Elevated Numbers of Tissue-Factor Exposing Microparticles Correlate With Components of the Metabolic Syndrome in Uncomplicated Type 2 Diabetes Mellitus

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Background—Type 2 diabetes is associated with accelerated atherosclerosis. Because cell-derived microparticles support coagulation and inflammation, they may be involved in atherogenesis. We characterized circulating microparticles both in patients with uncomplicated, well-regulated type 2 diabetes and in healthy subjects, as well as their relationship with coagulation and metabolic control.

Methods and Results—Microparticles were isolated from plasma, stained with annexin V, cell-specific monoclonal antibodies (MoAbs) and a MoAb directed against tissue factor (TF), and analyzed by flow cytometry. Microparticle numbers and origin were comparable in the two groups, but the median number of TF-positive microparticles was twice as high in patients than in controls (P=0.018). Patients had higher percentages of TF-positive microparticles from T-helper cells (P=0.045), granulocytes (P=0.004), and platelets (P=0.002). Subpopulations of TF-positive microparticles from platelets and T-helper cells exposed granulocytic markers. Correlations were found between the numbers of various TF-positive microparticle subpopulations and body mass index, fasting plasma glucose and insulin, or tumor necrosis factor-α and serum HDL cholesterol. Microparticles from patients generated less thrombin in vitro (P=0.007). Microparticle numbers did not correlate with in vivo coagulation markers prothrombin fragment F1+2 and thrombin-antithrombin complexes.

Conclusions—TF, possibly of granulocytic origin, is exposed on microparticle subpopulations in asymptomatic patients with well-regulated type 2 diabetes. TF-positive microparticles are associated with components of the metabolic syndrome but not with coagulation. Thus, TF on microparticles may be involved in processes other than coagulation, including transcellular signaling or angiogenesis. (Circulation. 2002;106:2442-2447.)

Key Words: diabetes mellitus ■ microparticles ■ tissue factor ■ atherosclerosis ■ coagulation

Microparticles (MP) are small membrane vesicles, released from blood cells or endothelial cells on activation or during apoptosis.1–3 MP support coagulation by exposure of negatively charged phospholipids and sometimes tissue factor (TF), the initiator of coagulation in vivo.4,5 MP bind to cells via specific adhesion receptors, thereby stimulating these cells to produce both TF and cytokines.5–8 Thus, cell-derived MP may affect coagulation and inflammation activation, both processes associated with atherogenesis. Recently, we demonstrated that MP of various cellular origin are present in vivo in the circulation of healthy subjects and in patients with vascular disease and disseminated intravascular coagulation (DIC), and that these MP support coagulation in plasma via various pathways.2,9,10

Type 2 diabetes is associated with accelerated atherosclerosis,11,12 which is evidenced already early in the course of the disease. Recently, increased numbers of platelet-derived MP (PMP) were reported in type 2 diabetic patients with poor metabolic control and microvascular complications.13 To date, there are no data regarding the presence and function of MP in well-controlled patients with uncomplicated, recently diagnosed type 2 diabetes. Therefore, in this group of patients, we characterized MP with regard to numbers, cellular origin, TF exposure, and procoagulant properties. In addition, the relation between MP subpopulations and metabolic abnormalities, and in vivo coagulation and inflammation status was studied.

Methods

Subjects Sixteen patients with uncomplicated type 2 diabetes and 18 age-matched healthy controls were studied. Patients (12 male, aged 35 to
65 years) were selected from general practices. Selection criteria included type 2 diabetes of short duration (<5 years, diagnosed by WHO criteria), no signs or symptoms or history of cardiovascular disease (CVD), normal ECG, body mass index (BMI) <28 kg/m², seated office blood pressure (BP) <150/90 mm Hg, good metabolic control (glycated hemoglobin [HbA1c] <7.8%), no history of smoking, no use of drugs other than sulfonylureas and/or metformin (6 were on diet only, 10 were taking oral antidiabetic drugs), and no diabetic complications including albuminuria (albumin/creatinine ratio [ACR] in a 24-hour urine collection was 0.5±0.5 mg/mmol in patients, 0.4±0.3 mg/mmol in controls; normal value <2.5 mg/mmol), retinopathy (fundus photography), and neuropathy (vibration sense and Ewing’s tests[15]). Healthy controls without history or clinical evidence of diabetes and CVD were included through advertisements. Eligibility was evaluated during a screening visit. The protocol was approved by the local Ethics Committee and written informed consent was obtained from all participants.

Blood Sample Collection
After an overnight fast, venous blood was collected into 3.2% trisodium citrate (Becton Dickinson, San Jose, Calif). Cells were removed by centrifugation (20 minutes, 1550g, 20°C). For flow cytometry, MP were isolated from fresh plasma samples. Samples for additional analysis were snap frozen in liquid nitrogen and stored at −80°C until assay.

Reagents and Assays
Reptilase was from Roche (Basel, Switzerland), thrombin substrate S2238 from Chromogenix AB (Möndal, Sweden), murine normal serum from the Central Laboratory of the Netherlands Red Cross Bloodtransfusion Service (CLB, Amsterdam, the Netherlands). Phycoerythrin (PE)-labeled antilymphocyte globulin A (JC159, IgG1) and anti-TF (4502; 1 mg/mL) from American Diagnostics, Inc (Greenwich, Conn). Prothrombin fragment F1 +2 (F1 +2), thrombin-antithrombin complex (TAT; Behring Diagnostics, Marburg, Germany), were measured on a Hitachi 911 analyzer by immunoturbidimetric assay (Roche), the hematologic parameters were assessed with the Technicon H-1 system (Bayer Diagnostics, München, Germany), blood glucose by the hexokinase method (BM/Hitachi 747 analyzer; Boehringer Mannheim Biochemica), and HbA1c by HPLC after hemolysis (reference range 4.3 to 6.3% in nondiabetic subjects; Bio Rad, Richmond, Calif). Serum creatinine, total cholesterol, HDL cholesterol, and triglyceride levels were determined with an RA-1000 analyzer (Technicon Instruments Corporation). LDL cholesterol levels were calculated with the Friedewald formula. Insulin was assayed by radioimmunoassay (Medenix, Brussels, Belgium) and microalbuminuria by immunonephelometry (Array Protein System, Beckman, Fullerton, Calif).

MP Isolation
MP were isolated as previously described.[2-10] After removal of cells, plasma (250 µL) was centrifuged (30 minutes, 17 500 g, 20°C) and 225 µL was removed. The remaining plasma was diluted with phosphate-buffered saline (225 µL PBS; 154-mmol/L NaCl, 1.4-mmol/L phosphate, pH 7.4), containing 10.9-mmol/L trisodium citrate. MP were resuspended and centrifuged (30 minutes, 17 500g, 20°C). The supernatant (225 µL) was removed and MP were resuspended. For thrombin generation experiments, 10 µL of this suspension was used per incubation. For flow cytometry, MP were diluted 4-fold with PBS/citrate buffer, of which 5 µL per incubation was used.

Flow Cytometry
Samples were analyzed in a FACScan flow cytometer with CellQuest software (Becton Dickinson).10 MP were triple-stained with annexin V, a cell-specific MoAb and anti-TF, analyzed for 1 minute, and identified by their characteristic forward and side scatter, and by their ability to bind annexin V and cell-specific MoAbs.2,9,10 By electron microscopy, the size of these in vivo MP ranged between 100 and 500 nm (unpublished data, Dr Nieuwland, Leiden University, Leiden, the Netherlands, 2001).

From the number of events (N) in the upper right (marker and annexin V-positive) quadrant of the flow cytometric analysis (FL-1 versus FL-2), corrected for isotype control and autofluorescence, the number of MP/L plasma was calculated: Number/L = N × [100/(5 × [55×60] [(10/250)], in which 5 (µL) is the volume of MP suspension, 100 is the total volume of washed MP suspension, 355 is the total volume in the tube before analysis, 60 is the sample volume analyzed, 10 is the number of µL/L, and 250 is the original volume of plasma.

To investigate the cellular origin of TF on MP from platelets and T-lymphocytes, respectively, MP from 6 patients and 5 controls were quadruple-stained with annexin V, CD61, anti-TF, and CD15 (ligand for P-selectin), CD66e (granulocytic marker), or CD62P (P-selectin), or with CD4 (T-lymphocytes), anti-TF, and CD11b (leukocyte marker).

Thrombin Generation Test
In vitro thrombin generation was determined as described previously.[10,11] MP were isolated from plasma, washed and added to defibrinated, MP-free normal plasma. On recalcification, thrombin was generated.[10,11] For quantitative analysis, results were determined as area under the curve. Inhibitory antibodies against TF, factor XI, and factor XII were used to determine the contribution of the various coagulation pathways to MP-induced thrombin generation.

Statistical Analysis
MP numbers and nonnormally distributed data are expressed as median (interquartile range or range), other data as mean±SD. T tests (or, when appropriate, nonparametric tests) were used for comparisons. The Bonferroni method was used to correct for multiple comparisons. Correlations were calculated in patients and controls together with the use of Spearman’s rank correlation test. P<0.05 was regarded as statistically significant. Statistical analysis was performed with SPSS (SPSS, Chicago, Ill).

Results
Subject Characteristics
Patients had significantly higher BMI, leukocyte counts, fasting plasma glucose and insulin levels, HbA1c, and lower HDL cholesterol concentrations than controls (Table 1). In patients, plasma TNFα levels were significantly elevated (P=0.016), whereas the increases in CRP, interleukin-6, elastase, F1 +2, and TAT levels were not significant (Table 2).

Numbers, Cellular Origin, and TF-Exposure of Circulating MP
Figure 1 shows the total number of MP in patients and controls (2345 versus 2408×10³/L; P=0.783). Both in patients and controls, PMP constituted the largest proportion of
TABLE 1. Characteristics of Patients and Controls

<table>
<thead>
<tr>
<th>Subjects, n</th>
<th>Patients</th>
<th>Controls</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female, n</td>
<td>12/4</td>
<td>14/4</td>
<td>NS</td>
</tr>
<tr>
<td>Age, y</td>
<td>54±8</td>
<td>55±6</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of disease, mo</td>
<td>26±22</td>
<td>...</td>
<td>NA</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.8±1.7</td>
<td>24.3±1.4</td>
<td>0.043</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>136/84±8/4</td>
<td>137/83±11/6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.3±1.9</td>
<td>5.2±1.8</td>
<td>0.043</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.1±0.4</td>
<td>1.5±0.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.7±0.2</td>
<td>1.5±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.8±0.8</td>
<td>3.7±0.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are mean±SD. NS indicates nonsignificant; NA, not applicable.
*Only significant P values are listed.

In all subjects, 35.3% (12.2 to 51.5) of TF-positive MP from platelets stained for P-selectin (data not shown). TF-positive MP subpopulations from both patients and controls co-localized CD61 and CD66e (38.5%, 10.8 to 76.8, and 27.4%, 19.4 to 53.8, respectively; P=0.537). Figure 4 shows the results of an experiment performed with MP from one patient. TF-positive MP were identified (4B), and their binding of annexin V (4C), anti-CD61 (4D), and anti-CD66e (4E) was assessed. Figure 4F shows the TF-positive MP that double-stain for CD61 and CD66e (upper right quadrant). The percentage of TF-positive MP co-localizing CD4 and CD11b was 29.2% (0 to 37.5) in patients and 33.3% (9.1 to 75.0) in controls (P=0.662).

**Thrombin Generating Capacity of MP**

Both patient and control MP initiated thrombin generation after 5 minutes (data not shown). However, the mean thrombin generating capacity during 15 minutes induced by patient MP was significantly lower than that of control MP (168.6±45.7 versus 230.8±65.3 arbitrary units, P=0.007). The in vitro thrombin generating capacity was positively associated with the total number of MP (r=0.645, P<0.001) and the number of PMP (r=0.673, P<0.001), but negatively with the total proportion of TF-exposing MP (r=−0.541, P=0.001).

Preincubation of both patient and control MP with anti-TF or anti-factor XII did not significantly affect thrombin generation (data not shown). Anti-factor XI MoAb reduced thrombin generation to a greater extent in controls than in patients (26.7±10.5% versus 19.0±5.2%, P=0.013). No staining of tissue factor pathway inhibitor (TFPI) or thrombomodulin was observed on TF-positive MP (data not shown).

**Association of MP with Components of the Metabolic Syndrome**

We correlated total as well as cell-specific TF-positive MP with features of the metabolic syndrome and inflammation and coagulation markers. Both the total number of TF-positive MP and those of platelet origin correlated with fasting plasma glucose (r=0.438, P=0.01; r=0.350, P=0.043) and insulin levels (r=0.435, P=0.01; r=0.451, P=0.007). TF-positive MP from T-helper cells correlated with BMI (r=0.338, P=0.049), HDL cholesterol (r=−0.429, P=0.011), and TNFα (r=0.412, P=0.017).

TABLE 2. Markers of Inflammation and Coagulation in Type 2 Diabetic Patients and Controls

<table>
<thead>
<tr>
<th>Marker</th>
<th>Patients</th>
<th>Controls</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase, μg/L</td>
<td>21.9±9.2</td>
<td>21.4±7.2</td>
<td>NS</td>
</tr>
<tr>
<td>C-reactive protein, mg/L †</td>
<td>0.9 (0.3–13.3)</td>
<td>0.7 (0.1–4.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Interleukin-6, ng/L †</td>
<td>1.1 (0.5–7.4)</td>
<td>0.8 (0.3–2.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Tumor necrosis factor a, ng/L</td>
<td>3.0±0.8</td>
<td>2.4±0.6</td>
<td>0.016*</td>
</tr>
<tr>
<td>Prothrombin fragment F1+2, nmol/L</td>
<td>1.0±0.4</td>
<td>0.8±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Thrombin-antithrombin complex, μg/L †</td>
<td>2.8 (1.4–54.6)</td>
<td>2.0 (0.9–10.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Only significant P values are listed. Data are mean±SD or median (range).†
TF-positive MP from T-suppressor cells correlated with BMI ($r=0.406, P=0.017$), TNF-α ($r=0.390, P=0.025$), and TAT ($r=0.387, P=0.026$). TF-positive MP from granulocytes were associated with BMI ($r=0.414, P=0.015$) and HDL cholesterol ($r=-0.387, P=0.024$). No significant correlations were found between MP subpopulations and blood pressure, triglycerides, CRP, interleukin-6, or F1.2. MP from granulocytes were associated with elastase levels ($r=0.384, P=0.028$).

**Discussion**

Similar numbers of cell-derived MP were found both in asymptomatic patients with well-controlled, uncomplicated type 2 diabetes and in controls. Higher proportions of MP derived from T-helper cells, granulocytes, and platelets exposed TF in patients. Despite the presence of more TF, MP from patients generated less thrombin than MP from controls. Significant associations were found between distinct TF-positive MP subpopulations and components of the metabolic syndrome.

PMP almost always constitute the majority of in vivo cell-derived MP, and elevated numbers occur in diseases associated with thromboembolic complications, including DIC, heparin-induced thrombocytopenia, atherosclerotic vascular disease, and type 2 diabetes.9,13,18–21 Here, we found similar numbers of PMP in patients with uncomplicated type 2 diabetes and controls. The differential findings in diabetic patient populations may be explained by the presence of vascular complications, in particular diabetic nephropathy,13 poor metabolic control, and the use of various drugs, including insulin.21 In those studies, the authors suggested that PMP may play a role in vascular injury, as they reflect platelet activation and may stimulate endothelial cells to produce soluble vascular molecules.21

Circulating MP of nonplatelet origin were found in patients suffering from vascular diseases of various etiology but also in healthy controls.2,3,10,22 In patients with meningococcal sepsis elevated levels of MP from platelets, granulocytes, and monocytes,9 and in subjects with acute coronary syndromes and in patients with lupus anticoagulant, MP from endothelial cells were demonstrated.3,22 Furthermore, in HIV-infected patients, increased levels of MP from T-helper cells were identified.23 Recently, monocyte-derived MP were reported in patients with type 2 diabetes, with the highest numbers occurring in patients with nephropathy.24 In our patients with uncomplicated diabetes, the numbers of circulating non-PMP were similar to controls. The major difference between patients and controls was that higher numbers of patient MP exposed TF.

TF is the major in vivo initiator of coagulation.5,25 Increased expression of TF promotes thrombotic events in patients with a variety of clinical disorders.25 More recently, a role for TF in signal transduction, tumor metastasis, growth, wound-healing, and angiogenesis has been reported.25–27

Under physiological conditions, the transmembrane form of TF is exclusively expressed by extravascular cells. How-
ever, expression of TF, not only by monocytes or endothelial cells, but possibly also by granulocytes and platelets, has been proposed.25,26,28,29 In the present study, TF was also exposed on MP from platelets, lymphocytes, and granulocytes. Rauch et al29 reported transfer of TF by leukocyte-derived MP to platelets in vitro, via interaction between granulocytic CD15 and platelet P-selectin. In our study, subpopulations of TF-positive PMP from patients and controls stained for CD15 and P-selectin. A high percentage of TF-positive PMP co-localized granulocytic CD66e. Similarly, leukocytic CD11b was detected in a subset of TF-positive MP from T-lymphocytes. Thus, the observed transfer of TF from leukocytic MP to platelets in vitro may also apply to platelets and other cells in vivo, and this phenomenon may occur in both healthy and diabetic subjects.

Despite the more abundant presence of TF antigen on patient MP, no increased TF-mediated procoagulant activity was observed. In fact, MP from patients generated less thrombin than controls. We evaluated the presence of TFPI and thrombomodulin on MP, but no staining of these antigens was detected (data not shown). Alternatively, an “encryption/de-encryption” phenomenon has been described for cellular TF. Cell surface TF activity is normally “encrypted,” in which state it binds anti-TF antibody and factor VII/VIIa, but lacks procoagulant activity.25 To become activated, TF has to be “de-encrypted” by stimuli causing perturbation of the cell membrane, such as low concentrations of detergent. To investigate whether this phenomenon also applies for MP-associated TF, we incubated MP with triton (0.05% [v/v]) and observed that TF exposure on MP was still detectable, whereas most cell-specific antigens could no longer be visualized. In contrast to findings in intact cells, treatment of MP with detergent resulted in a complete loss of MP-induced procoagulant activity. TF activity is critically dependent on the microenvironment within the membrane and a phosphatidylermine (PS)-enriched envelope is essential.30 In preliminary experiments, addition of artificial phospholipid vesicles potentiated thrombin generation induced by both patient and control MP, which was in part TF dependent (data not shown). This observation supports the hypothesis that MP-associated TF activity may become “de-encrypted” in the presence of an appropriate microenvironment. In view of the absence of association between TF-positive MP and circulating coagulation markers, however, it is uncertain whether these conditions occur in vivo. Therefore, although the role for MP-associated TF in in vivo coagulation cannot be excluded, its function remains speculative and may involve other processes including angiogenesis, cell adhesion, and/or signal transduction.

TF exposure by MP suggests activation of the respective cells of origin. The observed association between granulocyte MP and elastase suggests granulocyte activation. Although the elevations of CRP and interleukin-6 were not significant, the elevated leukocyte count, TNFα levels, and the association between TF-positive MP from T-lymphocytes and TNFα are compatible with the presence of low-grade inflammation in patients with diabetes.

Cellular TF expression may be induced by the various metabolic abnormalities observed in patients with type 2 diabetes. Hyperglycemia and hyperinsulinemia activated the TF coagulation pathway in healthy subjects.31 Also, adipose tissues in obese mice produced TF.32 Furthermore, remnant lipoproteins and oxidized LDL induced expression of TF in human endothelial and smooth muscle cells, respectively.33,34 In our study, TF-positive MP subpopulations rather than the total, predominantly TF-negative, number of MP, were associated with components of the metabolic syndrome, including BMI, fasting plasma glucose, and insulin levels and low HDL cholesterol.

In conclusion, although comparable numbers of circulating MP of various cellular origin were observed in asymptomatic patients with uncomplicated well-controlled type 2 diabetes and controls, a larger proportion of MP from patients exposed TF, which was not associated with coagulation, suggesting that TF on MP may rather be involved in other processes, such as transcellular signaling or angiogenesis. Additional studies should clarify the biological role of circulating MP and TF in diabetes.

Acknowledgment
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References
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