

# Type 1 And Type 2 Diabetic Patients Display Different Patterns of Cellular Microparticles

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**The development of vasculopathies in diabetes involves multifactorial processes including pathological activation of vascular cells. Release of microparticles by activated cells has been reported in diseases associated with thrombotic risk, but few data are available in diabetes. The aim of the present work was to explore the number and the procoagulant activity of cell-derived microparticles in type 1 and 2 diabetic patients. Compared with age-matched control subjects, type 1 diabetic patients presented significantly higher numbers of platelet and endothelial microparticles (PMP and EMP), total annexin V-positive blood cell microparticles (TMP), and increased levels of TMP-associated procoagulant activity. In type 2 diabetic patients, only TMP levels were significantly higher without concomitant increase of their procoagulant activity. Interestingly, in type 1 diabetic patients, TMP procoagulant activity was correlated with HbA<sub>1c</sub>, suggesting that procoagulant activity is associated with glucose imbalance. These results showed that a wide vesiculation process, resulting from activation or apoptosis of several cell types, occurs in diabetes. However, diabetic patients differ by the procoagulant activity and the cellular origin of microparticles. In type 1 diabetic patients, TMP-procoagulant activity could be involved in vascular complications. Moreover, its correlation with HbA<sub>1c</sub> reinforces the importance of an optimal glycemic control in type 1 diabetes. *Diabetes* 51:2840–2845, 2002**

**L**ong-term micro- and macrovascular complications represent the main cause of morbidity and mortality in both type 1 and type 2 diabetes. Microangiopathy is a common feature of both types of diabetes, whereas macroangiopathy occurs more frequently in type 2 diabetes on account of the clustering of other traditional risk factors of atherosclerosis, i.e.,

hypertension, dyslipidemia, or obesity. The pathogenesis of diabetic vascular complications is complex and multifactorial (1). Early alterations of endothelial function may be involved in the development of both micro- or macroangiopathy in diabetic patients. Hyperglycemia plays a crucial role in vascular injury since it leads to the accumulation of polyol compounds and advanced glycation end products and contributes to an increased oxidative stress. Furthermore, hyperlipidemia and hypertension may contribute in a large part to the changes in endothelial function and to the development and the progression of atherosclerosis, especially in type 2 diabetes. Diabetes is also associated with the activation of blood and vascular cells. This cellular activation constitutes one of the major mechanisms leading to accelerated atherosclerosis. Polymorphonuclear cells present abnormal chemotaxis (2,3), as seen through their spontaneous adhesion and increased expression of adhesion molecules like CD11b/CD18 or CD11c. Moreover, an activation of a procoagulant pathway, reflected by tissue factor expression induced on monocytes, has been proposed as a marker of diabetic microangiopathy (4,5). Activated platelets expressing adhesive molecules (P-selectin, thrombospondin, CD63, and activated glycoprotein IIb/IIIa) circulate in type 1 diabetic patients with clinical signs of vascular complications (6). Thus the thrombotic potency of platelets adhering to other cell types could contribute to circulatory disturbances. A large body of evidence indicates that endothelial dysfunction is closely associated with microangiopathy and atherosclerosis in diabetes as shown by a great variety of markers including poor endothelial cell-dependent vasodilatation and increased levels of soluble markers released by endothelial cells including von Willebrand factor, thrombomodulin, E-selectin, plasminogen-activator inhibitor (PAI)-1, tissue plasminogen activator (t-PA), vascular cell adhesion molecule (VCAM)-1, or intracellular adhesion molecule (ICAM)-1 (7–11).

It is now well accepted that in response to activation or apoptosis, all eukariotic cells shed microparticles (12). These elements are produced from the plasma membrane after the flip-flop of the membrane phospholipids leading to a loss of membrane asymmetry. The blebs formed at the cell surface are then shed in the circulation under the form of vesicles ranging in size from 0.1 to 1  $\mu\text{m}$ . Increased levels of microparticles, mainly derived from platelets and to a lesser extent from leukocytes and endothelial cells, have been described in several pathologies associated with prothrombotic and proinflammatory tendencies like

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EMP, endothelial microparticle; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PE, phycoerythrin; PMP, platelet microparticle; TMP, total annexin V-positive blood cell microparticle; TNF, tumor necrosis factor.

TABLE 1  
Demographic description of patients and healthy donors

	Healthy donors	Diabetic patients		P*
		Type 1	Type 2	
N	47	24	52	—
Men/Women	18/29	16/8	26/26	—
Mean age (years)	44 ± 13	34 ± 12	57 ± 10	0.0001
Duration of disease (years)	—	12 ± 7	10 ± 8	NS
BMI (kg/m <sup>2</sup> )	22 ± 3	23.5 ± 4.1	28.8 ± 4.4	p < 0.0001
Biological parameters				
Fasting glycemia (mmol/l)	4.0 ± 1.5	8.0 ± 4.4	8.9 ± 2.4	NS
Total cholesterol (mmol/l)	4.20 ± 0.80	4.91 ± 0.80	4.94 ± 0.98	NS
LDL cholesterol (mmol/l)	2.52 ± 0.4	2.87 ± 0.65	2.92 ± 0.93	NS
HDL cholesterol (mmol/l)	1.32 ± 0.25	1.53 ± 0.49	1.29 ± 0.36	0.032
Triglycerides (mmol/l)	0.8 ± 0.33	0.96 ± 0.49	1.6 ± 0.89	0.0016
Albuminuria (mg/24 h)	15 ± 20	204 ± 709	165 ± 470	NS
HbA <sub>1c</sub> (%)	—	9.2	8.0	0.034
Vascular complications				
Macrovascular complications	—	8%	14%	ND
Retinopathy	—	37.5%	27%	ND
Nephropathy	—	12.5%	21%	ND
Neuropathy	—	33%	47%	ND

Data are means ± SD. \*Type 1 diabetic patients vs. type 2 diabetic patients. ND, not determined; NS, not significant.

heparin-induced thrombocytopenia, thrombotic thrombocytopenic purpura, paroxysmal nocturnal hemoglobinuria, HIV infection, and acute coronary syndromes (13–17). Moreover, a recent work has shown that, in atherosclerotic plaques, procoagulant microparticles result from an apoptotic phenomenon involving monocytic and vascular cells (18).

The present study focused on microparticles originating from platelet and endothelial cells, defined as GPIIb/IIIa and  $\alpha$ v $\beta$ 3-positive microparticles, respectively, and microparticles expressing phosphatidylserine defined as total annexin V-positive microparticles. Our initial aim was to investigate the level and procoagulant activity of circulating microparticles in type 1 and type 2 diabetic patients. These microparticles were then explored in relation with type of diabetes and with biological and clinical features of diabetic patients.

## RESEARCH DESIGN AND METHODS

**Patients and control subjects.** All of the subjects gave informed consent in accordance with local ethics committee recommendations. A total of 47 healthy subjects aged 18–60 years were recruited. They had a normal biological check-up and BMI and presented no history of vascular complications (either micro- or macrovascular). Patients were distributed into two groups: type 1 diabetic individuals ( $n = 24$ ; age range 16–55 years, mean 34) and type 2 diabetic individuals ( $n = 52$ ; age range 36–69 years, mean 57).

Subjects with <16 years or >70 years and a previous history of neoplasm, auto-immune disease other than type 1 diabetes, liver disease, or acute infections were excluded from the study.

Retinal vascular morphology was evaluated by direct ophthalmoscopy and fluorescein angiography. Retinopathy was scored as absent, background, preproliferative, or proliferative. Nephropathy was diagnosed by confirmed microalbuminuria (30–300 mg/day) or macroalbuminuria (>300 mg/day) on two 24-h samples of urine collected over a 6-month period in the absence of urinary infections or other renal diseases. Peripheral neuropathy was assessed for sensory and motor function by clinical examination and classified as normal or impaired.

Macroangiopathy was considered in subjects who met one of the following conditions: 1) history and/or absence of angina and/or permanent ischemic electrocardiogram abnormalities at rest or ischemic abnormalities in a stress, 2) claudication and/or abolished peripheral pulses and/or foot lesions due to vascular disease demonstrated by Doppler echography, and 3) history of

stroke and/or significant carotid stenosis (>50%) as assessed by Doppler echography.

All type 1 diabetic patients were insulin-treated, whereas 13 of 52 type 2 diabetic patients (25%) received insulin, alone or in combination with oral hypoglycemic agents (metformin and/or sulfonylureas and/or acarbose). Moreover, the proportion of subjects who were undergoing treatment with aspirin, ACE inhibitors, or statins was greater in type 2 than in type 1 diabetic patients (19.2 vs. 8.3, 26.9 vs. 12.5, and 21.2 vs. 8.3%, respectively), but those differences were not statistically significant.

**Reagents.** Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against  $\alpha$ v $\beta$ 3 (FITC-CD51, clone AMF7) and phycoerythrin (PE)-conjugated mAb against platelet glycoprotein GPIIb/IIIa (PE-CD41, clone P2) were used to identify endothelial microparticle (EMP) and platelet microparticle (PMP), respectively. FITC-conjugated mAbs against CD45 and CD66b were used to identify microparticles from leukocytes and neutrophils, respectively. PE-conjugated mAb against CD14 was used to identify microparticles from monocytes. PE- and FITC-conjugated isotype controls (PE-IgG1, FITC-IgG1) were used to define the background noise of the labeling. FITC-conjugated annexin V was used to label total microparticles. All labeling reagents were purchased at Immunotech Beckman Coulter (Marseille, France). Microparticle absolute values were determined using Flowcount beads (Beckman Coulter, Margency, France).

**Blood sampling for microparticle quantitation and procoagulant activity measurements.** Venipuncture was performed on 0.129 mol/l sodium citrate tubes (Vacutainer; Becton Dickinson) on informed healthy donors and patients. Within 2 h, whole blood was treated as previously described (19). Briefly, samples were centrifuged 15 min at 1,500g, and the plasma was then harvested and spun down at 13,000g during 2 min. Supernatants were then immediately frozen at  $-80^{\circ}\text{C}$  until used.

**Microparticle quantitation.** Numeration of platelet- and endothelial-derived microparticles was performed, as previously described (19), using anti-CD41 and anti-CD51 labeling, respectively. After thawing, 30  $\mu$ l of plasma was incubated with either FITC-CD51 or PE-CD41PE and their corresponding isotype control for EMP and PMP quantitation, respectively. For total annexin V-positive blood cell microparticle (TMP) quantitation, 30  $\mu$ l of plasma was incubated with FITC-annexin V. After a 30-min incubation at room temperature, samples were diluted in 1.5 ml of PBS (Dulbecco's; Life Technologies, Paisley, U.K.) or binding buffer (Beckman Coulter Immunotech) for mAbs and annexin V labeling, respectively. Then, 30  $\mu$ l of Flowcount beads was added to each sample for calculation of microparticle absolute value.

**Flow cytometric analysis.** After labeling and dilution, samples were analyzed by flow cytometry on an EPICS XL (Beckman Coulter). Microparticles present in plasma were analyzed according to their parameters of size and fluorescence. Briefly, on a LogFS-LogSS dotplot, the microparticle upper size limit was defined using 0.8- $\mu$ m beads, and a gate was drawn around the population. The lower limit of the gate excludes the first channels that contain

TABLE 2  
Microparticle levels and procoagulant activity in the total control population and in two subgroups adjusted to the age of diabetic patients

	C (n = 47)	C1 (n = 19)	C2 (n = 28)	P*
Healthy donors				
Mean age (years)	44	34	51	
EMP (per $\mu\text{l}$ plasma)	14 $\pm$ 16	13 $\pm$ 17	16 $\pm$ 15	NS
PMP (per $\mu\text{l}$ plasma)	625 $\pm$ 493	564 $\pm$ 398	702 $\pm$ 631	NS
TMP (per $\mu\text{l}$ plasma)	752 $\pm$ 612	691 $\pm$ 300	810 $\pm$ 839	NS
Procoagulant activity (equivalent nmol/l phosphatidylserine)	14 $\pm$ 9	15 $\pm$ 9	12 $\pm$ 8	NS

Data are means  $\pm$  SD. C, whole healthy donor group; C1, control subjects adjusted to the age of type 1 diabetic patients; C2, control subjects adjusted to the age of type 2 diabetic patients.

the electronic background noise of the machine. Only the events included in this gate were further analyzed for their fluorescence on a LogSS-LogFL dotplot. The Flowcount beads were counted in a third dotplot LogSS-LogFL3. Using the absolute value of this calibrant, microparticle count was expressed as microparticles per microliter of plasma.

**Microparticle-linked procoagulant activity measurement.** The microparticle procoagulant activity was determined, as previously described (16), using a prothrombinase assay after capture on a microtitration plate coated with annexin V. The blood clotting factor concentrations were determined to ensure that phosphatidylserine is the rate-limiting parameter of the reaction, and results were expressed as nanomolar phosphatidylserine equivalent by reference to a standard curve constructed by the use of liposomes of defined composition.

**Statistical analysis.** Differences between groups were evaluated by the nonparametric Mann-Whitney *U* test; *P* < 0.05 was considered significant.

## RESULTS

**Demographic description of patients.** Biological parameters and clinical features of diabetic patients and healthy donors are presented in Table 1. Patients suffering from type 1 or type 2 diabetes differed by their age, BMI, and levels of HDL cholesterol, triglycerides, and HbA<sub>1c</sub>.

**Microparticle levels in the healthy population.** Microparticles derived from endothelial cells and platelets (EMP and PMP, respectively) were enumerated by flow cytometry in the plasma of healthy subjects using anti-CD51 and anti-GpIIb/IIIa, respectively. Because type 1 diabetic patients were younger than type 2 diabetic patients, we first analyzed the levels of microparticles in healthy donors according to age (Table 2). We divided our control popu-

lation into two subgroups (C1 and C2) adjusted on the age of the corresponding diabetic patients (type 1 and type 2, respectively). Elderly healthy subjects (C2) presented higher levels of EMP and PMP but in a nonsignificant manner. Since no methodology allows to isolate and evaluate specifically the procoagulant activity of microparticles derived from one cell type, the whole annexin V-positive population (TMP) was considered for procoagulant activity measurement. Results showed that elderly healthy subjects (C2) presented slightly higher levels of TMP than younger (C1) subjects, whereas an inverse tendency was observed regarding their procoagulant activity. Although the differences were not significant, these two control groups were used for comparative analysis between type 1 and type 2 diabetic patients.

**Analysis of microparticle pattern according to the type of diabetes.** Compared with healthy subjects, the whole diabetic group displayed higher levels of EMP (Fig. 1A) (*P* = 0.0032). EMP levels were significantly elevated in type 1 diabetic patients versus C1 control subjects (*P* = 0.016), whereas no difference was observed in type 2 diabetic patients versus C2 control subjects or between type 1 and type 2 diabetic patients.

In the whole diabetic group, PMP numbers were similar to those of healthy donors. Nevertheless, when patients were analyzed according to the type of diabetes, PMP levels were significantly elevated only in type 1 diabetic patients, as compared with C1 control subjects (Fig. 1B, *P* = 0.04). No difference was observed between type 1 and type 2 diabetic patients.

Compared with healthy donors, numbers of TMP were significantly elevated in the whole diabetic group (Fig. 2A) (*P* = 0.0021). In reference with their respective control subjects, TMP was also significantly elevated in both type 1 and type 2 diabetic patients (Fig. 2A) (*P* = 0.0028 and *P* = 0.04, respectively), with levels significantly higher in type 1 than in type 2 diabetic patients (1,238  $\pm$  556 and 988  $\pm$  690 TMP/ $\mu\text{l}$ , respectively, *P* = 0.024). Interestingly, the procoagulant activity of TMP was significantly increased in the whole diabetic group (Fig. 2B, *P* = 0.009) and in type 1 but not in type 2 diabetic patients (*P* = 0.0013). Among diabetic patients, we found a higher procoagulant activity in type 1 than in type 2 diabetic patients (*P* < 0.0001). No correlation was observed between num-

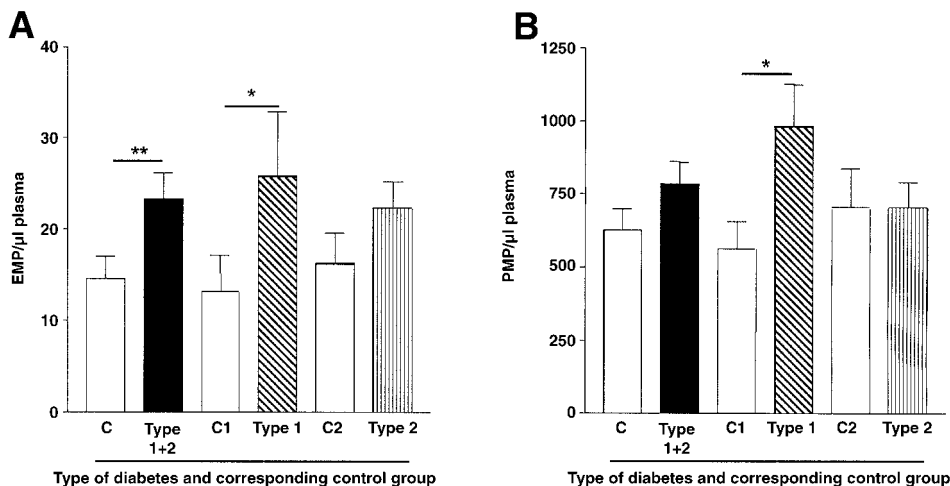
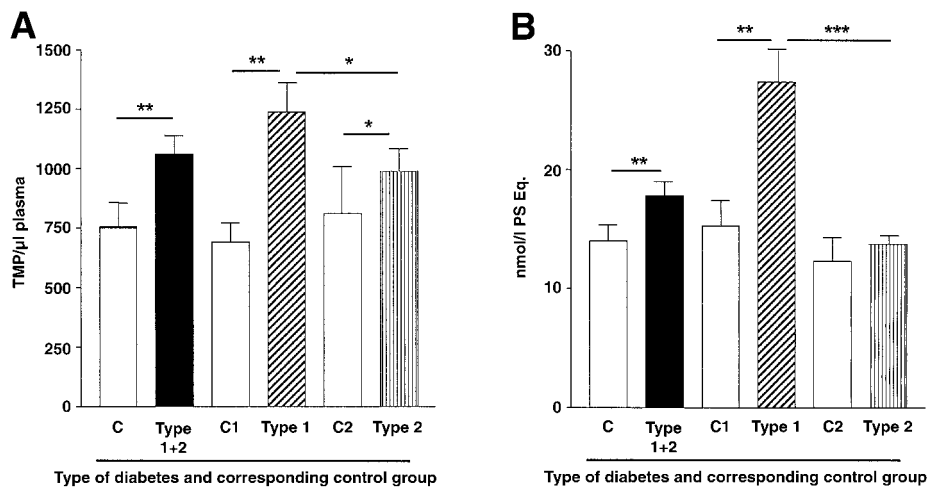


FIG. 1. Flow cytometric enumeration of microparticles. Flow cytometric analysis of microparticles in the platelet-free plasma from healthy donors (C: *n* = 47,  $\square$ ) and all diabetic patients (type 1 + 2, *n* = 76,  $\blacksquare$ ) was performed using mAb directed against either  $\alpha\text{v}\beta 3$  (FITC-CD51) or GPIIb/IIIa (PE-CD41) for labeling of EMP (A) and PMP (B), respectively. Microparticles were quantitated in plasma as described in RESEARCH DESIGN AND METHODS. Then, young and elderly healthy subjects (C1: *n* = 19,  $\square$ , and C2: *n* = 28,  $\square$ ) were compared with their corresponding patient groups (type 1: *n* = 24,  $\text{▨}$ , and type 2: *n* = 52,  $\text{▨}$ ). Results were expressed as number of microparticles per microliter of platelet-free plasma. The difference between groups was analyzed using a Mann-Whitney *U* test (\**P* < 0.05; \*\**P* < 0.005).



**FIG. 2.** Total microparticle number and procoagulant activity in diabetic patients. Annexin V was used for both TMP quantitation by flow cytometry (A) and TMP capture for determination of the procoagulant activity (B). Number and TMP procoagulant activity were measured in healthy donors (C:  $n = 47$ , □) and all diabetic patients (type 1 and 2:  $n = 76$ , ■). Then, young and elderly healthy subjects (C1:  $n = 19$ , □, and C2:  $n = 28$ , □) were compared with their corresponding patient groups (type 1:  $n = 24$ , ▨, and type 2:  $n = 52$ , ▩, respectively). TMP count was expressed as TMP per microliter of plasma and TMP procoagulant activity as nanomolar of equivalent phosphatidylserine. The difference between groups was analyzed using a Mann-Whitney  $U$  test (\* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0005$ ).

ber and procoagulant activity of TMP. In summary, analysis of the pattern of cellular microparticles showed that EMP, PMP, and TMP numbers and procoagulant activity were elevated in type 1 diabetic patients, whereas only TMP numbers were increased in type 2 diabetic patients.

To investigate the cellular component giving rise to the significant increase in annexin V-positive microparticles observed in type 2 diabetic patients, we quantitated microparticles derived from total leukocytes, neutrophils, and monocytes using CD45, CD66b, and CD14 labeling, respectively. As shown in Fig. 3, leukocyte-derived microparticles were significantly higher in type 1 and type 2 diabetic patients ( $38 \pm 44$  and  $37 \pm 43/\mu\text{l}$ , respectively) compared with control subjects ( $14 \pm 18/\mu\text{l}$ ). This increase was associated with a significant elevation of microparticles derived from neutrophils in type 1 and type 2 diabetes ( $22 \pm 23$  and  $21 \pm 15/\mu\text{l}$ , respectively) compared with control subjects ( $9 \pm 8/\mu\text{l}$ ), whereas monocyte microparticles were increased but in a nonsignificant manner.

**Correlations between microparticles and clinical and biological parameters.** Among diabetic patients, no cor-

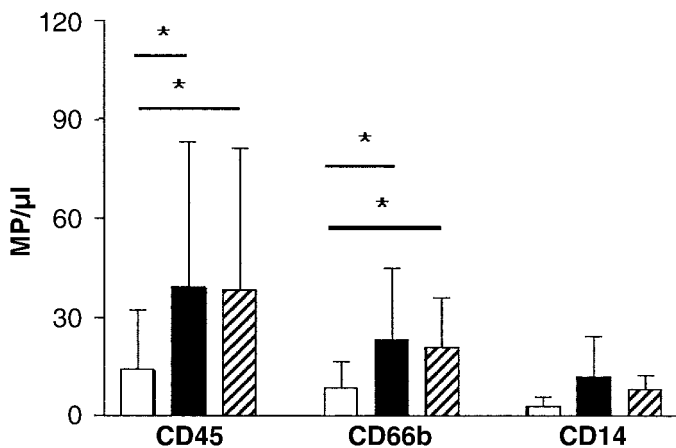
relation was observed between microparticle levels (whatever their cellular origin) and fasting glycemia, HDL, LDL, triglycerides, fibrinogen, PCR, and clinical onset of vascular complications or treatment (insulin, aspirin, ACE inhibitors, or statins). Interestingly, the procoagulant activity of TMP was positively correlated with levels of HbA<sub>1c</sub> in both the whole diabetic group and the type 1 diabetic patients ( $r = 0.27$ ,  $P = 0.018$  and  $r = 0.48$ ,  $P = 0.03$ , respectively). The levels of EMP were positively correlated with the albuminuria in type 1 diabetic patients ( $r = 0.52$ ,  $P = 0.025$ ) and with total cholesterol in type 2 diabetic patients ( $r = 0.51$ ,  $P < 0.0001$ ).

Although the proportion of patients with vascular complications was not sufficient to perform extensive correlations, type 1 diabetic patients suffering from one or more microvascular complications (retinopathy, nephropathy, or neuropathy) displayed higher EMP mean levels than those who did not (data not shown). It is important to note that the highest amounts of EMP were found in patients suffering from microvascular complications.

## DISCUSSION

The present study showed that both type 1 and type 2 diabetes are associated with increased levels of circulating microparticles. However, diabetic patients differ by the procoagulant activity and the cellular origin of microparticles. Indeed, only TMP numbers were increased in type 2 diabetes, whereas EMP, PMP, and TMP levels and procoagulant activity were elevated in type 1 diabetes. Interestingly, this procoagulant activity was correlated with levels of HbA<sub>1c</sub>.

A wide vesiculation process, probably involving several cell types, is associated with diabetes, as shown by the elevated numbers of TMP in both type 1 and type 2 diabetes. The mechanisms of vesiculation are not completely elucidated. A large body of evidence obtained from in vitro studies and animal models has shown that this process can be triggered by activating factors such as the complement membrane attack complex (MAC) (20) or by cytokines like tumor necrosis factor (TNF) and interleukin-1 $\beta$  (21). Vesiculation can also be induced in conditions like apoptosis or shear stress disturbance (18,21). All of these parameters are potentially implicated in the development of diabetic vascular complications and may thus



**FIG. 3.** Numeration of leukocyte microparticles. Flow cytometry analysis of microparticles derived from leukocytes, neutrophils, and monocytes was performed using CD45, CD66b, and CD14 labeling, respectively. Microparticles were numerated in plasma from healthy donors (C: □), type 1 diabetic patients (■) and type 2 diabetic patients (▨) as described in RESEARCH DESIGN AND METHODS. Results are expressed as number of microparticles per microliter of platelet-free plasma. The difference between groups was analyzed using a Mann-Whitney  $U$  test (\* $P < 0.05$ ).

account for a part of the vesiculation process (22–25). Previous studies have documented an increased number of PMP in diabetic patients, but none investigated vesiculation according to the type of diabetes (26–28). Our results showed that a significant elevation of PMP occurs in type 1 diabetes, whereas no significant increase was observed between type 2 diabetic patients and their age-matched control subjects. This observation differs from data reported by Nomura et al. (29) showing that PMP levels are increased in type 2 diabetic patients and therefore may be used as a marker of vascular disease. Differences in both patients and control population recruitment and/or in microparticle analysis may account for this discrepancy. Indeed, our population included patients with or without microvascular complications, whereas the study of Nomura et al. has focused on complicated forms of diabetes. Moreover, our detection of PMP was performed on platelet-free plasma in which PMP were labeled with anti-GPIIb/IIIa mAb, whereas Nomura et al. used PMP extracted from a platelet suspension and labeled together with additional washed platelets using anti-GPIb mAb. In our study, we can rule out an effect of aspirin treatment on PMP levels since no significant difference in PMP number was noted between patients who were or were not undergoing this treatment. In addition, we have previously reported the lack of effect of aspirin on PMP levels in healthy donors and in patients undergoing coronary angioplasty (30).

Endothelial dysfunction is a common feature of diabetic patients who have early or advanced nephropathy. Recently, we and others have shown that the release of EMP reflects endothelial alterations in pathologies such as antiphospholipid syndrome (31), meningococcal sepsis (32), thrombotic thrombocytopenic purpura (33), and acute coronary syndromes (17). Moreover, we have demonstrated that cultured endothelial cells stimulated by TNF released microparticles that display the same procoagulant receptors and adhesion molecules as the cells of origin (31). A novel finding of the present study is that EMPs are found in higher amounts in type 1 diabetic patients, whereas the increase does not reach statistical significance in type 2 diabetic patients. The highest levels of EMP in type 1 diabetic patients with microvascular complications, together with the positive correlation between EMP levels and albuminuria, suggest that EMP could be a marker of the endothelial damage associated with type 1 diabetic nephropathy.

In type 2 diabetes, the TMP number was increased, whereas PMP and EMP numbers were not significantly modified. Enhanced vesiculation of leukocyte could account for the result, as shown by increased levels of CD45- and CD66-b–positive microparticles in these patients. Consistent with the involvement of neutrophil activation, several studies have reported upregulation of adhesion molecules on neutrophil from diabetic patients (34,35).

At present, no study has explored the procoagulant activity of the microparticles present in the circulation of diabetic patients. Numerous works reported that microparticles are rich in membrane receptors for coagulation factor Va and provide a catalytic surface for the assembly of the prothrombinase complex. Thus, microparticles produced by circulating blood cells could play a role in the

dissemination of a procoagulant potential. Surprisingly, despite high numbers of TMP, type 2 diabetes was not associated with an elevation of microparticle procoagulant activity, whereas type 1 diabetes was characterized by a concomitant increase of numbers and procoagulant activity of TMP. The mechanism underlying the difference in this intrinsic procoagulant activity is actually unknown. In our functional assay, the amount of thrombin generated by microparticles strictly depends on phosphatidylserine exposure. Therefore, heterogeneity in aminophospholipid content could account for the differences observed in the procoagulant phenotype of microparticles released in the two diabetic populations. In addition, several studies have described changes of tissue factor pathway in diabetes and their involvement in the vascular complications (36). Accordingly, another procoagulant mechanism could result from tissue factor expression by microparticles. However, this hypothesis can be ruled out because no significant difference in tissue factor activity of the circulating microparticles has been observed between the two diabetic groups (data not shown).

Interestingly, a positive correlation was found between this total procoagulant activity and HbA<sub>1c</sub> levels but not with fasting glycemia. The procoagulant activity of microparticles could be indicative of a chronic lack of balance of glycemia rather than an acute phenomenon and could enhance blood thrombogenicity in type 1 diabetic patients. This observation is sustained by the higher thrombotic risk of poorly controlled HbA<sub>1c</sub> in diabetic patients (37).

In addition to coagulation receptors, microparticles carry adhesion molecules at their surface. They have the capacity to bind to circulating cells or endothelium and therefore to participate in a “long range” transmission of inflammatory information. Recently, we demonstrated that microparticles released by stimulated endothelial cells can activate tissue factor procoagulant pathway by adhering to monocytes (38). In the same way, leukocyte-derived microparticles interact with endothelial cells and induce tissue factor expression (39,40). Thus, we can speculate that microparticles released in both types of diabetes may adhere to circulating cells or endothelium, exacerbating cell activation and thus contributing to worsen vasculopathy and disease progress.

The different patterns of microparticle phenotype and procoagulant activity observed in both types of diabetes may have different causes and biological consequences. Among these consequences, the procoagulant activity carried by microparticles is potentially involved in type 1 diabetic vascular complications. Moreover, its relation with the impaired glucose balance suggests that it could be useful in monitoring disease equilibrium.

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#### REFERENCES

1. Kirpichnikov D, Sowers JR: Diabetes mellitus and diabetes-associated vascular disease. *Trends Endocrinol Metab* 12:225–230, 2001
2. Wierusz-Wysocka B, Wysocki H, Siekierka H, Wykretowicz A, Szczepanik A, Klimas R: Evidence of polymorphonuclear neutrophils activation in patients with insulin dependent diabetes. *J Leukoc Biol* 42:519–532, 1987
3. Rao KM, Hatchell DL, Cohen HJ, De La Paz MA: Alterations in stimulus-

- induced integrin expression in peripheral blood neutrophils of patients with diabetic retinopathy. *Am J Med Sci* 313:131-135, 1997
4. Reverter JL, Reverter JC, Tassies D, Rius F, Monteagudo J, Rubies-Prat J, Escolar G, Ordinas A, Sanmarti A: Thrombomodulin and induced tissue factor expression on monocytes as markers of diabetic microangiopathy: a prospective study on hemostasis and lipoproteins in insulin-dependent diabetes mellitus. *Am J Hematol* 56:93-99, 1997
  5. Patino R, Ibarra J, Rodriguez A, Ruiz-Yague M, Pintor E, Fernandez-Cruz A, Figueredo A: Circulating monocytes in patients with diabetes mellitus, arterial disease, and increased CD14 expression. *Am J Cardiol* 85:1288-1291, 2000
  6. Tschöpe D, Driesch E, Schwippert B, Nieuwenhuis HK, Gries FA: Exposure of adhesion molecules on activated platelets in patients with newly diagnosed IDDM is not normalized by near-normoglycemia. *Diabetes* 44:890-894, 1995
  7. Calles-Escandro J, Cipolla M: Diabetes and endothelial dysfunction: a clinical perspective. *Endocr Rev* 22:36-52, 2001
  8. Cosentino F, Luscher TF: Endothelial dysfunction in diabetes mellitus (Review Article). *J Cardiovasc Pharmacol* 32 (Suppl. 3): S54-S61, 1998
  9. Huszka M, Kaplar M, Rejto L, Tornai I, Palatka K, Laszlo P, Udvardy M: The association of reduced endothelium relaxing factor: NO production with endothelial damage and increased in vivo platelet activation in patients with diabetes mellitus. *Thromb Res* 86:173-180, 1997
  10. Huvers FC, De Leeuw PW, Houben AJ, De Haan CH, Hamulyak K, Schouten H, Wolffenbuttel BH, Schaper NC: Endothelium-dependent vasodilatation, plasma markers of endothelium function, and adrenergic vasoconstrictor responses in type 1 diabetes under near-normoglycemic conditions. *Diabetes* 48:1300-1307, 1999
  11. Makimattila S, Virkamaki A, Groops PH, Cockcroft J, Utriainen T, Fagerudd J, Yki-Jarvinen H: Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation* 94:1276-1282, 1996
  12. Zwaal RF, Comfurius P, Bevers EM: Platelet procoagulant activity and microvesicle formation: its putative role in hemostasis and thrombosis. *Biochim Biophys Acta* 1180:1-8, 1992
  13. Warkentin TE, Sheppard JI: Generation of platelet-derived microparticles and procoagulant activity by heparin-induced thrombocytopenia IgG/serum and other IgG platelet agonists: a comparison with standard platelet agonists. *Platelets* 10:319-326, 1999
  14. Galli M, Grassi A, Barbui T: Platelet-derived microvesicles in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome. *Thromb Haemost* 75:427-431, 1996
  15. Hugel B, Socie G, Vu T, Toti F, Gluckman E, Freyssinet JM, Scrobohaci ML: Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. *Blood* 93:3451-3456, 1999
  16. Aupeix K, Hugel B, Martin T, Bischoff P, Lill H, Pasquali JL, Freyssinet JM: The significance of shed membrane particles during programmed cell death in vitro, and in vivo, in HIV-1 infection. *J Clin Invest* 99:1546-1554, 1997
  17. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A: Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation* 101:841-843, 2000
  18. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A: Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. *Circulation* 99:348-353, 1999
  19. Combes V, Dignat-George F, Mutin M, Sampol J: A new flow cytometry method of platelet-derived microvesicle quantitation in plasma (Letter). *Thromb Haemost* 77:220, 1997
  20. Wiedmer T, Hall SE, Ortel TL, Kane WH, Rosse WF, Sims PJ: Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. *Blood* 82:1192-1196, 1993
  21. Nomura S, Imamura A, Okuno M, Kamiyama Y, Fujimura Y, Ikeda Y, Fukuhara S: Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines. *Thromb Res* 98:257-268, 2000
  22. Acosta J, Hettinga J, Fluckiger R, Krumrei N, Glodfine A, Angarita L, Halperin J: Molecular basis for a link between complement and the vascular complications of diabetes. *Proc Natl Acad Sci U S A* 97:5450-5455, 2000
  23. Giordano C, Stassi G, Todaro M, De Maria R, Richiusa P, Scorsone A, Giordano M, Galluzzo A: Low bcl-2 expression and increased spontaneous apoptosis in T-lymphocytes from newly-diagnosed IDDM patients. *Diabetologia* 38:953-958, 1995
  24. Green EA, Flavell RA: The temporal importance of TNF alpha expression in the development of diabetes. *Immunity* 12:459-469, 2000
  25. Srinivasan S, Stevens M, Wiley JW: Diabetic peripheral neuropathy: evidence for apoptosis and associated mitochondrial dysfunction. *Diabetes* 49:1932-1938, 2000
  26. Shouzu A, Nomura S, Hayakawa T, Omoto S, Shimizu H, Miyake Y, Yonemoto T, Fukuhara S, Iwasaka T, Nishikawa M, Inada M: Effect of sarpogrelate hydrochloride on platelet-derived microparticles and various soluble adhesion molecules in diabetes mellitus. *Clin Appl Thrombosis/Hemostasis* 6:139-143, 2000
  27. Nomura S, Shouzu A, Omoto S, Hayakawa T, Kagawa H, Nishikawa M, Inada M, Fujimura Y, Ikeda Y, Fukuhara S: Effect of cilostazol on soluble adhesion molecules and platelet-derived microparticles in patients with diabetes. *Thromb Haemost* 80:388-392, 1998
  28. Omoto S, Nomura S, Shouzu A, Hayakawa T, Shimizu H, Miyake Y, Yonemoto T, Nishikawa M, Fukuhara S, Inada M: Significance of platelet-derived microparticles and activated platelets in diabetic nephropathy. *Nephron* 81:271-277, 1999
  29. Nomura S, Suzuki M, Katsura K, Xie GL, Miyazaki Y, Miyake T, Kido H, Kagawa H, Fukuhara S: Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus. *Atherosclerosis* 116:235-240, 1995
  30. Combes V, Joly P, Ambrosi P, Sabatier F, Camoin L, Pili J, Sampol J, Dignat-George F: Platelet vesiculation in angina treated with coronary angioplasty (Letter). *Thromb Haemost* 83:518, 2000
  31. Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, Mutin M, Sanmarco M, Sampol J, Dignat George F: In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest* 104:93-102, 1999
  32. Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romjin F, Hack CE, Sturk A: Microparticles from patients with multiple organ dysfunction and sepsis support coagulation through multiple mechanisms. *Thromb Haemost* 85:810-820, 2001
  33. Jimenez JJ, Jy W, Mauro LM, Horstman LL, Ahn YS: Elevated endothelial microparticles in thrombotic thrombocytopenic purpura: findings from brain and renal microvascular cell culture and patients with active disease. *Br J Haematol* 112:1-11, 2000
  34. Berliner S, Rogowski O, Rostein R, Fusman R, Shapira I, Bornstein NM, Prochorov V, Roth A, Keren G, Eldor A, Zeltser D: Activated polymorphonuclear leukocytes and monocytes in the peripheral blood of patients with ischemic heart and brain conditions correspond to the presence of multiple risk factors for atherothrombosis. *Cardiology* 94:19-25, 2000
  35. Chello M, Mastroberroto P, Cirillo F, Bevacqua E, Carrano A, Perticone F, Marchese AR: Neutrophil-endothelial cells modulation in diabetic patients undergoing coronary artery bypass grafting. *Eur J Cardiothorac Surg* 14:373-379, 1998
  36. Ichikawa K, Yoshinari M, Iwase M, Wakisaka M, Doi Y, Lino K, Yamamoto M, Fujishima M: Advanced glycosylation end products induced tissue factor expression in human monocyte-like U937 cells and increased tissue factor expression in monocytes from diabetic patients. *Atherosclerosis* 136:281-287, 1998
  37. Stratton IM, Adler AI, Neil HA, Matthews DR, Manley SE, Cull CA, Hadden D, Turner RC, Holman RR: Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* 321:405-412, 2000
  38. Sabatier F, Roux V, Anfosso F, Camoin L, Sampol J, Dignat-George F: Interaction of endothelial microparticles with monocytic cells induces tissue factor-dependent procoagulant activity. *Blood* 99:3962-3970, 2002
  39. Mesri M, Altieri DC: Endothelial cell activation by leukocyte microparticles. *J Immunol* 161:4382-4387, 1998
  40. Mesri M, Altieri DC: Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem* 274:23111-23118, 1999