

Cell-derived Microparticles Circulate in Healthy Humans and Support Low Grade Thrombin Generation

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Key words

Microparticles, coagulation, thrombin generation, flow cytometry, tissue factor

Summary

We determined the numbers, cellular origin and thrombin-generating properties of microparticles in healthy individuals (n = 15). Microparticles, isolated from fresh blood samples and identified by flow cytometry, originated from platelets [237 × 10^6/L (median; range 116-565)], erythrocytes (28 × 10^6/L; 13-46), granulocytes (46 × 10^6/L; 16-94) and endothelial cells (64 × 10^6/L; 16-136). They bound annexin V, indicating surface exposure of phosphatidylserine, and supported coagulation *in vitro*. Interestingly, coagulation occurred via tissue factor (TF)-independent pathways, because antibodies against TF or factor (F)VII were ineffective. In contrast, in our *in vitro* experiments coagulation was partially inhibited by antibodies against FXII (12%, p = 0.006), FXI (36%, p <0.001), FIX (28%, p <0.001) or FVIII (32%, p <0.001). Both the number of annexin V-positive microparticles present in plasma and the thrombin-generating capacity inversely correlated to the plasma concentrations of thrombin-antithrombin complex (r = -0.49, p = 0.072 and r = -0.77, p = 0.001, respectively), but did not correlate to prothrombin fragment F_{1+2} (r = -0.002, p = 0.99). The inverse correlations between the number of microparticles and their thrombin-forming capacity and the levels of thrombin-antithrombin complex in plasma may indicate that microparticles present in the circulation of healthy individuals have an anticoagulant function by promoting the generation of low amounts of thrombin that activate protein C. We conclude that microparticles in blood from healthy individuals support thrombin generation via TF- and FVII-independent pathways, and which may have an anticoagulant function.

Introduction

Traditionally, activation of the coagulation system can occur via an intrinsic or via an extrinsic pathway, i. e., the contact-activation pathway initiated via factor XII and the tissue factor/factor VII-dependent pathway, respectively. The importance of the contact activation pathway for hemostasis *in vivo* is debated, amongst others because persons with a genetic deficiency of factor XII have no bleeding tendency, in contrast to those with a deficiency of factor XI or particularly factor VIII or IX, but tend to have an increased risk for thromboembolic disorders (1, 2). To explain these discrepancies, the coagulation cascade has been revisited: factor IX is now considered to be activated predominantly via factor VIIa (3), and factor XI by thrombin (4-6). In this view most clotting reactions in (patho)physiological conditions are triggered by extrinsic pathway activation and are amplified by factors VIII, IX and XI. Several lines of evidence support this view. First, the severity of bleeding tendency in persons with genetic deficiencies of clotting factors, as discussed above. Second, infusion of factor VIIa in chimpanzees induced an increase of the plasma concentrations of activation peptides of factor IX, X and II, and these were all reduced by infusion with an anti-tissue factor antibody (7). Third, thrombin generation in experimental endotoxemia is reduced by infusion of anti-factor VIIa (8). Moreover, blocking factor XII in a baboon model for sepsis-induced diffuse intravascular coagulation did not affect factor V or fibrinogen consumption (9), whereas anti-tissue factor antibodies did (10).

Previously, Wagenvoord et al. demonstrated that blood as well as platelet-rich plasma contain procoagulant phospholipids (11). In 1997, we reported the presence of thrombin-generating microparticles in blood from the pericardial cavity of patients undergoing cardiac surgery (12). These microparticles were predominantly of platelet and erythrocyte and to a lesser extent of monocyte origin. They exposed negatively charged phospholipids – as demonstrated by annexin V binding – and supported coagulation via the tissue factor/factor VII-dependent pathway. Additionally, we observed increased numbers of microparticles from platelet, granulocyte, monocyte and endothelial cell origin in the circulation of patients with meningococcal sepsis (13). These microparticles also supported thrombin generation via the tissue factor pathway and could be involved in the diffuse intravascular coagulation occurring in such patients.

The coagulation system is not only activated in disease states. Low-grade activation of the coagulation system has been shown to occur in healthy individuals. Bauer and coworkers showed that plasma levels of activation peptides of factors IX, X and prothrombin (F_{1+2}) are always detectable in healthy individuals (14). These levels are all decreased in patients with a hereditary factor VII deficiency (14, 15), but not in patients with a factor XI deficiency, indicating that this low grade basal activation of the clotting system is dependent on tissue factor/factor VII, but not on factor XI (3). Although these studies thus illustrated the important role of the tissue factor/factor VII-complex in the basal activation of coagulation in healthy individuals *in vivo*, they did not elu-

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cidate how this pathway was activated. One possibility is the exposure of blood to minute amounts of tissue factor *in vivo*. Indeed normal individuals have low levels of soluble tissue factor in the circulation (16). Whether this originates from monocytes, endothelial cells or granulocytes, which are all capable of expressing tissue factor on their surface (17-19), is not known. *In vitro*, tissue factor can also be released from cell surfaces by shedding of tissue factor-exposing microparticles, as demonstrated for both monocytes (20) and endothelial cells (21). In view of our previous findings on microparticles in cardiac surgery and in meningococcal sepsis patients (12, 13), an alternative hypothesis to explain a basal activation of the coagulation system could be that low grade thrombin generation is induced by microparticles. In the present study we tested this hypothesis and assessed the number, cellular origin and thrombin-generating capacity of microparticles in the circulation of healthy individuals and their relationship to basal activation of the coagulation system.

Materials and Methods

Reagents and Assays

Reptilase was obtained from Roche (Mannheim, Germany) and the chromogenic substrate S2238 from Chromogenix AB (Mölndal, Sweden). CD66e-PE (clone CLB-gran/10, IH4Fc, IgG_1) and murine normal serum were obtained from the Central Laboratory of the Netherlands Red Cross Bloodtransfusion Service (Amsterdam, The Netherlands), anti-Glycophorin A-FITC (clone JC159, IgG_1) and CD61-FITC (clone Y2/51, IgG_1) from Dako A/S (Glostrup, Denmark), CD14-FITC (clone CRIS-6, IgG_1) and CD45-FITC (clone HI30, IgG_1) from Biosource (Camarillo, CA, USA), IgG_1-FITC and PE (clone X40) from Becton Dickinson (San Jose, CA, USA), and CD62E-FITC (clone 1.2B6, IgG_1) from Serotec Ltd. (Oxford, England). Phosphatidylserine (PS) P-7769, phosphatidylcholine (PC) P-7212 and thrombin (EC 3.4.21.5; T-7009, 1570 NIH-units/mg) were obtained from Sigma (St. Louis, Missouri, USA). Artificial phospholipid vesicles containing PS (20%) and PC (80%) were prepared according to Brunner (22). The concentration of phospholipids of the preparations was determined by phosphate analysis. Kaolin was provided by B.L.B. Laboratoires du Bois de Boulogne (Puteaux, France). Thromborel S, a commercial preparation of thromboplastin containing tissue factor and phospholipids from human placenta, was used to activate coagulation via the extrinsic pathway [Behring Diagnostics GmbH (Marburg, Germany)], hirudin [recDNA y(Tyr^{63})] (0.1 mg/mL) from Ciba-Geigy (Basel, Switzerland), annexin V-PE from PharMingen (San Jose, CA, USA). OT-2 (0.71 mg/mL), a monoclonal antibody (MoAb) which inhibits the activity of factor XII(a) was prepared as described earlier (23). MoAbs directed against factor VIIa (VII-1[1.46 mg/mL], VII-15 [0.53 mg/mL]) and XI (XI-1 [0.92 mg/mL]) were from the Netherlands Red Cross Bloodtransfusion Service (Amsterdam, The Netherlands). MoAb VII-1 and VII-15 were mixed at 1:1 ratio. Each individually inhibited thromboplastin-induced thrombin generation. Anti-factor VIII (2.4 mg/mL) was a gift from the Department of Hematology (Leiden University Medical Center, Leiden) and anti-factor IX (12 mg/mL) from Dako A/S (Glostrup, Denmark). Anti-tissue factor-FITC (4508CJ) and polyclonal rabbit anti-human tissue factor (1 mg/mL; 4502) were from American Diagnostics inc. (Greenwich, CT, USA). Plasma concentrations of prothrombin fragment F_{1+2} (F_{1+2}) and thrombin-antithrombin III complexes (TAT) were determined by ELISA (Enzygnost) as described by the manufacturer (Behring Diagnostics GmbH, Marburg, Germany). Factor II-deficient plasma was obtained from Biopool AB (Umeå, Sweden). All other chemicals were of the highest grade commercially available.

Collection of Blood Samples

Venous blood from healthy male individuals (with informed consent) was collected into 3.2% trisodium citrate (Becton Dickinson, San Jose, CA, USA). The individuals (n = 15) had not taken any medication during the previous ten days. Blood cells were removed by centrifugation for 20 min at 1,550 × g at room temperature. For flow cytometry and thrombin generation experiments, microparticles were isolated (see below) from fresh plasma samples. Aliquots of plasma (first snap frozen in liquid nitrogen) were stored at –70° C and used for determinations of the F_{1+2} and TAT concentrations, and, where indicated, for thrombin generation experiments.

Isolation of Microparticles

After removal of cells, 250 μL plasma were centrifuged for 30 min at 17,570 × g at 20° C. The plasma from which microparticles were isolated was not defibrinated to avoid a potential loss of platelet-derived microparticles (24). After centrifugation, 225 μL of (microparticle-free) plasma were removed. PBS (225 μL; 154 mmol/L NaCl, 1.4 mmol/L phosphate, 10.9 mmol/L trisodium citrate; pH 7.4) was added to the microparticle pellet and the remaining plasma (25 μL). The microparticles were subsequently resuspended and centrifuged for 30 min at 17,570 × g at 20° C. Again, 225 μL of the microparticle-free supernatant were removed and microparticles were resuspended in the remaining 25 μL. For the thrombin generation experiments 20 μL of this suspension was used. For flow cytometry, the 25 μL microparticle suspension was diluted 4-fold with PBS/citrate buffer, of which 5 μL were used per incubation.

Flow Cytometric Analysis

The samples were analyzed in a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA). Forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain and microparticles were identified as described previously (12). To distinguish microparticles from events due to noise, microparticles were identified not only on FSC and SSC, but also by binding of annexin V and a MoAb directed against a cell-specific antigen. To identify annexin V-positive microparticles, a threshold was placed in a microparticle sample prepared without any additions to correct for autofluorescence. To identify microparticles that bound cell-specific MoAbs, microparticles were incubated with identical concentrations of isotype-matched control antibodies to set the threshold. FITC-fluorescence was measured in the FL-1 channel and PE-fluorescence in the FL-2 channel. Microparticles (5 μL) were diluted in 35 μL PBS/calcium buffer (154 mmol/L NaCl, 1.4 mmol/L phosphate and 2,5 mmol/L $CaCl_2$, pH 7.4), containing an additional 5 μL of 500-fold prediluted normal mouse serum. After incubation for 15 min at room temperature, 5 μL annexin V-PE and 5 μL FITC-labeled cell-specific MoAbs or isotype-matched control antibody (IgG_1-FITC) were added. For the CD66e-PE analysis an IgG_1-PE control antibody was used. The mixtures were incubated in the dark for 15 min at room temperature. Subsequently, 200 μL PBS/calcium buffer were added and the suspensions centrifuged for 30 min at 17,570 × g and 20° C. Finally, 200 μL of (microparticle-free) suspension were removed. The microparticles were resuspended with 300 μL PBS/calcium buffer before flow cytometry. All samples were analyzed for 1 min during which the flow cytometer analyzed about 150 μL of the microparticle suspension. To estimate the number of microparticles/L plasma, the number of microparticles (N) found in the upper right quadrant of the flow cytometry analysis (FL1 versus FL2, corrected for isotype control and autofluorescence) was used in the formula: $\text{Number/L} = N \times [100/5] \times [355/150] \times [10^6/250]$.

Thrombin Generation Assay

The thrombin generation test (TGT) as described by Béguin et al. (25) was used to assess the thrombin-generating capacity of the microparticles. In brief, isolated microparticles were reconstituted in defibrinated normal (microparticle-free) plasma, or, where indicated, in factor II-deficient plasma. Defibrinated plasma was prepared by incubating normal plasma (a pooled plasma from 20 healthy individuals) with reptilase for 10 min at 37° C and, subsequently, for 10 min on melting ice. The fibrin clot was removed by centrifugation for 30 min at 17,570 × g at 20° C. In addition, this normal plasma was centrifuged for 30 min at 17,570 × g at 20° C to remove microparticles. To this plasma (120 μL), the microparticles (20 μL) and 10 μL buffer A (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.05% bovine serum albumin, pH 7.35) were added.

At t = 0, thrombin generation was triggered by the addition of 30 μL $CaCl_2$ (16.7 mmol/L final concentration) to a prewarmed (37° C) mixture of plasma, microparticles and buffer. At fixed intervals after t = 0, 3 μL aliquots were removed from this mixture and added to 147 μL prewarmed (37° C) chromogenic substrate S2238 in buffer B (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 20 mmol/L EDTA and 0.05% bovine serum albumin, pH 7.90). After 3 min, the conversion of S2238 was stopped by the addition of 90 μL citric acid (1 mol/L) and the generated amount of p-nitroaniline was determined at λ = 405 nm. To convert the observed optical density (OD) into thrombin concentration, a reference curve was prepared using purified human thrombin (0-600 nmol/L). For quantitative analysis, the results were expressed as the area under the thrombin generation curve (AUC), calculated for the time interval between 0 and 15 min after addition of calcium chloride. In some experiments, microparticles in the reaction mixture were replaced by 10 μL artificial phospholipid vesicles (PS:PC ratio 20:80) plus 10 μL kaolin, yielding final concentrations of 2.06 μmol phospholipid/L and 1.39 kaolin μg/mL, respectively. Alternatively, microparticles were replaced by 20 μL thromboplastin (20-fold prediluted in buffer A). In the inhibition experiments, the mixture of plasma and buffer A, and separately the microparticles, were incubated with 20 μL and 10 μL of antibodies, respectively. In preliminary experiments the concentration yielding maximal inhibition of thrombin generation was determined for each antibody. These were anti-TF (1 mg/mL initial concentration), anti-FVII (0.2 mg/mL), anti-FVIII (0.24 mg/mL), anti-FIX (1.2 mg/mL), anti-FXI (0.92 mg/mL) and anti-FXII (0.71 mg/mL). After 30 min pre-incubation at room temperature, the microparticles were added and incubated for 10 min at 37° C, whereupon the thrombin generation was started by addition of $CaCl_2$. Thrombin generation experiments were performed on fresh and frozen microparticle samples. The specificity of this assay for thrombin was demonstrated in experiments where the thrombin inhibitor hirudin (in normal plasma) or prothrombin-deficient plasma was used. In preliminary experiments several concentrations of hirudin were tested and a final concentration of 1.2 μmol/L proved optimal. After freezing and thawing of microparticle samples, the area under the curves increased about twofold, but the proportion of inhibition by the antibodies was unchanged.

Statistical Methods

Data were analyzed with SPSS for Windows, release 9.0. Differences in inhibitory capacity by the antibodies were evaluated by GLM univariate multiple variation analysis at overall significance level of p = 0.05, followed by post hoc analysis using the Scheffé test.

Results

Circulating Microparticles in Healthy Individuals

Flow cytometric analysis of the normal plasma samples revealed that all individuals tested had circulating microparticles. A representative example of these experiments and the overall results are presented in Fig. 1. Fluorescence thresholds were established by measuring samples in the absence of annexin V and in the presence of an IgG_1 isotype control antibody (Fig. 1A). The corrected data of each sample were then obtained as the number of events above these thresholds, for instance platelet-derived microparticles (Fig. 1B).

The majority of the microparticles thus identified in the 15 healthy individuals originated from platelets [237 × 10^6/L (median; range 116-565 × 10^6/L)]. Other microparticles were derived from erythrocytes (28 × 10^6/L; 13-46 × 10^6/L), granulocytes (46 × 10^6/L; 16-94 × 10^6/L) or endothelial cells (64 × 10^6/L; 16-136 × 10^6/L). Part of the microparticles stained positive for tissue factor (47 × 10^6/L; 15-108 × 10^6/L), as shown in Fig. 1C. Substantial numbers of lymphocyte- or monocyte-derived microparticles were not observed in the healthy individuals.

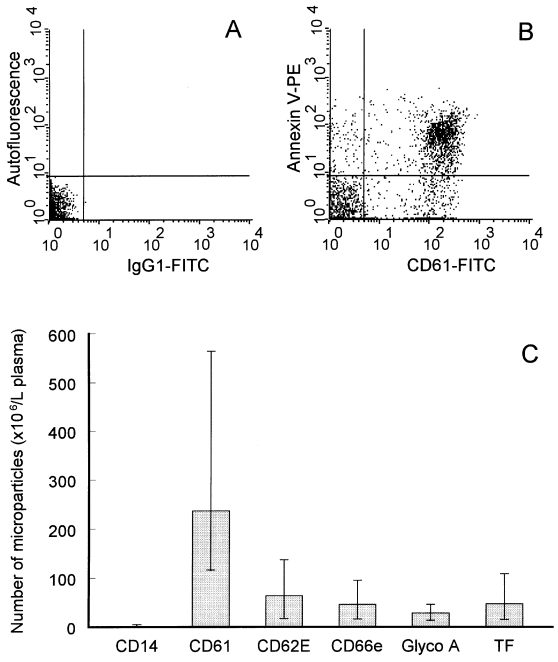

Fig. 1 Representative FACS dot plots of microparticles in plasma from a healthy individual and overall results in all individuals. Microparticles were isolated from citrate-anticoagulated plasma, and analyzed by flow cytometry as described in Methods. A) Threshold settings with control antibody (IgG_1-FITC) and in the absence of annexin V-PE; B) Platelet-derived microparticle analysis with annexin V-PE and CD61-FITC; C) The numbers of microparticles in plasma from healthy individuals (n = 15), positive for the indicated cell-specific marker: CD14 (monocytes), CD61 (GPIIIa, platelets), CD62E (E-selectin, endothelial cells), CD66e (granulocytes), and glycophorin A (glyco A; erythrocytes). The number of tissue factor (TF) expressing microparticles is also indicated. Data are presented as median and range

Thrombin Generation by Microparticles In Vitro

Thrombin generation was absent when plasma from healthy individuals was first subjected to high-speed centrifugation to remove the microparticles. Reconstitution of microparticles in autologous plasma resulted in thrombin generation. These results were obtained with each of the 15 individuals. When plasma samples from the healthy in-

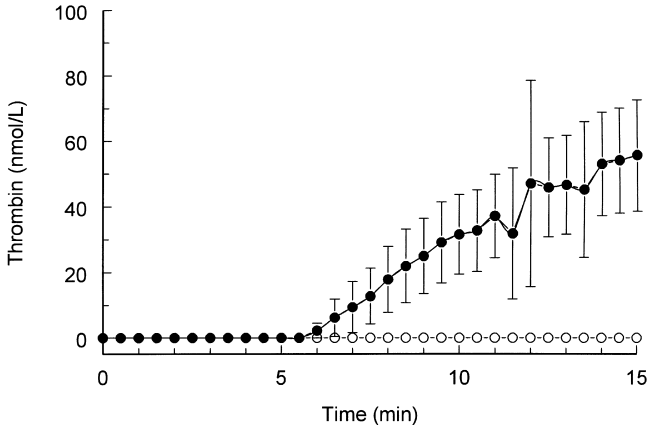

Fig. 2 Thrombin generation by microparticles from healthy individuals. Defibrinated, microparticle-free normal plasma in the presence (●) or absence (○) of microparticles from healthy individuals. Data are presented as mean ± SD, n = 15

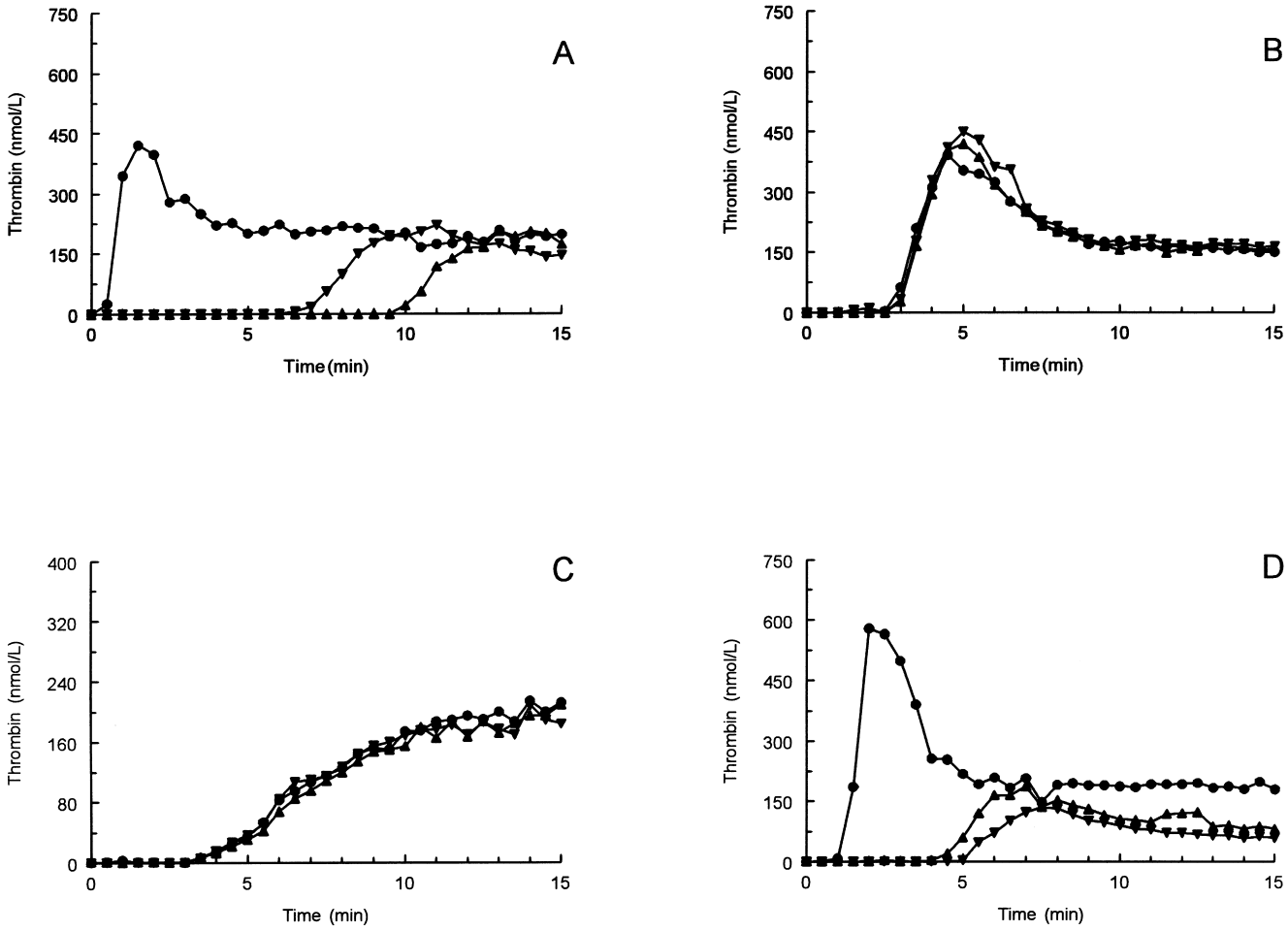

Fig. 3 Effect of inhibitors of the extrinsic coagulation pathway on thrombin generation by microparticles in normal defibrinated plasma. Thrombin generation was initiated by (A) thromboplastin (20-fold prediluted) or (B) kaolin (final concentration in the reaction mixture 1.39 μg/mL) in defibrinated, microparticle-free normal plasma in the absence (●) or presence of anti-tissue factor (▲) or anti-factor VII (▼). Figs. C and D show representative thrombin generation curves, with microparticles from (C) blood of a healthy individual or (D) material from the pericardial cavity of a patient undergoing cardiopulmonary bypass, reconstituted in defibrinated microparticle-free normal plasma

dividuals were not subjected to high-speed centrifugation but directly recalcified, a comparable generation of thrombin was observed (data not shown). Fibrin is known to bind thrombin. Fibrin formation will thus lead to an erroneous detection of the amount of generated thrombin. Also the clotting process would interfere with the subsampling of the 3 μL aliquots to measure the available thrombin. We therefore chose to use a system as shown in Fig. 2 and described in the Materials and Methods section. Conversion of the chromogenic substrate in the present study was indeed due to thrombin, because hirudin (1.2 μmol/L, final concentration) completely inhibited this conversion, and reconstituted microparticles displayed no activity in factor II-deficient plasma (data not shown).

Involvement of Intrinsic and Extrinsic Pathways in Microparticle-dependent Thrombin Generation

We first assessed optimal concentrations for the inhibiting antibodies used to inhibit the various clotting factors. Results of antibodies directed against extrinsic pathway factors are presented in Fig. 3. As expected, a tissue thromboplastin preparation rapidly induced a substantial amount of thrombin formation in the microparticle-free, defibrinated plasma, which was strongly delayed by the antibodies against tissue factor or factor VII (Fig. 3A). When both anti-factor VII and anti-tissue factor were present, the extent of inhibition was identical to that observed in the presence of anti-tissue factor alone (data not shown). These antibodies did not affect the thrombin generation by kaolin, a known activator of the contact activation system (Fig. 3B). Hence, at the concentrations used the antibodies specifically inhibited thrombin generation via the extrinsic pathway. Similar concentrations of anti-factor VII, anti-tissue factor or their combination (latter not shown) did not affect the thrombin generation by the microparticles from the healthy individuals (Fig. 3C). As we did not anticipate these results, we did an additional experiment to show the specificity of the antibodies. Thrombin generation by microparticle fractions from the pericardial cavity of patients undergoing cardiopulmonary bypass (CPB), which we previously reported to promote this generation via tissue factor/factor VII (12), was indeed inhibited by these antibodies (Fig. 3D).

Results of the studies on the intrinsic pathway are presented in Fig. 4. Kaolin induced a substantial generation of thrombin, which was considerably reduced and delayed by antibodies against factors VIII, IX, XI or XII (Fig. 4A). These antibodies were indeed specific inhibitors of the activation of the contact system or the intrinsic pathway, because they did not affect the thrombin generation induced by the thromboplastin (Fig. 4B). The thrombin generation by the microparticles from the healthy individuals was slightly inhibited by the antibodies against factor XII and to a somewhat larger extent by those

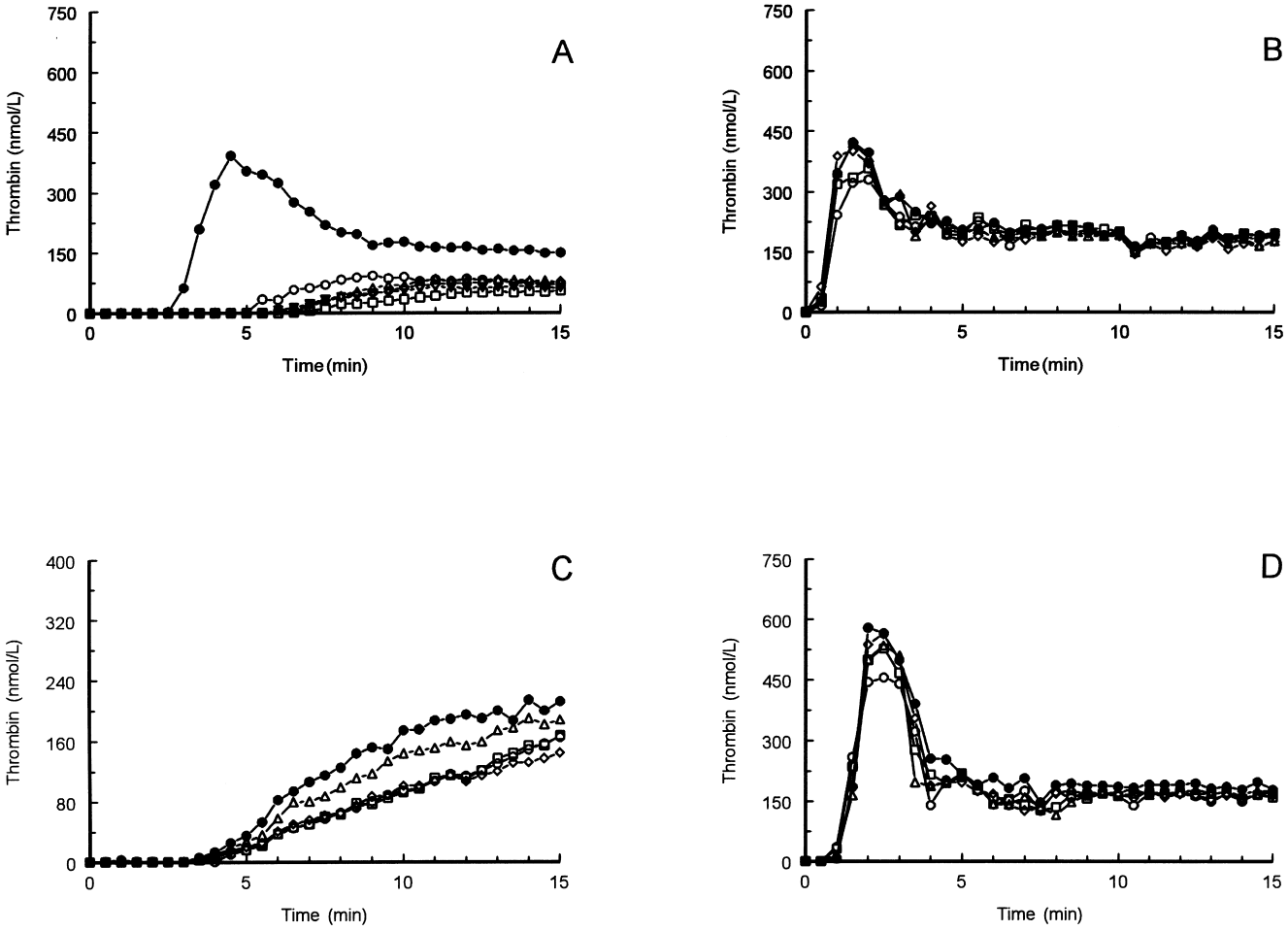

Fig. 4 Effect of inhibitors of the intrinsic coagulation pathway on thrombin generation by microparticles in normal defibrinated plasma. Thrombin generation was initiated by (A) kaolin (final concentration in the reaction mixture 1.39 μg/mL) or (B) thromboplastin (20-fold prediluted) in defibrinated, microparticle-free normal plasma in the absence (●) or presence of anti-factor XII (△), anti-factor XI (◇), anti-factor IX (□) or anti-factor VIII (○). Figs. C and D show representative thrombin generation curves, with microparticles from (C) blood of a healthy individual or (D) material from the pericardial cavity of a patient undergoing cardiopulmonary bypass, reconstituted in defibrinated microparticle-free normal plasma

against factors VIII, IX and XI (Fig. 4C). Conversely, these antibodies did not affect the thrombin generation by the microparticles from the CPB patients (Fig. 4D), in agreement with the observation that this latter generation mainly occurs via the extrinsic pathway (see above).

Quantitative data of the inhibition studies are given in Table 1. Antibodies against factor VII or tissue factor did not inhibit the generation of thrombin by the microparticles obtained from normal individuals, whereas antibodies against factors XII, XI, IX or VIII inhibited by 12, 36, 28 and 32%, respectively. Additional inhibitory effects with anti-VII plus anti-tissue factor, anti-VII plus anti-XI, anti-VII plus anti-XII or anti-tissue factor plus anti-XI, were not observed.

Relation of Microparticles to Coagulation Activation In Vivo

To analyze the correlation between the number of microparticles and their thrombin generating capacity *in vitro* and the activation status of the coagulation system *in vivo*, plasma concentrations of TAT complexes and F_{1+2} were measured in the healthy individuals. Results are presented in Fig. 5. We did not observe a correlation between microparticle numbers and F_{1+2} (r =-0.08, p = 0.79; Fig. 5A), and a substantial but not statistically significant correlation with TAT complex concentrations (r = -0.49, p = 0.07; Fig. 5B). The thrombin generating capacity of the microparticles, however, correlated with levels of TAT comple-

Table 1 Effect of antibodies against extrinsic and intrinsic pathway factors on thrombin generation by microparticles from healthy individuals

	MoAb	n	Thrombin generation (% of control)[1]	P[2]
Extrinsic	VII	15	97 ±14	0.854
	TF	15	98 ±14	0.908
Intrinsic	XII	15	88 ±15	0.006
	XI	6	64 ± 3	< 0.001
	IX	6	72 ± 6	< 0.001
	VIII	6	68 ± 6	< 0.001
Combination	VII/ TF	6	98 ± 12	0.893
	VII/ XII	6	88 ± 6	0.047
	VII/ XI	6	71 ± 10	< 0.001
	TF/ XI	6	65 ± 10	< 0.001

1. The area under the thrombin generation curve (AUC), calculated for the time interval between 0 and 15 minutes after addition of $CaCl_2$, was determined in the absence of inhibitors and for each individual set at 100% (control). The effect of MoAbs is expressed as % remaining thrombin generation. Data are expressed as mean ± SD.
2. Differences in inhibitory capacity by the antibodies were evaluated by the Scheffé test and were considered statistically significant at P<0.05.

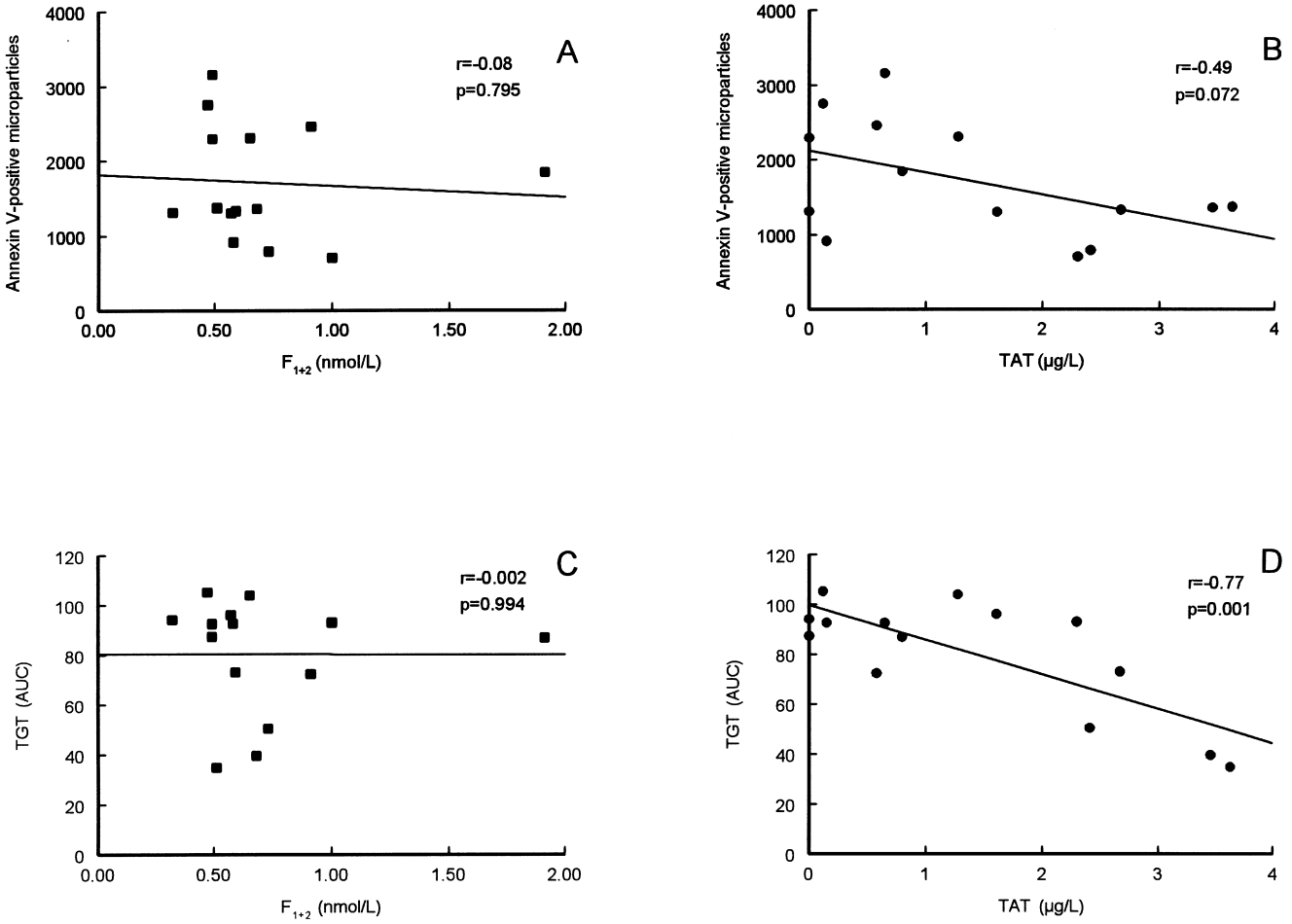

Fig. 5 Correlations between the *in vivo* activation status of coagulation activation, microparticle numbers and their *in vitro* thrombin generating activity. Plasma concentrations of prothrombin fragment F_{1+2} and TAT complexes were correlated with the number of annexin V-positive microparticles (panels A and B, respectively) and their ability to generate thrombin (expressed as area under curve; panels C and D, respectively) when reconstituted in defibrinated normal plasma

xes (r = -0.77, p = 0.001; Fig. 5D), but not with F_{1+2} (r = -0.002, p = 0.99; Fig. 5C).

Discussion

In the present study we detected low numbers of microparticles from several cell types in the circulation of healthy individuals. Most of the particles were from platelets, some from erythrocytes or granulocytes and possibly endothelial cells (see below). The microparticles supported thrombin generation *in vitro* upon reconstitution in normal plasma. They were essential for this generation, because microparticle-free plasma did not generate thrombin. The microparticles, however, did not initiate the thrombin generation via the extrinsic pathway, as antibodies against either tissue factor or factor VII did not inhibit thrombin generation in plasma triggered by these microparticles. In contrast, either antibody significantly delayed the thrombin generation induced by thromboplastin or by microparticles from pericardial blood from patients undergoing coronary bypass surgery. Thus, the antibodies are obviously specific and sufficiently potent to establish inhibition. Conversely, the antibodies against coagulation factors of the intrinsic pathway inhibited the thrombin-generating capacity of the microparticles to a various extent. This was not anticipated in view of the results of Bauer and coworkers on the role of tissue factor/factor VII in the activation of the basal coagulation and the extensive studies on the role of this pathway in disease (3, 7, 14, 15, 26). However, the antibodies used to block the intrinsic pathway were also obviously specific and sufficiently potent, since they inhibited the kaolin-induced generation of thrombin, whereas they did not affect the generation induced by thromboplastin or by isolated microparticles from pericardial cavity blood. Very recently, Santucci et al. reconstituted blood cells in FXI- or FXII-deficient plasma, which resulted in prolonged clotting times, indicating the involvement of these factors in coagulation (27). In addition, elevated levels of FXI were recently demonstrated to be an independent risk factor for deep venous thrombosis (28).

An intriguing and unexplained observation in the present study was that combinations of anti-factor XII and anti-factor VII antibodies or anti-factor XI and anti-factor VII antibodies never yielded complete inhibition of thrombin generation by the microparticles of the healthy individuals. Actually, none of these antibody combinations appeared to be superior to the single antibody effects regarding inhibition of thrombin generation by these microparticles. It is unlikely that binding of the antibodies to their respective clotting factor was impaired by the microparticles, since we did not observe such a phenomenon in the control experiments with kaolin or the commercial thromboplastin. Hence, the question remains how this thrombin formation by the microparticles of the healthy individuals is initiated and subsequently propagated. Alternative pathways to initiate coagulation do exist, such as the macrophage fgl-2 prothrombinase (29) and monocyte Mac-1 integrin (30). Clearly, the present findings challenge our understanding of the clotting mechanism, and require further studies.

From the present study it is evident that most of the microparticles found in healthy individuals are of platelet-origin, similar to the findings of Combes et al. (31). Activated platelets and platelet-derived microparticles express high affinity receptors for factor VIII (32) and factor Va (33), and have Xa activity (34), which at least on the platelet surface is preferentially activated by platelet-bound factor IXa (35). Platelets also contain factor XI, which was shown to be a splice variant of the plasma factor XI (36). Approximately 40% of factor XI is already present on the outer surface of resting platelets, and this surface exposure increases upon platelet activation (37). Factor XI has functional activity since platelets are able to overcome the clotting defect in plasma factor XI-deficient plasma. To what extent platelet-derived microparticles circulating *in vivo* similarly may expose or bind these clotting factors is not clear from our present data, because we did not perform studies to measure their surface exposure. Holme et al. (34) showed that in vitro generated microparticles from washed platelets demonstrated factor Xa activity. Since also in vivo microparticles may exhibit Xa activity, we reconstituted microparticles from healthy individuals in factor X-deficient plasma. Under these conditions, no thrombin generation was observed. Also in factor V-deficient plasma no thrombin generation was detectable (data not shown). These experiments do not exclude that some factor Xa is present on the surface of these microparticles. They show that this amount is insufficient to explain the total thrombin formation in normal plasma. However, we cannot exclude that some Xa activity – present on the microparticles – is sufficient to initiate trace amounts of thrombin which then propagate ongoing thrombin formation in plasma, but insufficient to propagate the observed thrombin generation itself. Such an involvement may be resolved by performing studies with specific anti-Xa inhibitors added to the microparticle suspension.

Combes and colleagues described the presence of endothelial cell-derived microparticles in healthy individuals and to a larger extent in patients with a lupus anticoagulant (31). In that study the possible presence of tissue factor on these vesicles was not addressed. Giesen et al. observed TF-positive membrane vesicles in native human blood under flow conditions (17). We did observe endothelial cell-derived microparticles in the healthy individuals, as well as tissue factor-bearing microparticles. However, those observations have to be interpreted with caution. On the one hand, the signals of detection were not conclusive. On the other hand, our choice to use E-selectin as a marker to detect endothelial cell-derived microparticles may not be optimal (31). Thus, data regarding the presence of tissue factor-exposing microparticles as well as endothelial cell-derived microparticles require further experimental support, such as the use of better and more specific antibodies.

Elevated numbers of especially platelet microparticles have been reported in a variety of illnesses or diseases, such as patients undergoing cardiac surgery (12), plasmapheresis (38), or coronary angiography (39), as well as in patients suffering from diabetes (40, 41), heparin-induced thrombocytopenia (42, 43), infarctions (44), uremia (45), idiopathic thrombocytopenic purpura (46), thrombotic thrombocytopenic purpura (47), and meningococcal septic shock (13), which all have in common an increased risk for thromboembolic events. Possibly, the numbers of such microparticles are the limiting factor in the propagation of the coagulation activation induced by the tissue factor/factor VII pathway. In agreement herewith, patients who suffer from the Scott syndrome, who have an increased risk for bleeding, have an impaired capacity to generate microparticles and have an impaired binding of factor VIIIa to their microparticles (18, 48).

One might infer from our observations that microparticles play a role in coagulation activation, both in the basal activation status in healthy individuals (this study) and under pathophysiological conditions, such as cardiac surgery and meningococcal sepsis. However, our finding that the thrombin generating capacity of reconstituted microparticles i*n vitro* inversely correlated with the concentration of circulating TAT in the healthy individuals seems to contradict this view. We propose that the inverse correlation of TAT with the number of microparticles reflects another fundamental principle of coagulation, i. e. that low concentrations of thrombin are anti-coagulant by virtue of their capacity to generate activated protein C. Experiments in baboons have shown that low doses of thrombin protect against thrombosis by activating protein C (49). This is likely to occur under basal conditions as infusion of an antibody that blocks protein C triggers an increase in circulating TAT complexes in baboons. Hence, we suggest that low grade thrombin generation by circulating microparticles under basal conditions is involved in the activation of protein C. *In vitro*, platelet microparticles indeed facilitate inactivation of factor Va by activated protein C (50). We are currently testing this hypothesis for these *in vivo* generated microparticles.

To summarize, we demonstrate that healthy individuals have significant numbers of cell-derived microparticles in the circulation that support low grade thrombin-generation by tissue factor-independent pathways. We suggest this thrombin to be involved in activation of protein C under basal conditions. Also, the initiation pathways of thrombin formation by microparticles from healthy individuals evidently differ from those of patients undergoing CPB or suffering from meningococcal disease.

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