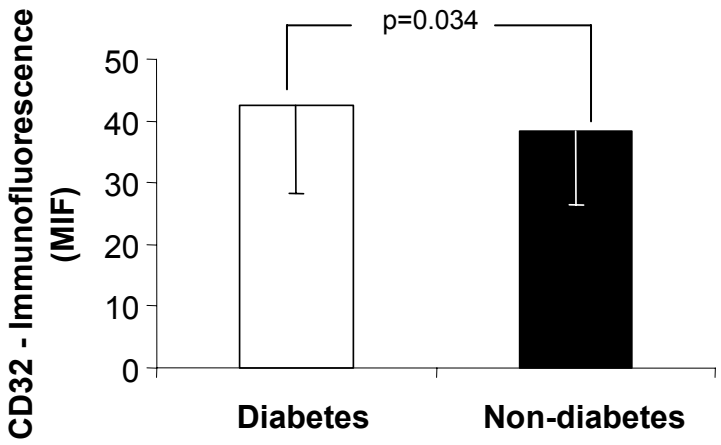

Figure 4-2. Surface expression of platelet CD32 in diabetic and non-diabetic patients of CAD subgroup. *Data are presented as mean intensity of CD32-Immunofluorescence (MIF). n=76 for diabetic group and n=220 for non-diabetic group.*

4.2.2 FcγRIIA expression is associated independently with diabetes

To examine 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). It is conceivable that FcγRIIA expression acts as a potential independent risk factor for diabetes.

4.2.3 Surface expression of platelet GPVI

In a subpopulation of patients (n=122) we additionally analyzed platelet surface expression of GPVI that forms a complex with the γ-chain of the Fc receptor at the platelet plasma membrane (101). There was no significant difference between diabetic and non-diabetic individuals with regard to the expression of platelet GPVI (31.4 ± 8.1 vs. 30.1 ± 10.1, p=0.202) (Fig. 4-3). Similarly, in the CAD group or further SAP subgroup, we failed to find the differences between diabetes and non-diabetes regarding the platelet surface expression of GPVI (data not shown).

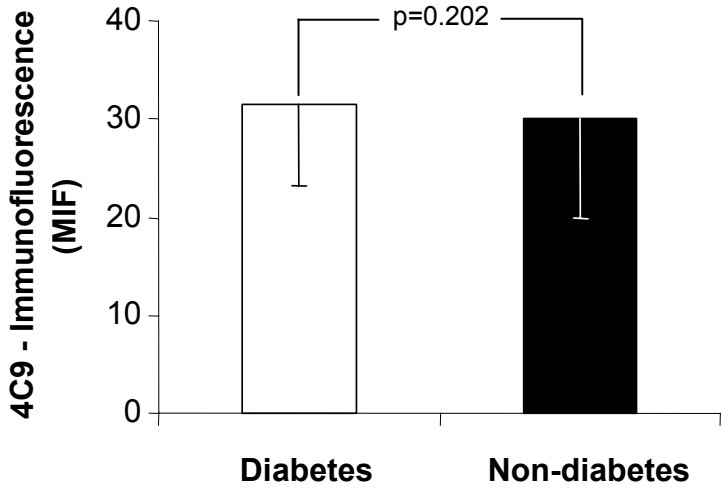

Figure 4-3. Surface expression of platelet GPVI in diabetic and non-diabetic patients. *Platelet GPVI expression was evaluated using mAb 4C9-FITC by flow cytometry. Data are presented as mean intensity of 4C9-Immunofluorescence (MIF). n=36 for diabetic group and n=86 for non-diabetic group.*

We have performed a Pearson correlation to test the association between platelet FcγRIIA expression and GPVI surface expression. There was a strong positive correlation between expression of the FcγRIIA and GPVI (r=0.529, p<0.001) (Fig. 4-4). The subjects in the lower FcγRIIA expression demonstrated the less platelet expression of GPVI. Conversely, subjects in the higher FcγRIIA had the higher expression of GPVI.

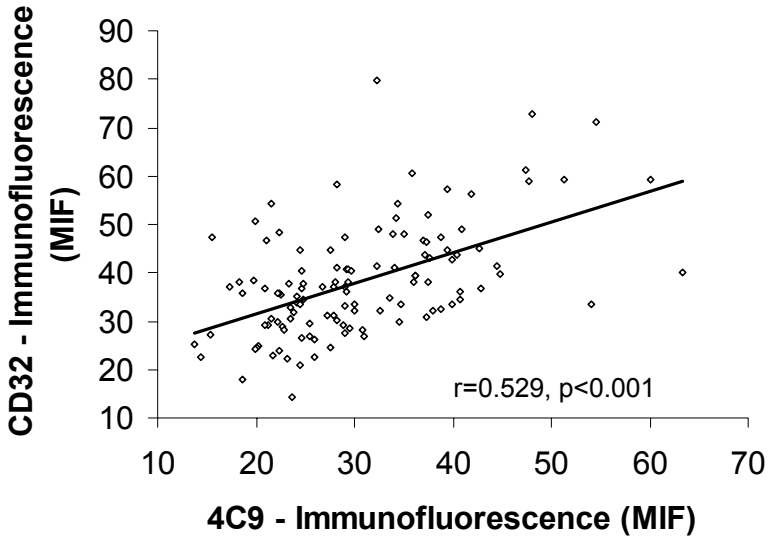

Figure 4-4. Correlation between surface expression of platelet FcγRIIA (CD32) and of GPVI (mAb anti-4C9) in a consecutive population of diabetic and non-diabetic patients. *mAb anti-4C9 is directed against the human GPVI and specifically detects surface bound GPVI. mAb CD32 recognizes the platelet FcγRIIA. Both were determined by whole-blood flow cytometry. A statistically significant relationship was observed between GPVI (abscissa) and FcγRIIA (ordinate) levels: r=0.529, p<0.001. Data are presented as mean intensity of CD32- or 4C9-Immunofluorescence (MIF).*

When we examined the correlation in diabetic subjects and non-diabetic subjects respectively, GPVI was significantly and positively correlated with FcγRIIA expression in both groups (r=0.381, p=0.026 for diabetes and r=0.558, p<0.001 for non-diabetes, respectively) (Fig. 4-5 and Fig. 4-6).

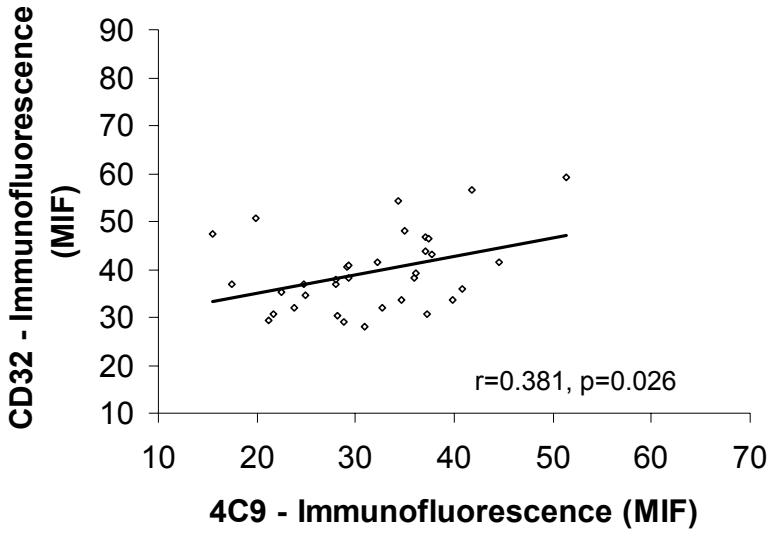

Figure 4-5. Correlation between surface expression of platelet FcγRIIA (CD32) and of GPVI (mAb anti-4C9) in diabetic patients. *Expression levels of GPVI and FcγRIIA were measured by flow cytometry as described in the methods. Data are presented as mean intensity of CD32- or 4C9-Immunofluorescence (MIF).*

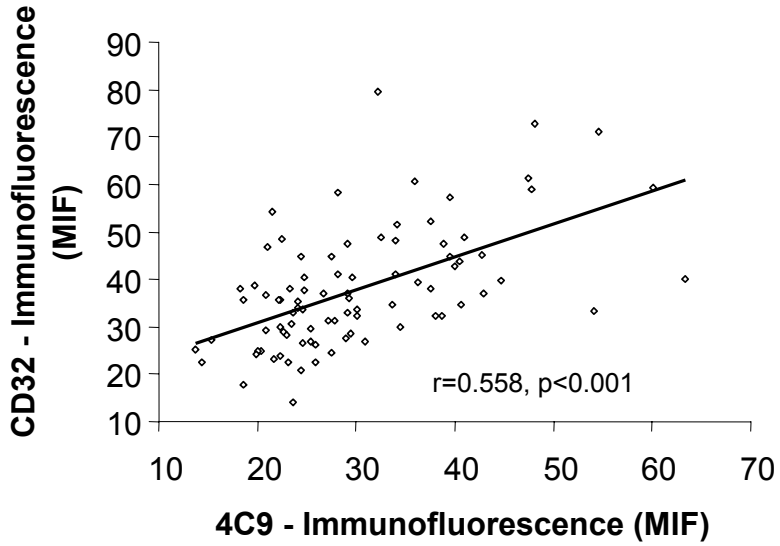

Figure 4-6. Correlation between surface expression of platelet FcγRIIA (CD32) and of GPVI (mAb anti-4C9) in non-diabetic patients. *Expression levels of GPVI and FcγRIIA were measured by flow cytometry as described in the methods. Data are presented as mean intensity of CD32- or 4C9-Immunofluorescence (MIF).*

4.2.4 Correlation between platelet surface expression of collagen receptor and HbA1c and blood glucose values

The correlation between platelet surface expression of CD32 or 4C9 and hemoglobin A1c (HbA1c) or fasting blood glucose was investigated. Among the diabetic patients, we did not find a significant correlation between surface expression of the collagen receptor (FcγRIIA or GPVI) and HbA1c value (data not shown). But in the studied patients, there is a slight correlation betweeen platelet surface expression of FcγRIIA (CD32) and blood glucose value (r=0.121, p=0.027) (Figure 4-7). The correlation between platelet surface expression of GPVI and blood glucose value were not observed (data not shown).

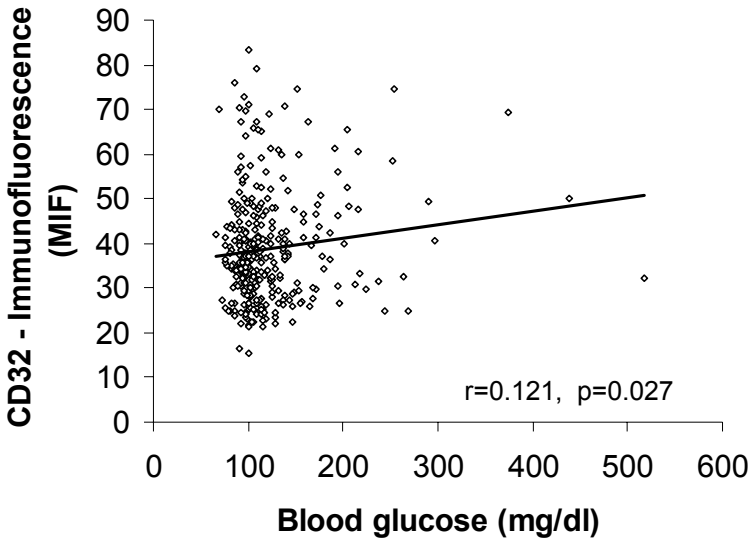

Figure 4-7. Correlation between surface expression of platelet FcγRIIA (CD32) and fasting blood glucose in the studied patients. *Expression level of FcγRIIA was measured by flow cytometry as described in the methods. Data are presented as mean intensity of CD32-Immunofluorescence (MIF).*

4.3 Platelet secretion in diabetes

4.3.1 Platelet CD61 surface expression

CD61 is directed against the GPIIIa (β_3 chain) in the glycoprotein complex IIb-IIIa (GPIIb-IIIa) and detects the receptor regardless of whether it is in its resting or activated form. In the present study, PE-labeled anti-CD61 antibody was used as a platelet identifier. Platelets were incubated for 60 minutes with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter surface expression of CD61 was analyzed on non- and GPVI-stimulated platelets by flow cytometry. As shown in Fig. 4-8, diabetes had a significantly decreased CD61 surface expression on GPVI-mediated platelets as compared with non-diabetic patients (271.7 ± 65.8 vs. 313.0 ± 72.4, p=0.005). The two groups were homogeneous with respect to the platelet GPIIIa surface expression when platelets were not stimulated (Fig.4-8).

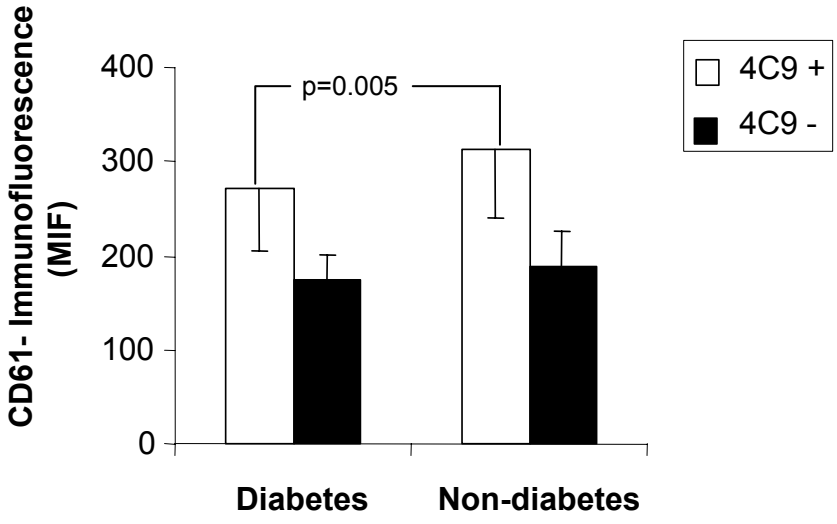

Figure 4-8. Comparison of platelet CD61 surface expression in diabetic and non-diabetic patients. *Platelets were incubated for 60 minutes with mAb 4C9 or control mAb 2D1, then, surface expression of CD61 was analyzed by flow cytometry. Data are presented as mean intensity of CD61-Immunofluorescence (MIF). (n=36 for diabetic group and n=86 for non-diabetic group).*

We divided all the enrolled patients into CAD and non-CAD groups. Among the CAD patients, surface expression of CD61 was assessed in diabetic and non-diabetic patients. As shown in Fig. 4-9, CAD subjects with diabetes showed a markedly decreased surface expression of CD61 on both non- and GPVI-stimulated platelets compared with CAD patients with non-diabetes (271.3 ± 65.1 vs. 313.2 ± 70.1, p=0.008 for GPVI-stimulated platelets, 171.7 ± 28.9 vs. 190.9 ± 36.2, p=0.026 for non-stimulated platelets, respectively).

All the CAD patients were divided into SAP and ACS groups, SAP subjects further into diabetic and non-diabetic groups. Similarly, in SAP subgroup, diabetic subjects showed a significantly lower surface expression of CD61 on GPVI-stimulated platelets compared with non-diabetic patients (257.4 ± 52.2 vs. 312.4 ± 73.1, p=0.002) (Fig. 4-10). In SAP patients, CD61 did not show significant difference on non-stimulated platelets between the two groups. In the ACS patient population, which is a mixture of UAP and MI patients, no significant difference was observed regarding the surface expression of CD61 on GPVI-stimulated platelets between diabetic and non-diabetic patients (Data were not shown).

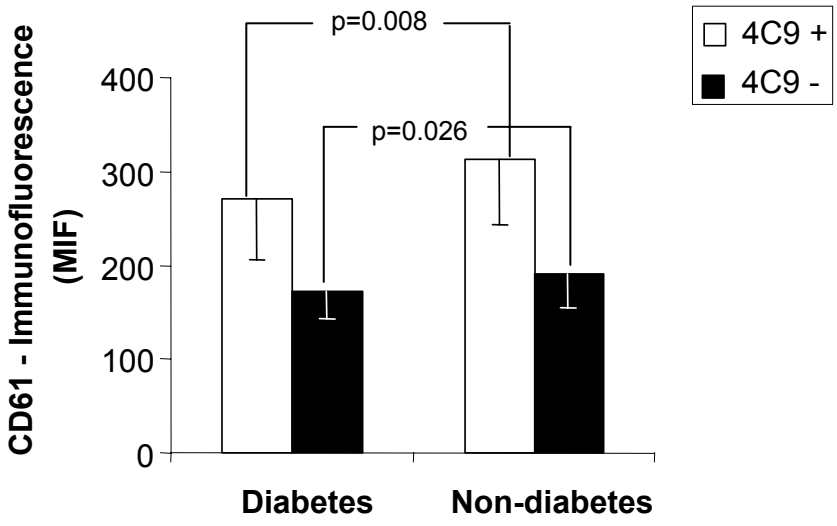

Figure 4-9. Comparison of platelet CD61 surface expression between diabetic and non-diabetic patients in CAD subgroup. *Data are presented as mean intensity of CD61-Immunofluorescence (MIF). (n=29 for diabetic group and n=57 for non-diabetic group).*

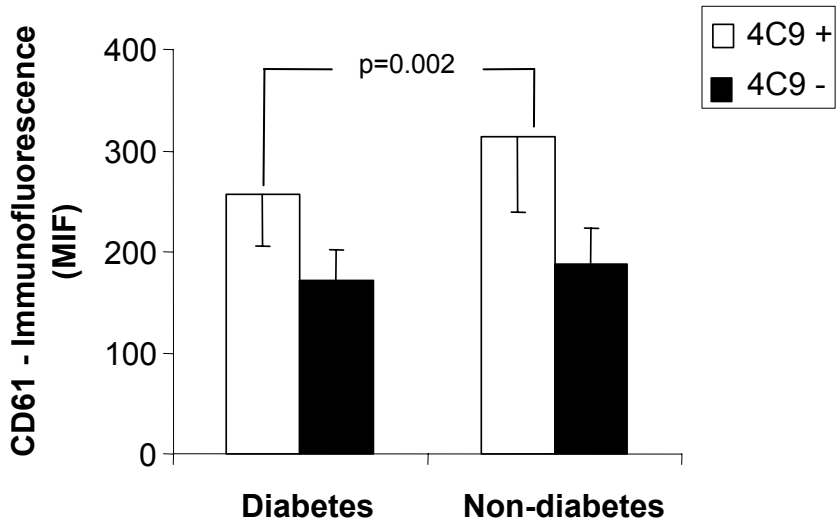

Figure 4-10. Comparison of platelet CD61 surface expression between diabetic and non-diabetic patients in SAP subgroup. *Data are presented as mean intensity of CD61-Immunofluorescence (MIF). (n=25 for diabetic group and n=47 for non-diabetic group).*

4.3.2 Platelet CD62P surface expression

Anti-CD62P recognizes P-selectin that is expressed on the activated platelet surface as a consequence of alpha-degranulation. In the present study platelets were incubated for 60 minutes with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter surface expression of CD62P was detected by flow cytometry. As shown in Fig. 4-11, a significantly reduced GPVI-dependent platelet expression of P-selectin was seen in subjects with diabetes as compared with non-diabetes (142.5 $\pm$ 63.7 vs. 162.5 $\pm$ 53.2, p=0.042). There was no significant difference with regard to the platelet CD62P surface expression between the two groups when platelets were not stimulated (Fig. 4-11).

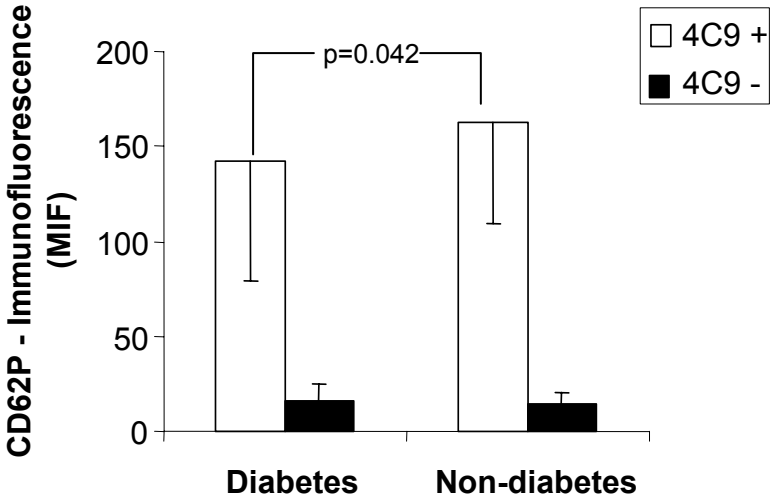

Figure 4-11. Comparison of platelet P-selectin surface expression in diabetic and non-diabetic patients. *Platelets were incubated for 60 minutes with mAb 4C9 or control mAb 2D1, then, surface expression of CD62P was analyzed by flow cytometry. Data are presented as mean intensity of CD62P-Immunofluorescence (MIF). (n=36 for diabetic group and n=86 for non-diabetic group).*

We divided all the enrolled patients into CAD and non-CAD groups, then CAD patients into SAP and ACS groups. Diabetic and non-diabetic patients were compared in the SAP and ACS subgroups regarding the surface expression of CD62P on GPVI-stimulated platelets. In the SAP subgroup diabetic subjects showed a significantly decreased surface expression of CD62P on GPVI-stimulated platelets compared with non-diabetic patients (137.7 $\pm$ 55.8 vs. 157.1 $\pm$ 44.8, p=0.05) (Fig. 4-12). However, in the ACS subgroup there was no difference (data not shown).

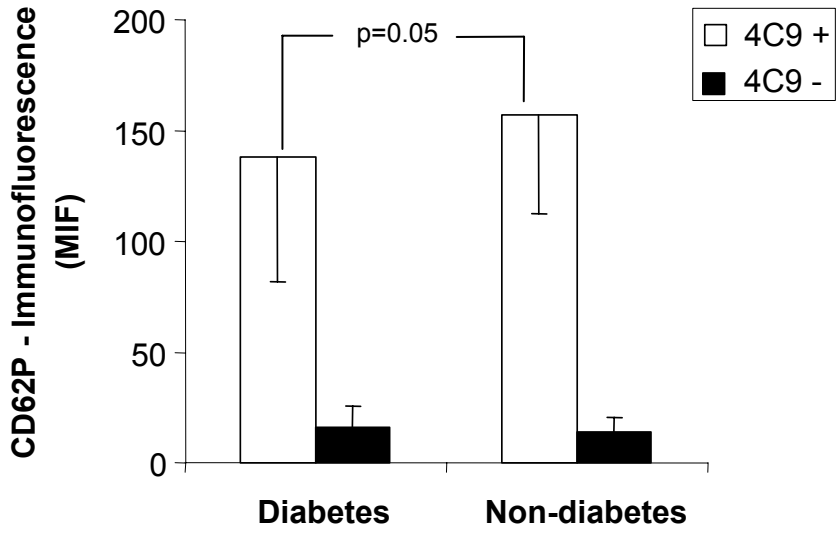

Figure 4-12. Comparison of platelet P-selectin surface expression in diabetic and non-diabetic patients with SAP. *Data are presented as mean intensity of CD62P-Immunofluorescence (MIF). (n=25 for diabetic group and n=47 for non-diabetic group).*

4.3.3 Platelet CD40L surface expression

Anti-CD40L monoclonal antibody recognizes the transmembrane signaling protein CD40L on platelets after activation, which mediates inflammatory cascades. As shown in Fig. 4-13, patients with type 2 diabetes showed a dramatically enhanced surface expression of CD40L on GPVI-stimulated platelets compared with non-diabetic patients (21.9 ± 5.3 vs. 18.0 ± 5.9, $p=0.003$) (Fig. 4-13).

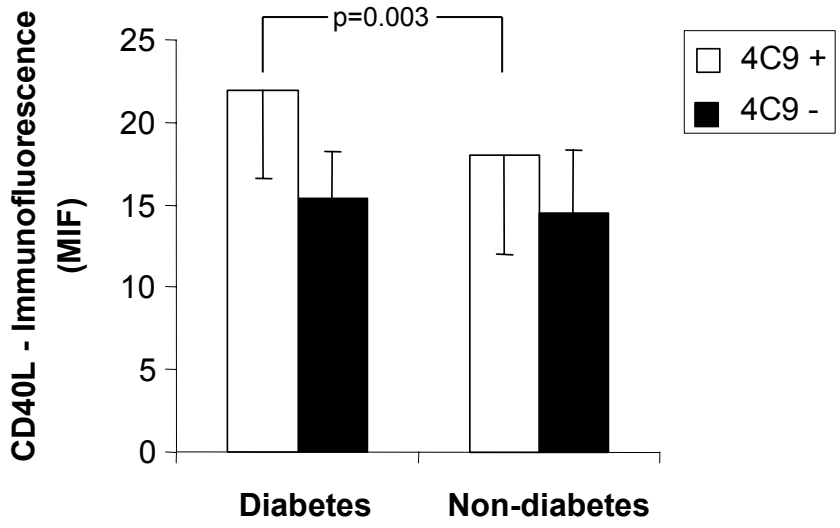

Figure 4-13. Effects of ligation of GPVI on platelet secretion of CD40L in diabetic and non-diabetic patients. *Platelets were incubated for 60 min with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter, surface expression of CD40L was analyzed by flow cytometry. Data are presented as mean intensity of CD40L-Immunofluorescence (MIF). (n=26 for diabetic group and n=55 for non-diabetic group).*

We divided all the enrolled patients into CAD and non-CAD groups. The patients with CAD were further divided into diabetic and non-diabetic groups. In the patients with CAD, we found the same situation, that was to say, diabetic subjects were more likely to show a significantly increased surface expression of CD40L on GPVI-stimulated platelets compared with non-diabetic patients (22.2 $\pm$ 5.0 vs. 18.2 $\pm$ 6.1, p=0.007) (Fig. 4-14).

We divided all the CAD patients into SAP and ACS groups. In the patient group with SAP, we also found that diabetic subjects showed a significantly elevation of CD40L surface expression on GPVI-stimulated platelets compared with non-diabetic patients (22.6 $\pm$ 4.8 vs. 17.1 $\pm$ 5.8, p=0.001) (Fig. 4-15). In the ACS population, the situation was not detected (Data were not shown).

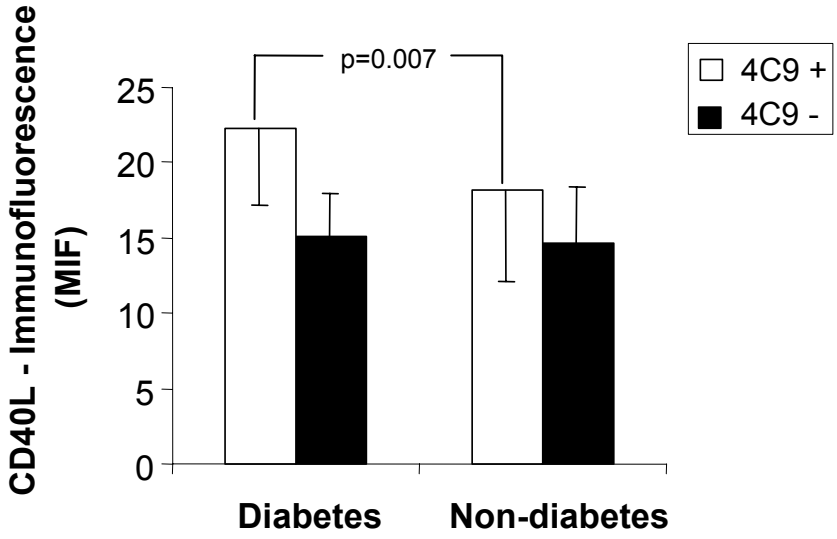

Figure 4-14. Effects of ligation of GPVI on platelet secretion of CD40L in diabetic and non-diabetic patients, which also suffered from CAD. *Data are presented as mean intensity of CD40L-Immunofluorescence (MIF). (n=22 for diabetic group and n=35 for non-diabetic group).*

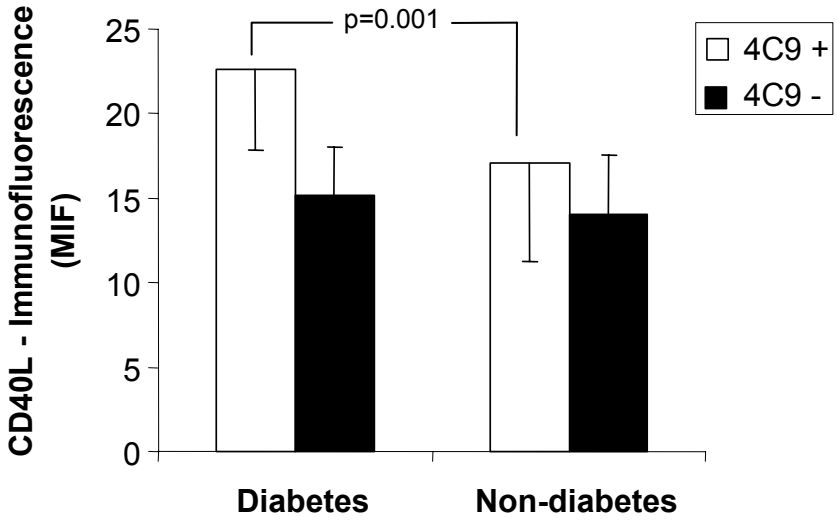

Figure 4-15. Effects of ligation of GPVI on platelet secretion of CD40L in diabetic and non-diabetic patients, which belonged to SAP subgroup. *Platelets were incubated for 60 min with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter, surface expression of CD40L was analyzed by flow cytometry. Data are presented as mean intensity of CD40L-Immunofluorescence (MIF). (n=21 for diabetic group and n=28 for non-diabetic group).*

4.4 Effects of ligation of GPVI on platelet secretion of P-selectin and CD40L

Interaction of collagen with platelets induces aggregation and secretion (101). To evaluate the role of GPVI for platelet secretion we stimulated GPVI with the specific mAb 4C9 or an irrespective control mAb (2D1). After 30 minute incubation, the surface expression of CD62P and CD40L was analyzed by flow cytometry. As shown in Fig. 4-16, ligation of GPVI through mAb 4C9 resulted in substantial release of P-selectin (CD62P) and CD40L (both $p<0.01$) (Fig. 4-16).

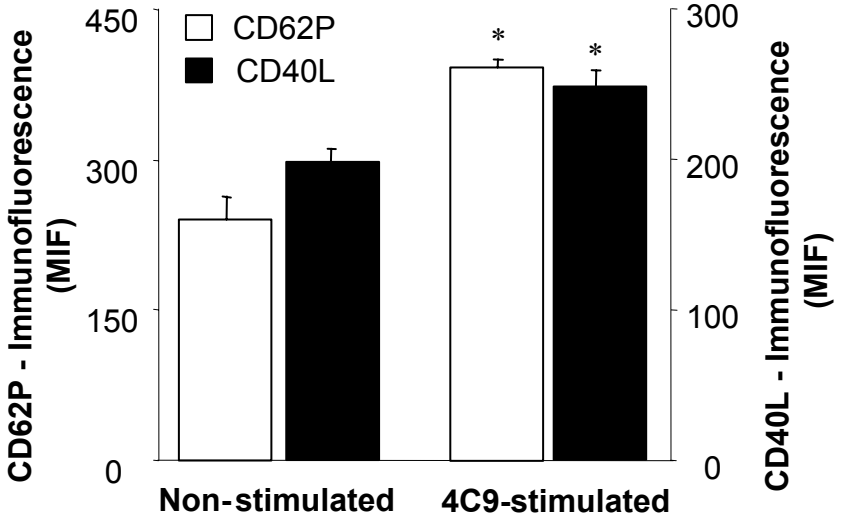

Figure 4-16. Effects of ligation of GPVI on platelet secretion. *Platelet surface glycoproteins were measured in PRP using specific monoclonal antibodies. PRP was incubated for 30 min with the GPVI-specific mAb 4C9 or an irrelevant control mAb 2D1. Thereafter, surface expression of P-selectin and CD40L was analyzed by flow cytometry. Data are presented as mean intensity of Immunofluorescence (MIF). Asterisks indicate statistical significance ($p<0.01$) between non- and 4C9-stimulated platelets. (n=4 independent experiments).*

To analyze the effect of inhibition of GPVI on GPVI-mediated platelet CD40L secretion, platelets were incubated with mAb 4C9 in the presence or absence of the soluble human GPVI. As shown in Fig. 4-17, in the presence of soluble GPVI, the GPVI/ligation-induced CD40L release was substantially attenuated both in diabetic and non-diabetic patients (21.9 ± 5.3 vs. 14.9 ± 2.4, p<0.001 for diabetes, and 18.0 ± 5.9 vs. 12.7 ± 3.0, p<0.001 for non-diabetes, respectively) (Fig. 4-17).

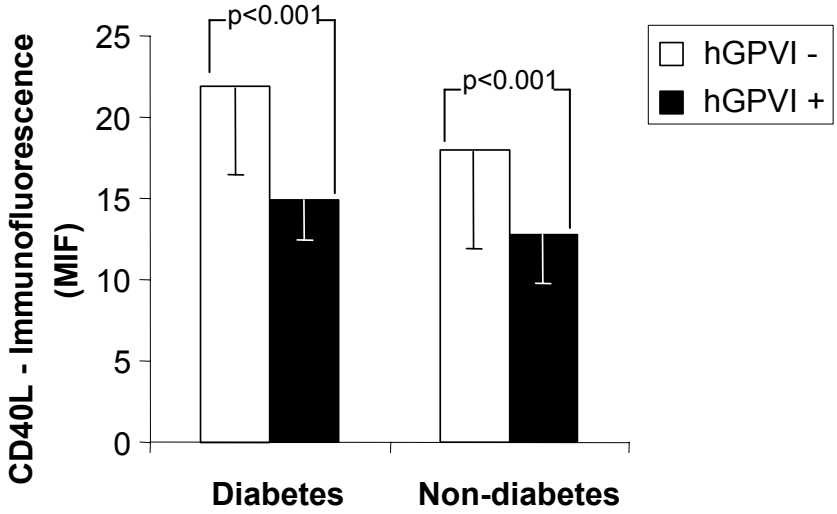

Figure 4-17. Effect of recombinant soluble GPVI on GPVI/ligation-induced secretion of CD40L. *Platelets were incubated for 60 min with the GPVI-specific mAb 4C9 in the presence or absence of recombinant soluble GPVI. Thereafter, surface expression of CD40L was analyzed by flow cytometry. Data are presented as mean intensity of CD40L-Immunofluorescence (MIF). (n=26 for diabetic group and n=55 for non-diabetic group).*

4.5 Effects of GPVI/ligation-stimulated platelets on activation of endothelial cells

Activated platelets release CD40L, a major platelet-derived proatherogenetic substance. To analyze the effects of GPVI-dependent platelet CD40L release on endothelial activation, monolayers of cultured HUVECs were co-incubated with

platelets pre-treated with the specific mAb 4C9 or a control mAb 2D1. We investigated the effects of GPVI-stimulated platelets on secretion of monocyte chemotactic protein-1 (MCP-1) and on surface expression of intercellular adhesion molecule-1 (ICAM-1) of cultured endothelial cells.

4.5.1 Secretion of MCP-1 on endothelial cells

HUVEC monolayers were co-incubated with platelets for 1 hour in the presence of mAb 4C9 or mAb 2D1, respectively. Thereafter, platelets were removed, and HUVECs were additionally incubated with medium for 10 hours. HUVEC supernatant was stored and ELISA was performed. Pretreatment of cultured monolayers of endothelial cells with GPVI-stimulated platelets significantly enhanced secretion of MCP-1 compared with control ($p<0.01$) (Fig. 4-18).

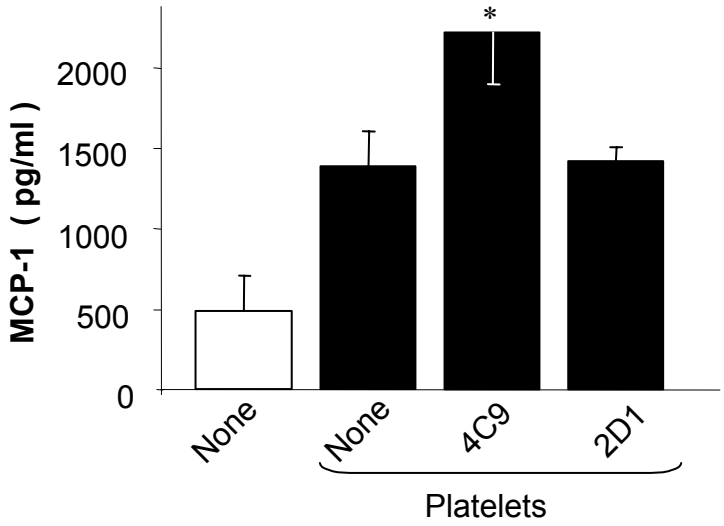

Figure 4-18. Effects of co-incubation of HUVECs with GPVI/ligation-stimulated platelets on endothelial secretion of MCP-1. *Monolayers of HUVEC were co-incubated for 1 hour with platelets in the presence of mAb 4C9 or mAb 2D1, respectively. Thereafter, platelets were removed and secretion of MCP-1 was determined by ELISA after 10 hours of further cultivation. Data are presented as mean ± SD of 3 independent experiments. Asterisk indicates a significant difference ($p<0.01$) between 4C9 and 2D1 values.*

4.5.2 ICAM-1 surface expression of endothelial cells

As shown in Fig. 4-19, endothelial ICAM-1 expression values did not differ between HUVECs incubated with the GPVI- and non-stimulated platelets. There was the trend that co-incubation of GPVI-stimulated platelets with HUVECs resulted in an increase in endothelial surface expression of ICAM-1 compared with control, although the difference failed to reach a statistical significance ($p>0.05$) (Fig. 4-19).

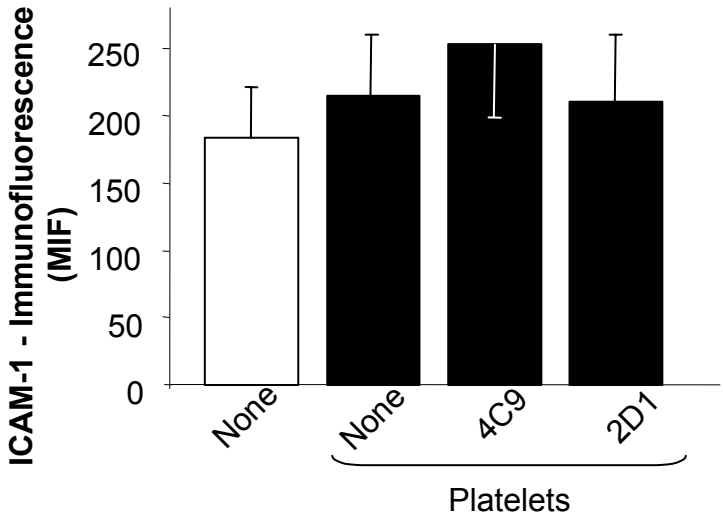

Figure 4-19. Effects of co-incubation of HUVECs with GPVI/ligation-stimulated platelets on endothelial surface expression of ICAM-1*. Monolayers of HUVEC were co-incubated for 1 hour with platelets in the presence of mAb 4C9 or mAb 2D1, respectively. Thereafter, platelets were removed and surface expression of ICAM-1 on HUVECs was determined after 10 hours of further cultivation. Data are presented as mean intensity of ICAM-1-Immunofluorescence (MIF). Depicted are mean and SD of three independent experiments.*

5 Discussion

5.1 Major findings in the present analysis

To the best of authors´ knowledge, the present analysis is the first study to investigate the role of GPVI for platelet activation and platelet-mediated endothelial activation in patients with type 2 diabetes and to assess the possible effect of platelet GPVI expression on atherogenesis and thrombosis in diabetes. The major findings of the present study are: a) patients with type 2 diabetes have an enhanced platelet surface expression of FcγRIIA that correlates with GPVI expression compared with non-diabetic patients; b) stimulation of GPVI results in substantial secretion of CD40L in normal control platelets, GPVI-dependent CD40L release is enhanced in type 2 diabetes as compared with non-diabetes; c) soluble GPVI inhibits GPVI-induced secretion of platelet CD40L; d) co-incubation of cultured endothelial cells with GPVI/ligation-stimulated platelets induces substantial endothelial activation. The present findings indicate that an enhanced surface expression of platelet collagen receptor GPVI in diabetic patients results in an increased activation of circulating platelets and thus, enhances platelet-dependent thrombus formation and platelet-induced proatherogenetic changes of the vascular wall.

5.2 Increased consumption of activated platelets in diabetes

Secondary vascular complications are frequently observed in patients with diabetes mellitus. The role of diabetes as an independent risk factor for cardiovascular disease has been well established. In type 2 diabetic patients, there is a 2-3 fold increase of mortality attributed to coronary artery disease (atherosclerosis and its thrombotic complications) (64). In the present clinical study, we evaluated a total of 385 consecutive patients and 22.6% (n=87) of these patients suffered from type 2 diabetes. 87.4% (n=76) of diabetic patients suffered from CAD.

A variety of platelet functional changes have been described in diabetic patients including abnormalities of signal transduction events (11, 53, 150), enhanced thromboxane A_2 formation (50), an increased surface expression of platelet membrane glycoproteins such as GPIIb-IIIa (137), and an enhanced sensitivity of diabetic platelets to collagen (11, 150). Antiplatelet therapy with aspirin is recommended as a primary and secondary prevention strategy in patients with diabetes and has been shown to effectively reduce morbidity and mortality of CAD in diabetes. In addition, chronic aspirin therapy reduces complications of diabetic retinopathy possibly by reducing microthrombosis in retinal capillaries. Thus, effective antiplatelet therapy might be a promising therapeutic strategy in diabetes.

Anti-CD61 is a monoclonal antibody representing the surface expression of the β_3 chain of the platelet surface antigen GPIIb-IIIa and the vitronectin receptor $\alpha_v\beta_3$. GPIIb-IIIa is a membrane protein present exclusively on all resting and activated platelets. P-selectin, which is also referred to as granule membrane protein-140 (GMP-140), or PADGEM protein (platelet activation-dependent granule-external membrane protein), or CD62P, is a 140 kDa glycoprotein that is a component of the α-granule membrane of resting platelet (102). Platelet activation leads to its fusion with the surface connecting system and expression on the surface of platelet plasmatic membrane after α-granule secretion (102). Therefore, a P-selectin-specific mAb only binds to degranulated platelets and not to resting platelets. An increased binding of anti-CD62P indicates an irreversible degranulation of the platelets. However, it was also reported that *in vivo* circulating degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function (92). As a major surface receptor of activated platelets (102), P-selectin is a platelet activation surface marker. P-selectin mediates interaction of activated platelets to neutrophils and monocytes. Thus, the expression of P-selectin is able to mediate both activation and local recruitment of leukocytes (29). In addition, P-selectin induces inflammatory reactions in leukocytes (91).

Huo et al (57) reported that circulating activated platelets promote formation of atherosclerotic lesions. They showed that the role of activated platelets in atherosclerosis is attributed to platelet P-selectin-mediated delivery of platelet-derived proinflammatory factors to monocytes/leukocytes and the vessel wall. These observations suggest that P-selectin expressed on activated platelets may be involved in the initial process of atherosclerotic lesions *in vivo*.

Circulating activated platelets induce atherosclerosis and vascular complications by promoting microthrombus formation. Our group has recently shown that platelets are critical for the development of atherosclerosis (87). Chronic inhibition of platelet adhesion to the arterial wall attenuates substantially development of atherosclerotic lesions in mice (87).

Platelets from diabetic subjects exhibit numerous features which make these individuals more prone to thrombosis. Platelets from diabetic subjects show an increased adhesiveness and increased spontaneous aggregation or aggregation on extracellular matrices (83, 120, 153). However, some authors indeed found enhanced adhesion (67), while others reported unchanged platelet adhesion (130).

There is also evidence for activation of circulating platelets *in vivo* in diabetes (32). Most reports suggest that there may be a special "priming" of hypersensitive platelets of diabetes in response to agonists, but also there were reports on depressed platelet reactivity to agonists under *ex vivo* conditions, probably as a result of the enhanced activation in the circulation (60, 139, 143, 147). This apparent discrepancy may be explained as follows: as a result of platelet hypersensitivity in platelets with diabetes (151), the circulating blood platelets go through more frequent episodes of granule release. These episodes result in the formation of three distinguished subpopulations: (a) non-activated platelets; (b) activated platelets, partly exhausted (with lesser reactivity); (c) reticulated platelets (rich with residual RNA derived from the precursor cell), which replace the exhausted and consumed cells. Augmented granule release may imply the reduced platelet survival (because of the accelerated sequestration in the circulation), the increased platelet turnover and may reflect the state of thrombopoiesis in diabetic individuals (136, 138, 147).

The decrease in membrane-exposed glycoproteins may, thus, reflect sequestration of high adhesive circulating platelets rather than platelet deactivation. This explanation is supported by the findings that P-selectin expression is correlated with decreased platelet survival (113). In addition, the decrease in surface glycoproteins may be caused by the generation of microparticles that are shed from the platelet surface during the activation process, resulting in loss of membrane glycoproteins (40). Thus, when testing platelet reactivity *in vitro*, one might expect either enhanced or depressed reactivity (147).

Rauch and colleagues evaluated platelet activation by flow cytometric detection of special platelet surface marker, such as P-selectin, thrombospondin, or the active

complex of glycoprotein IIb-IIIa in patients with type 1 diabetes mellitus with and without microangiopathy (110). They found reduced expression of these markers in diabetic subjects with microangiopathy.

In the present study, we also found decreased surface expression of CD61 and CD62P on GPVI-mediated platelets in subjects with type 2 diabetes mellitus. Together, our results may reflect the increased consumption of activated platelets in type 2 diabetes by showing the decreased expressions of the markers of platelet activation in these patients. Activation of platelets is involved in atherogenesis and the development of the thrombotic complications of atheroma (36, 116).

5.3 Platelet surface expression of collagen receptor in diabetes

5.3.1 Platelet surface expression of FcγRIIA

Platelets play a fundamental role in atherogenesis and development of ischemic complications (35, 116). Platelets adhere to the vascular endothelium of the arterial wall prior to the development of manifest atherosclerotic lesions (87). In the process of atherogenesis, enhanced collagen synthesis by intimal smooth muscle cells and fibroblasts has been shown to significantly contribute to luminal narrowing (111). Thus the matrix exposed on plaque rupture is enriched in collagen. Fibrillar collagen is the most thrombogenic constituent of the vascular subendothelium as it not only supports platelet adhesion but also acts as a strong activator of platelets *in vitro* (9, 22, 119), inducing integrin activation through GPVI (101).

There is compelling evidence for a crucial role of GPVI in arterial thrombosis from studies on mice. The group of Gawaz and co-workers demonstrated that thrombus formation in the injured carotid artery in mice is virtually abolished in the absence of functional GPVI (88). This agrees with a recent study by Konishi et al (68) who found markedly reduced platelet attachment and subsequent neointimal hyperplasia at sites of vascular injury in FcR γ-chain deficient mice, which lack GPVI (98).

One of the major clinical problems in diabetic patients is coronary atherosclerosis and coronary arterial thrombosis caused by rupture or erosion of an atherosclerotic plaque causing angina pectoris or myocardial infarction.

Collagen-mediated platelet activation contributes significantly to coronary vascular thrombus formation associated with atherosclerotic plaque destabilization, leading to unstable angina and myocardial infarction (8, 34). Recent clinical and laboratory observations support a potential role for the platelet Fc receptor (FcγRIIA) in this process (15). The 40 kDa FcγRIIA (CD32) receptor is one of three biochemically distinct class of the Fcγ family and the only Fcγ receptor expressed by megakaryocytes and platelets (18). Fcγ receptors in immune effector cells bind to the Fc structure of IgG and link humoral and cellular immune components.

Most recently, Calverley DC et al. have observed that patients with diabetes have a significantly increased platelet Fc receptor expression over those without diabetes (15). The increased platelet Fc receptor expression was considered as a potential contributing cause of platelet hypersensitivity to collagen in diabetes mellitus (16). It was also demonstrated that increased platelet FcR expression may contribute towards risk for atherothrombotic events (15).

In the present study, analysis of the platelet FcγRIIA expression in the randomly selected patients revealed that diabetic patients had a significantly increased FcγRIIA expression over a non-diabetic cohort (42.4 ± 14.0 vs. 38.4 ± 12.3, $p=0.02$). The increased platelet surface FcγRIIA in diabetes found in our study is consistent with the report of Calverley DC (15). Moreover, we showed that platelet FcγRIIA was an independent factor associated with diabetes.

The significantly increased platelet FcγR expression in persons with diabetes in this study suggests a potential role in this group´s predisposition to vascular occlusive events over non-diabetic individuals.

The biological mechanism responsible for increased platelet FcγR expression associated with acute atherothrombotic events and certain atherosclerosis risk factors such as diabetes is presently unclear. One proposed hypothesis would link upregulation of megakaryocyte FcγR activity due to increased gene transcription in response to altered levels of one or more cytokines or other inflammatory mediators (19, 65, 115). Future studies will help to further elucidate the mechanism responsible

for increased platelet FcγRIIA expression in collagen-mediated platelet activation associated with cardiovascular disease and diabetes.

5.3.2 Platelet surface expression of GPVI

GPVI has recently been established as the central platelet collagen receptor that is essential for platelet adhesion and aggregation on immobilized collagen *in vitro*, as it mediates the activation of different adhesive receptors, including integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ (88, 101). Inhibition of GPVI abrogates arterial thrombus formation substantially making GPVI an attractive pharmacological target. GPVI forms a complex with the FcR γ-chain at the platelet surface (101). Ligand binding to GPVI triggers platelet activation, aggregation, and secretion (101).

In the present study, GPVI expression did not differ between diabetes and non-diabetes. The result may be attributable to relatively small numbers of subjects (less than 50 in the diabetic group) enrolled in the subgroup. Although direct evidence of higher GPVI level in diabetes was not provided, we found that circulating platelets of patients with diabetes are characterized with enhanced surface expression of the FcγR that correlates with the expression of GPVI on the platelet plasma membrane ($r=0.529$, $p<0.001$). Enhanced surface expression of platelet Fc receptor has been shown to be associated with an increased aggregation response to collagen (15, 16). Moreover, variation of GPVI surface density regulates thrombus formation on collagen (12). Thus, the herein described platelet surface expression of GPVI 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 TNF-like cytokine CD40L.

5.4 GPVI-dependent platelet secretion of P-selectin and CD40L

Platelet stimulation and subsequent aggregation are known to cause the expression or release of several factors that could affect vascular pathology. These

include P-selectin, an α-granule protein that mediates platelet rolling, leukocyte adhesion and coagulation; and CD40L, a member of the tumor necrosis factor-α family of proteins.

During adhesion and shape change the platelet begins to release stored substances into its surroundings. P-selectin (CD62P) translocates from the membrane of α-granules to the plasma membrane (45). P-selectin is a well-characterized endothelial and platelet adhesion receptor mediating interactions of activated platelets and endothelial cells with leukocytes (105). This enhances inflammatory responses by initiating leukocyte cytokine production and secretion (97).

In the present study we have found that platelet surface expression of GPVI mediates platelet secretion of P-selectin in normal control platelets.

Both P-selectin and CD40L could contribute to long-term vascular pathologies, CD40L appears to be particularly relevant because this protein is now known to be prothrombotic (3) and proinflammatory, to have a proven role in atherosclerotic lesion progression (124), and to be a risk factor for cardiovascular events (126).

When platelets are triggered by contact with collagen, platelet CD40L immediately links haemostasis to the vascular inflammatory system. CD40L led to enhanced platelet-leukocyte adhesion, which is important in the recruitment of leukocytes to sites of thrombosis or inflammation. Inflammation is now known to initiate and/or mediate the progression of atherosclerotic disease, and CD40L is increasingly recognized in this process. This was initially established in mouse models engineered for accelerated atherosclerosis, where disruption of CD40L function by administration of a blocking CD40L antibody (81) or targeting of the CD40L gene greatly inhibited lesion progression (78).

The interaction between CD40 and CD40L elicits diverse biological responses involved in atherosclerosis, such as secretion of pro-inflammatory cytokines and matrix metalloproteinases, and expression of adhesion molecules and tissue factor (70). The destabilization of the vulnerable fibrous cap of the atherosclerotic plaque seems to result from an imbalance of the plasminogen and matrix metalloproteinase (MMP) activation systems (37, 107). Endothelial cells secret urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), interstitial collagenase (MMP-1) and gelatinase A (MMP-2), and MMP-9 in an activation-dependent manner (51, 76). In line with these *in vitro* findings, enhanced levels of

uPA, urokinase-type plasminogen activator receptor (uPAR) and several MMPs have been found in atherectomy specimens from patients with unstable angina, suggesting a role in the rupture of the atherosclerotic plaque (37). Platelet CD40L mediates the inflammatory cascades, leading to matrix degradation and plaque rupture. These responses are known to make the plaque unstable. The expression of platelet CD40L may be related to the complexity or vulnerability of the plaques (73). Activated platelets expressing CD40L will also facilitate rapid formation of thrombus upon rupture of plaques. The finding that interruption of CD40/CD40L interaction enhances the content of interstitial collagen might be of particular clinical relevance, because this extracellular matrix component is considered the crucial determinant of fibrous cap integrity and thus plaque stability (72).

The generation of inflammatory signals by platelets may thus occur following acute mechanical damage of the endothelium in the pathogenesis of atherosclerosis and vascular infarction, in which monocytes and platelets have preeminent roles (26).

In the present study, we have shown that platelet surface expression of GPVI mediates release of CD40L in normal control platelets. We also quantitated platelet CD40L surface expression in diabetic patients and non-diabetic cohort, and we found diabetic subjects had a significant elevation in CD40L level of GPVI-stimulated platelets as compared with non-diabetes (21.9 ± 5.3 vs. 18.0 ± 5.9, $p=0.003$). The results of this study provide the first definitive evidence that platelets upregulate P-selectin and CD40L in direct response to GPVI. Moreover, we describe that inhibition of GPVI through soluble GPVI is effective in reducing platelet release of CD40L. Effective down-regulation of CD40L expression, a major proinflammatory stimulus, could be of considerable importance for the prevention of atherosclerosis and contribute to plaque stabilization in diabetes. Thus, platelet might be a promising pharmacological target for anti-inflammatory treatment in diabetes.

5.5 GPVI/ligation-stimulated platelets induce activation of endothelial cells

Interaction of activated platelets with the endothelium and consecutive inflammatory response within the vessel wall might contribute substantially to early steps of atherosclerosis (117). Recently, CD40L expression was found in activated

platelets in the thrombus *in vivo* and was reported to be responsible for the platelet mediated activation of endothelial cells *in vitro* (54). Like TNF-α and interleukin-1, CD40L on platelets induces endothelial cells to secrete chemokines and to express adhesion molecules, thereby generating signals for the recruitment and extravasation of leukocytes at the site of injury. Platelet CD40L binds to CD40 on endothelial cells inducing inflammatory genes in endothelial cells, including the most prominent monocytic chemotactic factor MCP-1 and the adhesion receptor ICAM-1 (41, 54). So CD40L causes decisive changes in the chemotactic and adhesive properties of endothelial cells.

Dysregulation of platelet-endothelium interaction has been implicated in atherogenesis and restenosis (144). On activation, platelets release a number of biologically highly active compounds from their granules that exert significant reactions within endothelial cells (146). Under pathophysiological conditions, platelets might adhere to the intact endothelial monolayer and might change the microenvironment of the vessel wall (114, 144).

Platelets and alterations of chemotactic and adhesive properties of endothelium play an important role in the pathophysiology of atherosclerosis. Activation of transcription factor nuclear factor-κB (NF-κB) which regulates transcription of early inflammatory response genes such as MCP-1 and ICAM-1, was significantly increased in endothelial cells treated with activated platelets. MCP-1, a potent chemotactic factor for monocytes, and the adhesion molecule ICAM-1, that supports monocyte adhesion to endothelium, have been found in abundance in atherosclerotic lesions, indicating their critical role in atherogenesis (116).

In the present study we evaluated the effects of GPVI-stimulated platelets on secretion of MCP-1 and surface expression of ICAM-1 on cultured endothelial cells. As a counterreceptor for leukocytes, ICAM-1 present on the luminal aspect of endothelium is critical for leukocyte binding to the endothelium and for concomitant extravasation to sites of inflammation or injury within the vessel wall (56). We found that co-incubation of HUVECs with GPVI/ligation-stimulated platelets increased ICAM-1 surface expression of endothelial cells compared with non-stimulated platelets, although the difference failed to reach a statistical significance. It may be speculated that this is the result of small numbers of independent experiments.

Apart from upregulating adhesion molecules, endothelial cells react to inflammatory stimuli by secreting various chemokines, including interleukin-8, the

principal chemoattractor for neutrophils, and MCP-1, which recruits and activates monocytes (28). Both chemokines can be used as indicators of the endothelial inflammatory reaction, because they are not stored in platelets.

It has been shown *in vivo* that platelets can adhere intermittently to intact endothelium via several mechanisms, including the adhesion molecule P-selectin (85), and by binding to fibrinogen immobilized to the surface of endothelial cells via glycoprotein IIb-IIIa (86). Thus, it seems likely that activated platelets come transiently into close contact with intact endothelium and release high concentrations of granule-stored cytokines into their thrombotic microenvironment and induce substantial MCP-1 secretion by endothelial cells. Localized platelets induce endothelial secretion of MCP-1 and subsequent accumulation and transmigration of monocytes might be an important trigger of atherogenetic responses within the vessel wall.

We demonstrate that GPVI-stimulated platelets can result in enhanced secretion of MCP-1 in endothelial cells, the major chemotactic factor for monocytes.

So the present study showed that GPVI-dependent stimulation of platelets induces CD40L upregulation on platelets and thereby stimulates endothelial cells to enhance chemotactic (secretion of MCP-1) and adhesive (surface expression of ICAM-1) activity. Platelet-induced secretion of MCP-1 and expression of ICAM-1 by endothelial cells may represent an initial regulatory step relevant to early atherosclerosis and also to plaque progression and destabilization, providing a mechanism by which the monocyte may first be attracted and adhere to, and then migrate through the endothelial barrier (117).

These findings in the present analysis imply a potential pathophysiological mechanism of platelets in an early stage of atherogenesis in diabetes.

CD40L-mediated endothelial activation substantially contributes to atherogenesis, because inhibition of CD40L retarded the progression of atherosclerosis in mice (124) and led to a collagen-rich stable plaque phenotype very likely attributable to decreased MMP-1 and MMP-2 activity (79). 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 (90). Moreover, drug resistance of current antiplatelet drugs like aspirin and clopidogrel has been described in high-risk patients (48, 96). Elucidation of mechanisms involved in platelet-induced inflammatory reactions within endothelial cells might disclose

pharmacological targets to interfere with early mechanisms of atherogenesis. 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 prevention of vascular complications in patients with diabetes.

In conclusion, our data indicate that GPVI is a major platelet receptor involved in the pathophysiological of atherosclerosis and thrombosis in diabetes. GPVI blockade may not only inhibit platelet adhesion to vascular lesions and thereby prevent physical vessel occlusion but also may prevent platelet-CD40L-mediated inflammatory cascades, accelerating atherosclerosis and plaque progression in diabetes.

5.6 Limitations of the study

The present study has some limitations. First, the findings of the present study indicate the effects of platelet GPVI on the platelet secretion and the activation of endothelial cells *in vitro*. However, we do not provide data that this phenomenon also occurs *in vivo*. The pathophysiological importance of platelet GPVI surface expression in the circulation in diabetes, remains to be assessed. Second, flow cytometry has some advantages for the study of platelet activation (93), for example, both the activation state of circulating platelets and the reactivity of circulating platelets can be determined; activation-dependent changes in multiple surface receptors can be detected. However, flow cytometry only measures the function of circulating platelets, and cannot reflect platelet activation at the blood vessel wall and recently cleared platelets. Thus, if activated platelets are rapidly cleared or are adherent to blood vessel walls or to extracorporeal circuits, flow cytometry may not detect evidence of platelet activation.

5.7 Pathophysiological considerations and therapeutic implications

This present study shows that platelet surface expression of FcγRIIA is enhanced in type 2 diabetes. Platelet surface expression of GPVI correlates significantly with surface expression of FcγRIIA and mediates release of CD40 ligand and activation of endothelial cells. This indicates that inhibition of GPVI/ligation interactions might represent a promising strategy to prevent atherogenesis and thrombosis in diabetes. However, antibodies directed against GPVI have been reported to induce platelet activation (127) and immune thrombocytopenia, or a complete loss of GPVI on circulating platelets (100, 132), hampering their use in the clinical setting. In the present study, we evaluated inhibitory effect of a soluble form of GPVI on GPVI-mediated platelet CD40L secretion. The soluble form of human GPVI specifically bound to collagen with high affinity (94) and attenuated the secretion of platelet CD40L, which was found increased in diabetes in our analysis. Hence, it is appealing to speculate that the GPVI therapy might be associated with a lower risk of clinical hemorrhage, compared with the anti-GPVI mAb-based strategies. These findings highlight the importance of soluble GPVI as a potential strategy to prevent platelet CD40L-mediated inflammatory cascades of atherosclerosis in patients with diabetes.

Furthermore, the evidences from the present study introduce a novel aspect of how platelets may contribute to atherosclerosis and thrombosis in type 2 diabetes. Contact of endothelial monolayers with GPVI/ligation-stimulated platelets might induce MCP-1, which enhances monocyte chemotaxis. Alteration of adhesive properties of endothelium through upregulated ICAM-1 expression might further support monocyte adhesion and transmigration. Thus, inhibition of GPVI-expression and platelet-endothelial interaction may be an effective strategy in downregulating atherosclerotic mechanisms in diabetes.

Here, we demonstrate for the first time that platelet GPVI plays a critical role in the initiation of atherosclerosis and thrombosis in diabetes. These data extend our knowledge of the vascular events in diabetes and point to the importance of platelets as a target for novel anti-atherosclerotic therapies in diabetes. Taken together, the present study emphasizes the fact that GPVI remains one of the promising pharmacological targets for antiplatelet therapy in diabetes.

6 Summary

Diabetes is associated with an enhanced collagen-mediated platelet activation that contributes significantly to thromboischemic complications. In this analysis, the platelet collagen receptor GPVI was studied in patients with type 2 diabetes. Surface expression of the platelet FcγR that forms a functional complex with GPVI was significantly increased in diabetes compared with non-diabetes (42.4 ± 14.0 vs. 38.4 ± 12.3, p=0.02). FcγR expression correlated with GPVI expression (r=0.529, p<0.001) and was found to be an independent risk factor of diabetes (coefficient 0.024; p=0.008). Stimulation of GPVI through a specific anti-GPVI monoclonal antibody significantly enhanced platelet surface expression of CD40L in normal control platelets. GPVI-dependent CD40L secretion was enhanced in type 2 diabetes compared with non-diabetes. Soluble recombinant GPVI substantially inhibited the GPVI-induced CD40L release (p<0.001). 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. Co-incubation of GPVI-stimulated platelets resulted in substantial enhanced secretion of MCP-1 (p<0.01) and surface expression of ICAM-1 of cultured HUVECs. These results suggest that function of collagen receptor GPVI is altered in type 2 diabetes and may play an important role in platelet-induced inflammation of endothelial cells, and thus, in atherothrombotic complications in diabetic patients. Inhibition of GPVI may be a promising pharmacological target in treatment of high-risk diabetic patients.

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J. Biol. Chem. 276 (2001) 12999-13006

8 Resume

Curriculum Vitae

Personal data:

Name	: Zhongyan Li	Gender	: Female
Birth date	: July 10, 1971	Birth place	: Heilongjiang, P.R.China
Nationality	: Chinese	Marital status	: Married

Education:

9/1978-7/1983 No.1 Primary School in Tieli City, Heilongjiang Province, P.R.China

9/1983-7/1986 No.1 High School in Tieli City, Heilongjiang Province, P.R.China (junior high school)

9/1986-7/1989 No.1 High School in Tieli City, Heilongjiang Province, P.R.China (senior high school)

9/1989-6/1994 Graduate student in Harbin Medical University, P.R.China a Bachelor's Degree of Medical Science awarded in June, 1994.

9/1994-6/1997 Postgraduate student in Dalian Medical University, P.R.China a Master's Degree of Medical Science (Internal Medicine with major in Cardiology) awarded in June,1997.

9/2001-4/2002 Has learned German in Tong-ji University in Shanghai (P.R. China) financed by China Scholarship Council.

6/2002-9/2002 Has learned German in Frankfurt/Main (Germany) financed by DAAD (Deutscher Akademischer Austauschdienst), and has passed the German language test "Mittelstufe II" and the "Test DaF".

10/2002-now A doctoral student supported by DAAD in the Department of Cardiology, German Heart Center Munich in Technical University Munich, Germany.

Working experience:

8/1997-8/2001: A resident physician (8/1997-8/1999) and an attending physician (9/1999-8/2001) in the division of Cardiology, Department of Internal Medicine, the Second Affiliated Hospital of Dalian Medical University, P.R.China. At the same time, a researcher in Internal Medicine laboratory and a lecturer in charge of Cardiology for the clinical practice of the students and in class in Dalian Medical University.

Publications:

1) Original papers

1. **Zhongyan Li**, Changyu Li, Chuanxun Li. Evaluation of sympathetic nervoussystem in insulin-resistant hypertensive rats. Chinese Journal of Hypertension, 2000, 8 (2): 159-161.
2. **Zhongyan Li**, Changyu Li, Yonge Liu. Evaluation of renin-angiotensin-aldosterone system in insulin resistant hypertension. Journal of Postgraduates of Medicine, 2000, 23 (12): 14-16.
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2) Oral presentations and posters

1. **Zhongyan Li**, Changyu Li. Evaluation of renin-angiotensin-aldosterone system in the insulin resistant hypertensive rats. the 9th China-Japan Joint Conference on Cardiovascular Diseases. Xi`an, P.R.China, Oct 9-10, 1997: P175-176.
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