Microparticles, coagulation and inflammation
Berckmans, R.J.

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Chapter 6

Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism


ABSTRACT

Aim. To determine the cellular origin of synovial microparticles, their procoagulant properties, and their relationship to local hypercoagulation.

Methods. Microparticles in synovial fluid and plasma from patients with rheumatoid arthritis (RA; n = 10) and patients with other forms of arthritis (non-RA; n = 10) and in plasma from healthy subjects (n = 20) were isolated by centrifugation. Microparticles were identified by flow cytometry. The ability of microparticles to support coagulation was determined in normal plasma. Concentrations of prothrombin fragment 1-2 (F$_{1,2}$) and thrombin-antithrombin (TAT) complexes were determined (by ELISA) as estimates of the coagulation activation status in vivo.

Results. Plasma from patients and healthy controls contained comparable numbers of microparticles, which originated from platelets and erythrocytes. Synovial microparticles from RA patients and non-RA patients originated mainly from monocytes and granulocytes; few originated from platelets and erythrocytes. Synovial microparticles bound less annexin V (which binds to negatively charged phospholipids) than did plasma microparticles, exposed tissue factor, and supported thrombin generation via factor VII. F$_{1,2}$ (median 66 nmol/L) and TAT complex (median 710 µg/L) concentrations were elevated in synovial fluid compared with plasma from the patients (1.6 nmol/L and 7.0 µg/L, respectively) as well as the controls (1.0 nmol/L and 2.9 µg/L, respectively).

Conclusion. Synovial fluid contains high numbers of microparticles derived from leukocytes that are strongly coagulant via the factor VII–dependent pathway. We propose that these microparticles contribute to the local hypercoagulation and fibrin deposition in inflamed joints of patients with RA and other arthritic disorders.
INTRODUCTION

The coagulation system in synovial fluid from inflamed joints is strongly activated, resulting in the deposition of fibrin; these fibrin deposits are called rice bodies [151-154]. Joint lavage removes the rice bodies and alleviates the pain.

Coagulation activation requires not only (activated) coagulation proteins and calcium ions, but also a procoagulant surface to which coagulation proteins can bind to assemble tenase and prothrombinase complexes. Membranes exposing negatively charged phospholipids, such as phosphatidylserine (PS), act as such a procoagulant surface. PS is not exposed on resting cells, but during cell activation or apoptosis it appears in the outer leaflet of the membrane. In vitro, platelets are known to release microparticles that expose PS and bind coagulation factors V, VIII, IX and XI and/or their activated forms [6,43,46,49]. Monocytes, endothelial cells, and erythrocytes also release PS-exposing microparticles upon appropriate activation, which support coagulation in vitro [51,53,58]. In vivo, microparticles can be found in the systemic circulation; their numbers and cellular origin are dependent on the state of health of the individual. Compared with healthy control subjects, patients at risk of thromboembolic complications have elevated numbers of platelet microparticles in their venous blood [12,14,15,17,22,24,29,138], and we and other investigators have recently demonstrated the presence of microparticles of non-platelet origin in the circulation of healthy subjects as well as patients with various clinical conditions [29,33,56,155].

Synovial fluid also contains cell-derived microparticles. These microparticles contain relatively high concentrations of lysophospholipids such as lysophosphatidylcholine and lysophosphatidylethanolamine [156]. Most likely, the presence of the lysophospholipids is due to secretory phospholipase A₂, a phospholipid-hydrolyzing enzyme found in elevated concentrations in synovial fluid [157]. The cellular origin of the microparticles in synovial fluid has not been reported, and the breakdown of the phospholipids to their lyso compounds is likely to inhibit their possible thrombin-generating capacity. The aims of the present study were to characterize the cellular origin of microparticles from synovial fluid and to study their
procoagulant properties and, thus, find a possible explanation for the coagulation activation occurring in synovial fluid of inflamed joints.

**PATIENTS AND METHODS**

**Patients and healthy individuals**

We studied 10 patients with rheumatoid arthritis (RA) and 10 patients with other forms of arthritis (non-RA), as follows: psoriatic arthritis (n = 3), undifferentiated arthritis (n = 2), reactive arthritis (n = 1), juvenile chronic arthritis (n = 1), systemic lupus erythematosus (n = 1), osteoarthritis (n = 1), and relapsing seronegative, symmetric synovitis with pitting edema (n = 1). Patient data are summarized in Table 1. The RA patients fulfilled the American College of Rheumatology criteria [158]. Twenty healthy, sex- and age-matched control subjects were also evaluated. Synovial fluid (SF) from inflamed joints as well as venous blood samples were obtained from the patients. Only venous blood samples were obtained from the healthy control subjects. This study was approved by the Medical Ethics Committee of the Leiden University Medical Center. All patients and controls gave their informed consent to participate.

**Reagents and assays**

Reptilase was obtained from Roche (Basel, Switzerland) and the chromogenic substrate Pefachrome TH-5114 from Pentapharm (Basel, Switzerland). Normal murine serum and fluorescein isothiocyanate (FITC)-labeled anti-CD4 (CLB-T4/1,10A12; immunoglobulin (Ig)G₂a) and anti-CD61 (Y2/51; IgG₁) and phycoerythrin (PE)-labeled anti-CD66e (CLB-gran/10 IH4Fc; IgG₁) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, The Netherlands). FITC-labeled anti-glycophorin A (JC159; IgG₁) and streptavidin-PE (R0438) were obtained from Dako (Glostrup, Denmark). FITC-labeled anti-CD8 (SK1; IgG₁), anti-CD20 (L27; IgG₁), and control antibodies (IgG₁ [X40] and IgG₂a [X39]) were obtained from Becton Dickinson (San Jose, CA). FITC-labeled anti-CD66b (80H3; IgG₁a) was obtained from Coulter/Immunotech (Marseilles, France) and FITC-labeled anti-CD14 (CRIS-6; IgG₁) from BioSource (Camarillo, CA).
Biotinylated annexin V (B700) was obtained from Nexins (Hoeven, The Netherlands) and FITC-labeled anti-tissue factor (TF; 4508CJ; IgG₁) from American Diagnostics (Greenwich, CT).

**Table 1. Demographic and clinical data of the RA and non-RA patients***.

<table>
<thead>
<tr>
<th></th>
<th>RA patients (n = 10)</th>
<th>Non-RA patients (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.5 (37 - 77)</td>
<td>52.5 (25 - 79)</td>
</tr>
<tr>
<td>Sex, no. of males/females</td>
<td>3/7</td>
<td>4/6</td>
</tr>
<tr>
<td>Disease duration, months</td>
<td>122 (6 - 300)</td>
<td>65 (6 - 192)</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>6 positive; 4 negative</td>
<td>All negative</td>
</tr>
<tr>
<td>Tender joint count</td>
<td>10.3 (2 - 19)</td>
<td>5.6 (2 - 14)</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>8.9 (1 - 17)</td>
<td>4.4 (1 - 12)</td>
</tr>
<tr>
<td>ESR, mm/hour</td>
<td>56.1 (4 - 83)</td>
<td>25.6 (2 - 63)</td>
</tr>
<tr>
<td>Erosive disease</td>
<td>6 positive; 4 negative</td>
<td>None</td>
</tr>
<tr>
<td>No. of DMARDs</td>
<td>2.6 (0 - 5)</td>
<td>1.5 (1 - 3)</td>
</tr>
<tr>
<td>SF leukocytes, x 10⁹/L</td>
<td>5.3 (0.0 - 12.3)</td>
<td>10.7 (0.7 - 30.3)</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean (range). RA = rheumatoid arthritis; ESR = erythrocyte sedimentation rate; DMARDs = disease-modifying anti-rheumatic drugs; SF = synovial fluid.
Elastase content was determined by ELISA, according to the manufacturer’s instructions (Milenia Biotec, Bad Nauheim, Germany).

In thrombin generation experiments, OT-2 (0.71 mg/mL) was used to determine the contribution of the factor XII-dependent coagulation pathway. This monoclonal antibody (MoAb) completely inhibits factor XIIa [85]. In contrast to kaolin-induced thrombin generation, thrombin generation in recalcified normal plasma initiated by the addition of Thromborel, a commercially available mixture of (brain) phospholipids and TF (Behring Diagnostics, Marburg, Germany), is unaffected by OT-2 [85,139]. The MoAb directed against factor VII (a 1:1 mixture of MoAb VII-1 and VII-15; initial concentrations 1.46 mg/mL and 0.53 mg/mL, respectively) were obtained from the CLB. These antibodies specifically inhibited Thromborel-induced coagulation but not kaolin-induced coagulation [139]. Triton X-100 (Baker, Deventer, The Netherlands) was prepared as a 0.5% stock solution in phosphate buffered saline (154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4) containing 10.9 mmol/L sodium citrate. Plasma and synovial fluid concentrations of prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin (TAT) complexes (Behring Diagnostics) were determined by ELISA, according to the manufacturer’s instructions.

Collection of synovial fluid and blood samples

Synovial fluid (4.5 mL) and venous blood were collected into tubes containing 0.5 mL of 3.2% sodium citrate (Becton Dickinson). Immediately after collection, an additional 0.5 mL of 3.2% sodium citrate was added to the synovial samples to prevent coagulation. Cells were removed by centrifugation (20 minutes at 1550g and 20 °C) within 10 minutes after collection. Microparticles were isolated from fresh samples, and their numbers and cellular source were determined immediately by flow cytometry. For all other determinations, aliquots of plasma, synovial fluid or isolated microparticles were snap-frozen in liquid nitrogen for 15 minutes and stored at -80 °C until use.
**Microparticle isolation**

Cell-free synovial fluid and plasma samples (250 μL) were centrifuged for 15 minutes at 17570g and 20 °C to obtain the microparticles. The supernatant (225 μL) was removed, and the microparticles were resuspended after addition of apopbuffer (225 μL; 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl₂, and 136 mmol/L NaCl, pH 7.4). After centrifugation, the supernatant (225 μL) was removed, and the microparticles were resuspended after addition of apopbuffer (90 μL). For flow cytometry 5 μL aliquots were used, and for thrombin generation 20 μL aliquots were used.

**Flow cytometric analysis**

Aliquots (5 μL) of the isolated and washed microparticles were further diluted with 35 μL apopbuffer/CaCl₂ (2.5 mmol/L CaCl₂) and microparticle-free normal mouse serum (5 μL; diluted 1:500 volume/volume), and incubated for 15 minutes at room temperature. Biotinylated annexin V (5 μL) and FITC-labeled MoAb (5 μL) were added and the mixtures were incubated for 15 minutes in the dark at room temperature. The following final concentrations of FITC-labeled antibodies were used: 0.5 μg/mL of anti-CD4, 0.25 μg/mL of anti-CD8, 0.25 μg/mL of anti-CD14, 0.5 μg/mL of anti-CD20, 0.5 μg/mL of anti-CD61, 0.25 μg/mL of anti-CD66b, and 0.25 μg/mL of anti-glycophorin A. FITC-labeled IgG₁ and IgG₂a (both 0.5 μg/mL) were used as controls.

After the incubation, 200 μL apopbuffer/CaCl₂ was added, and the suspensions were centrifuged for another 15 minutes at 17570g and 20 °C. The supernatant (200 μL) was removed, PE was added (5 μL). After another 15 minutes in the dark at room temperature, 200 μL apopbuffer/CaCl₂ was added, and the suspension was centrifuged for 15 minutes at 17570g and 20 °C. The supernatant (200 μL) was removed, apopbuffer/CaCl₂ (300 μL) was added, and microparticles were resuspended.

With the synovial microparticles, PE-labeled anti-CD66e and FITC-labeled anti-TF were tested in the absence and presence of 0.05% Triton X-100 in phosphate buffered saline, as described previously [159]. FITC-labeled anti-CD66b and PE-labeled anti-CD66e gave comparable results in the analysis of the synovial samples.
Chapters 6

Samples were analyzed in a FACScan flow cytometer with CellQuest software (Becton Dickinson). Both forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain. Microparticles were identified on FSC, SSC, and binding of cell-specific MoAb. Whereas in previous studies binding of both cell-specific MoAb and annexin V were used to identify microparticles, this was impossible in the present study since binding of annexin V to synovial microparticles was impaired.

The number of microparticles per liter plasma or synovial fluid was estimated by using the number of events (N) of cell-specific MoAb-binding microparticles in the flow cytometry after correction for IgG control antibody binding as follows: microparticles/L = N x [115/5] x [355/60] x [10⁶/250]. The lower detection limit of the particle count was established in the samples with the IgG control as 10 x 10⁶ microparticles/L.

**Thrombin generation test**

The thrombin generation test (TGT) was used to estimate the thrombin-generating capacity of the microparticles, as described previously [127,139]. Briefly, microparticles were reconstituted in defibrinated normal (microparticle-free) plasma. Thrombin generation was performed with synovial microparticles from 6 RA patients and 7 non-RA patients; only small volumes of synovial fluid were available from 3 of the patients (2 RA and 1 non-RA), and despite the relatively high concentration of anticoagulant, excessive clotting occurred during storage of 4 other synovial samples (2 RA and 2 non-RA).

Defibrinated normal plasma was prepared by incubating microparticle-free normal plasma (a pool of plasma from 20 healthy individuals that had been centrifuged for 30 minutes at 17570g and 20 °C) with reptilase for 10 minutes at 37 °C and, subsequently, for 10 minutes on melting ice. The fibrin clot was removed by centrifugation for another 5 minutes at 17570g at 20 °C. Since microparticles may, at least partly, adhere to fibrin, plasma and synovial fluid aliquots used for quantitation of microparticles were not defibrinated [87]. Microparticles (20 μL) were added to a mixture of defibrinated normal plasma (120 μL) and buffer A (10 μL; 50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.05% bovine serum albumin, pH 7.35). Thrombin
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generation was initiated at time 0 by addition of CaCl$_2$ (30 μL; 16.7 mmol/L final concentration). Experiments were performed at 37 °C.

At fixed intervals after time 0, aliquots (3 μL) were removed and added to 147 μL prewarmed chromogenic substrate Pefachrome TH-5114 in buffer B (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 20 mmol/L EDTA and 0.05% weight/volume bovine serum albumin, pH 7.9). After 3 minutes, citric acid (90 μL; 1 mol/L) was added to stop the conversion of Pefachrome TH-5114. The generated amount of p-nitroaniline was determined at $\lambda = 405$ nm. To convert the observed optical density (OD) into thrombin concentration, a reference curve was prepared using purified human thrombin [155].

For the inhibition experiments, the mixture of plasma plus buffer A, and separately the microparticles, was incubated for 30 minutes at ambient temperature with 20 μL and 10 μL of antibodies, respectively. The optimum concentration for each of the inhibitory antibodies was determined previously [139]. With those concentrations (i.e., 1 mg/mL initial concentration of anti-factor VII and 0.71 mg/mL of anti-factor XII), specific as well as maximum inhibition was achieved. Plasma and microparticles were pooled after the preincubation and incubated for an additional 10 minutes at 37 °C. Thrombin generation was then started by the addition of CaCl$_2$.

Statistics

Data were analyzed with SPSS for Windows, release 10.0 (SPSS, Chicago, IL). Differences between synovial fluid and plasma from the patients were analyzed with the Wilcoxon signed rank test. Differences between findings in plasma from the arthritis patients compared with the healthy controls were analyzed with the Mann-Whitney U test. One-tailed significance levels are provided, which were considered significant when $P < 0.05$. All data are presented as median (range), unless indicated otherwise.
RESULTS

Exposure of negatively charged phospholipids by synovial microparticles

In previous studies, microparticles from plasma were identified according to their characteristic FSC and SSC, as well as their binding of cell-specific antibodies and annexin V [29,33]. Figure 1B shows that all microparticles derived from plasma obtained from an RA patient strongly bound annexin V, and that the binding of annexin V to synovial microparticles was reduced compared with the plasma microparticles (Figure 1A).

Figure 1. Annexin V-labeling of microparticles derived from synovial fluid and plasma. Microparticles were isolated, labeled with annexin V-biotin / streptavidin-PE, and analyzed by flow cytometry (see Patients and Methods). Shown are representative data for microparticles from A, synovial fluid and B, plasma obtained from a rheumatoid arthritis patient, as well as from C, plasma obtained from a healthy control subject. The extent of annexin V binding is indicated on the x-axis and the side scatter on the y-axis. The fluorescence thresholds (vertical lines) were set using a sample without annexin V.
Similar dot plots were obtained for microparticles isolated from plasma and synovial fluid obtained from the non-RA patients. For comparison, microparticles isolated from plasma obtained from a healthy control are shown in Figure 1C.

Cellular origin of microparticles in synovial fluid

Figure 2 shows representative examples of dot plots for microparticles from synovial fluid and plasma obtained from an RA patient. Microparticles were stained with control antibody (Figures 2A and B) or cell-specific monoclonal antibodies (Figures 2C - H). Microparticles originating from platelets, i.e., those that bound anti-CD61, were almost completely absent in synovial fluid (Figure 2C), whereas in plasma, most microparticles were of platelet origin (Figure 2D). Synovial fluid contained considerable numbers of microparticles originating from granulocytes (CD66b) (Figure 2E) and monocytes (CD14) (Figure 2G), which were scarce in plasma (Figures 2F and 2H, respectively). In addition, synovial fluid contained low, but detectable, numbers of microparticles from CD4+ and CD8+ T cells, B cells, platelets, and erythrocytes.

All quantities of microparticles of a specific cellular origin were significantly higher or lower than those found in patient plasma. We did not observe any differences between RA and non-RA patients with regard to the numbers and cellular origin of the microparticles. The data for the 10 RA and the 10 non-RA patients were therefore combined. The data are summarized in Table 2, along with microparticle numbers in plasma from the healthy individuals. No differences between the plasma values in controls and patients were found.

The granulocyte origin of CD66b-positive microparticles was confirmed by measuring elastase concentrations in synovial fluid. Concentrations of elastase, a protease specifically secreted by activated granulocytes, strongly correlated with the numbers of CD66b-positive microparticles \( r = 0.76, P = 0.0004 \).
Figure 2. Cellular origin of microparticles (MP) in rheumatoid arthritis (RA) patients. MP isolated from the synovial fluid (A, C, E, and G) and plasma (B, D, F, and H) of a RA patient were stained with fluorescein isothiocyanate-labeled control antibody (A and B), anti-CD61 (platelet-derived MP) (C and D), anti-CD66b (granulocyte-derived MP) (E and F), and anti-CD14 (monocyte-derived MP) (G and H). All dot plots shown were obtained in one experiment with MP from a representative patient.
Table 2. Microparticles in synovial fluid and plasma from patients with arthritic inflamed joints, and in plasma from healthy controls.

<table>
<thead>
<tr>
<th>Origin</th>
<th>MoAb</th>
<th>Patient SF (n=20)</th>
<th>Plasma Patients (n = 20)</th>
<th>Plasma Healthy controls (n = 20)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T helper cells</td>
<td>CD4</td>
<td>44 (&lt; 10-224)</td>
<td>&lt; 10 (&lt; 10-40)</td>
<td>&lt; 10 (&lt; 10-36)</td>
<td>0.0001</td>
</tr>
<tr>
<td>T suppressor cells</td>
<td>CD8</td>
<td>40 (&lt; 10-197)</td>
<td>&lt; 10 (&lt; 10-23)</td>
<td>&lt; 10 (&lt; 10-33)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Monocytic cells</td>
<td>CD14</td>
<td>173 (46-708)</td>
<td>&lt; 10 (&lt; 10-77)</td>
<td>&lt; 10 (&lt; 10-67)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>B cells</td>
<td>CD20</td>
<td>&lt; 10 (&lt; 10-304)</td>
<td>&lt; 10 (&lt; 10-31)</td>
<td>&lt; 10 (&lt; 10-16)</td>
<td>0.041</td>
</tr>
<tr>
<td>Platelets</td>
<td>CD61</td>
<td>18 (&lt; 10-71)</td>
<td>593 (166-6027)</td>
<td>1615 (310-5026)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Glyc A</td>
<td>19 (&lt; 10-95)</td>
<td>68 (10-272)</td>
<td>61 (17-377)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>CD66b</td>
<td>96 (&lt; 10-649)</td>
<td>&lt; 10 (&lt; 10-224)</td>
<td>&lt; 10 (&lt; 10-22)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Values are the median (range) number of marker-positive microparticles (x 10⁶/L).
MoAb = monoclonal antibody; SF = synovial fluid; Glyc A = glycophorin A. †P values represent differences between synovial fluid and plasma microparticles from the patients, by the Wilcoxon's signed rank test.

Thrombin-generating capacity of microparticles from synovial fluid and plasma

Microparticles, isolated from synovial fluid and plasma obtained from the patients and healthy controls were reconstituted in normal, defibrinated plasma to test their thrombin-generating capacity. Reconstitution of synovial microparticles from the 6 RA patients tested and from 4 of the 7 non-RA patients tested resulted in massive thrombin generation compared with that of plasma microparticles from the patients and healthy controls. This thrombin generation by synovial microparticles was
completely blocked by anti-factor VIIa. A representative experiment is presented in Figure 3.

Thrombin generation by microparticle preparations from 1 RA and 1 non-RA patient was also partially blocked by anti-factor XI (25% and 29% inhibition, respectively), whereas anti-factor XII inhibited thrombin generation from 1 rheumatoid and 2 non-rheumatoid arthritis patients (20 - 27% inhibition). Three microparticle preparations of the 7 non-RA patients tested, however, were unable to initiate thrombin generation. Thrombin generation by plasma microparticles from both patients and controls was not affected by anti-factor VIIa, whereas anti-factor XII only slightly inhibited this thrombin generation. These data are summarized in Table 3.

Table 3. Thrombin generation by synovial microparticles in the absence and presence of inhibitory antibodies*.

<table>
<thead>
<tr>
<th></th>
<th>Anti-VII</th>
<th>Anti-XI</th>
<th>Anti-XII</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA patients</td>
<td>3.9 (0.3 - 42.5)</td>
<td>102.2 (75.6 - 111.7)</td>
<td>93.4 (73.0 - 100.0)</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-RA patients</td>
<td>1.7 (0.3 - 10.6)</td>
<td>94.7 (71.1 - 107.5)</td>
<td>83.2 (68.7 - 115.7)</td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The effect of monoclonal antibodies represents the remaining thrombin generation, expressed as a percentage of control (i.e., microparticle-induced thrombin generation in the absence of an antibody). Values are the median (range). No significant differences between the rheumatoid arthritis (RA) and non-RA patients were observed. However, microparticles from 3 other non-RA patients did not induce thrombin generation.

† The area under the thrombin generation curve, calculated for the time interval between 0 and 15 minutes.
Figure 3. Thrombin generation by microparticles from synovial fluid and plasma. Microparticles derived from synovial fluid and plasma (A and B, respectively) obtained from a rheumatoid arthritis (RA) patient, as well as from plasma (C) from a healthy control subject were reconstituted in normal, defibrinated plasma. Thrombin generation was performed in the absence (●) or presence of antibodies to factor VII (○), or factor XII (□). The curves shown were obtained in 1 representative experiment.
**Presence of tissue factor on synovial microparticles**

Because factor VII-mediated coagulation is initiated by TF, we stained synovial microparticles with anti-TF to verify whether synovial microparticles do indeed expose TF. Despite the fact that synovial microparticles strongly initiated TF-mediated thrombin generation, no TF antigen was visible on synovial microparticles (Figure 4C).

On cells, cryptic TF activity becomes de-encrypted when low concentrations of detergents such as Triton X-100 are added. In preliminary flow cytometry studies we recently performed on microparticles from patients with another clinical condition (from the pericardial cavity of patients undergoing cardiac surgery with cardiopulmonary bypass), we observed that the microparticles strongly generated thrombin via TF but only revealed TF antigen in the presence of Triton X-100 (data not shown). In the present study, Triton X-100 (0.05% v/v) did not affect control MoAb staining with the synovial microparticles (Figures 4A and B). Similar to the pericardial microparticles, the TF antigen was found on synovial microparticles in the presence of Triton X-100 (Figures 4C and D).

We then performed double label experiments to establish the cellular origin of these TF antigen-exposing microparticles. As shown in Figures 4E and F, part of the observed TF antigen was present on granulocyte-derived microparticles. For these experiments PE-labeled anti-CD66e was used to enable double staining with FITC-labeled anti-TF. Unfortunately, detection of several other cell-specific antigens was impaired by Triton X-100, thereby making a complete analysis of the cellular origin of TF-positive microparticles impossible (data not shown).
Figure 4. The presence of TF on synovial microparticles.

Microparticles from synovial fluid of a rheumatoid arthritis patient were isolated, stained and analyzed by flow cytometry (see Patients and Methods). Microparticles were stained with control antibody (MoAb; FITC-labeled IgG₁) (A and B), FITC-labeled anti-TF (C and D), and anti-TF plus phycoerythrin-labeled anti-CD66e (E and F), in the absence (A, C and E) or presence (B, D and F) of Triton X-100 (0.05% final concentration). All dot plots shown were obtained within one experiment with microparticles from a representative patient.
In vivo coagulation activation status

To establish the extent of ongoing coagulation activation in the plasma and synovial fluid, concentrations of F$_{1+2}$ and TAT complexes were determined. The data are summarized in Table 4. Compared to healthy control plasma, the coagulation system was clearly activated in the patient plasma, as shown by both increased concentrations of F$_{1+2}$ and TAT complexes ($P < 0.0001$ and $P = 0.0003$, respectively). The median concentrations of both coagulation activation markers were 40 and 100 times higher in synovial fluid than in plasma from the patients.

In these statistical analyses, the data for the RA and non-RA patients were pooled because the F$_{1+2}$ and TAT concentrations in the synovial fluid did not differ ($P = 0.16$ and $P = 0.26$, respectively) and both were increased compared with the concentrations in patient plasma (both $P < 0.0001$).

However, the plasma concentrations in RA and non-RA patients differed for both F$_{1+2}$ (2.0 nmol/L [range 1.5 - 3.7] and 1.5 nmol/L [range 0.9 - 2.1], respectively; $P = 0.004$) and TAT complexes (15.4 µg/mL [range 7.9 - 26.3] and 4.0 µg/mL [range 3.1 - 10.4], respectively; $P = 0.003$).
Synovial microparticles support coagulation

Table 4. In vivo coagulation activation status in patients with inflamed arthritic joints and in healthy control subjects.

<table>
<thead>
<tr>
<th></th>
<th>All (n = 20)</th>
<th>Patients RA (n = 10)</th>
<th>Non-RA (n = 10)</th>
<th>Healthy controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+2, nmol/L</td>
<td>66 (24-219)</td>
<td>45 (24-163)</td>
<td>76 (27-219)</td>
<td></td>
</tr>
<tr>
<td>TAT, µg/L</td>
<td>710 (44-14,242)</td>
<td>532 (80-14,242)</td>
<td>946 (44-2,372)</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+2, nmol/L</td>
<td>1.6 (0.9-3.7)</td>
<td>2.0 (1.5-3.7)</td>
<td>1.5 (0.9-2.1)</td>
<td>1.0 (0.4-2.3)</td>
</tr>
<tr>
<td>TAT, µg/L</td>
<td>7.0 (3.1-26.3)</td>
<td>15.4 (7.9-26.3)</td>
<td>4.0 (3.1-10.4)</td>
<td>2.9 (1.4-7.1)</td>
</tr>
</tbody>
</table>

*Values are the median (range). Differences were analyzed with the Mann-Whitney U test or the Wilcoxon signed rank test. There was no significant difference between synovial fluid (SF) from RA versus non-RA patients.

P < 0.001 versus synovial fluid from all patients, by one-tailed test.

P = 0.004 versus non-RA plasma. P = 0.0003 versus non-RA plasma.

P < 0.001 versus plasma from all patients. P = 0.0003 versus plasma from all patients.

**DISCUSSION**

The findings of the present study demonstrate that synovial fluid from inflamed joints of RA patients as well as non-RA patients contains cell-derived microparticles of mainly granulocyte (CD66b, CD66e) and monocyte/macrophage (CD14) origin, to a lesser extent, CD4+ and CD8+ T cells, B-cells (CD20), and erythrocytes (glycophorin A). Synovial microparticles from the 6 RA patients tested, and 4 of the 7 non-RA patients tested evoked thrombin generation via the TF/factor VII-initiating pathway. A subpopulation of synovial microparticles exposed TF, and we found colocalization of TF and the CD66e antigen (i.e., granulocyte origin of the microparticles) on part of this subpopulation. This suggests a role of granulocyte-
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derived microparticles in local hypercoagulation. The origin of the majority of the TF-exposing microparticles, however, could not be established.

TF antigen was demonstrated by flow cytometry only in the presence of a threshold concentration of the detergent Triton X-100. In previous studies, especially on microparticles from the pericardial cavity of patients undergoing cardiac surgery with cardiopulmonary bypass, we noticed discrepancies between the TF-mediated thrombin-generating activity and the apparent absence of the TF antigen. On cells, the presence of cryptic TF is a well-known phenomenon, where activity rather than antigen expression appears cryptic [160]. Upon incubation with detergent, the TF exposed on the cell surface becomes active; this has been explained by either changes in membrane phospholipid distribution, conformational changes in the TF molecule, or the multimerization of TF molecules [161-163]. In contrast to cells, however, incubation of the microparticles with Triton X-100 completely abolished their ability to induce thrombin generation (data not shown). The discrepancy between the presence of TF in thrombin generation experiments and the lack of detection by flow cytometry in the absence of Triton X-100 may simply be due to a lower detection limit of biological versus antigen assays, involving only a subclass of active TF molecules.

Our present findings of granulocyte-derived microparticles bearing TF may support the reports by other investigators that granulocytes are capable of producing TF [164]. However, we cannot exclude a possible transfer of TF from other cells (e.g. monocytes) to granulocytes or their microparticles, similar to the transfer between leucocytes and platelets [62]. The occurrence of microparticles of granulocyte origin in synovial fluid was confirmed by measuring concentrations of elastase (a protease secreted by activated granulocytes) and by the use of both CD66b and CD66e as granulocyte markers. We therefore propose that the microparticles originated from granulocytes.

As stated above, synovial microparticles generated thrombin via factor VII. In previous studies, we demonstrated that microparticles isolated from the pericardial cavity during cardiopulmonary bypass [33] and from plasma of a patient with meningococcal sepsis and disseminated intravascular coagulation [29] also triggered thrombin generation via factor VII and TF. In those studies, anti-TF and/or anti-factor
VII antibodies only delayed the onset of thrombin generation by 5 - 10 minutes, whereas in the present study, thrombin generation was permanently blocked. This may indicate that thrombin generation by these synovial fluid microparticles occurs continually through de novo activation of the coagulation system via TF/ factor VIIa, whereas in other conditions, other microparticle populations propagate thrombin generation, for instance, via their negatively charged phospholipid surface, and thus have the extra ability to form the tenase and prothrombinase complexes.

We recently showed that microparticles from plasma of healthy subjects slowly generated a modest quantity of thrombin [139]. This thrombin generation was independent from TF/factor VII and only partially sensitive to inhibition by factor XII (10%) or XI (40%). Since combinations of antibodies also failed to inhibit more than 40% of thrombin generation, we concluded that thrombin generation by microparticles is, at least in part, independent from the common intrinsic and extrinsic initiation pathways of coagulation activation. Due to the (almost complete) absence of these microparticles in synovial fluid, this alternative route of initiation of thrombin generation may be lacking. Whether this route only amplifies thrombin generation, e.g., via factor VIIIa/IXa and Va that are possibly already present on the surface of microparticles, or whether this is a completely novel coagulation initiation pathway, remains to be elucidated. However, we cannot exclude the involvement of elastase, cathepsins, (macrophage) fgl-2 prothrombinase or (monocyte) Mac-1 integrin in the initiation of coagulation under these conditions.

The high thrombin-generating capacity of synovial microparticles may contribute to the local hypercoagulable and account for the fibrin deposits called rice bodies. Actually, this procoagulant nature was not anticipated, because previous studies showed that secretory phospholipase A$_2$, which is present in high concentrations in synovial fluid, inhibits coagulation [156,157,165,166]. In line with such an effect of secretory phospholipase A$_2$ was the lower exposure of PS by synovial microparticles, as reflected by the reduced binding of annexin V, most likely due to the breakdown of PS to lyso-PS. Despite the reduced binding of annexin V, the synovial microparticles still efficiently supported thrombin generation. Thus, the presence of lyso-phospholipids apparently does not inhibit thrombin generation and the amount of
intact negatively charged phospholipids on the microparticles still appears to be sufficient to support coagulation.

At present, we have no explanation for the inability of 3 microparticle fractions (from 2 patients with undifferentiated arthritis and 1 patient with symmetric synovitis) to generate thrombin in normal plasma. The binding of annexin V to preparations of microparticle from these 3 patients was comparable to that of microparticle preparations from other patients indicating that the lack of thrombin-generating activity is not explained simply by an insufficient exposure of negatively charged phospholipids. However, preliminary experiments indicated that these microparticles expose sufficient negatively charged phospholipids to facilitate the propagation of thrombin generation.

Kaolin as an activator of factor XII requires the presence of negatively charged phospholipids, either microparticles or artificial phospholipid vesicles, to initiate and propagate thrombin generation. Addition of synovial microparticles from 1 of the 3 patients showing no thrombin generation was tested and did indeed propagate the kaolin-induced thrombin generation (data not shown). The absence of thrombin initiation by these microparticles could not be attributed to the absence of TF on the microparticles, which was detectable by flow cytometry and was similar to microparticles from the other patients, which did generate thrombin. We hypothesized that the synovial fluid from this particular patient may contain one or more substances that interfere with thrombin initiation Synovial microparticles from another patient lost more than 90% of their thrombin-generating capacity after preincubation in microparticle-free synovial fluid from this patient. Although these data are preliminary, they suggest that in some patients, one or more factors that hamper the initiation of thrombin generation by microparticles may be present in the synovial fluid.

Microparticles may also play a role in inflammation. Leukocyte-derived microparticles trigger endothelial cells to produce interleukin-6 [60,167]. P-selectin, which is present on platelet-derived microparticles, triggers monocytes to express not only TF, but also chemokines [168,169]. Platelet-derived microparticles transport arachidonic acid to endothelial cells and induce expression of cyclooxygenase-2 and
the increased production of prostacyclin [170], enhance the interaction between monocytes and endothelial cells [52], and enhance leukocyte aggregation and their accumulation on the endothelium [171]. Furthermore, thrombin, factor Xa and fibrin degradation products are all potent inducers of the inflammatory response [172-176]. Hence, the presence of microparticles in synovial fluid may facilitate not only coagulation, but also inflammation via direct and indirect mechanisms.

In summary, microparticles in the synovial fluid of arthritis patients differ in cellular origin from those found in plasma. In the majority of these patients, subpopulations of these microparticles expose TF on their surface, enabling them to generate thrombin. Synovial microparticles may underlie the extensive activation of the coagulation system in the inflamed arthritic joints, leading to local fibrin depositions which aggravate the condition in both RA and non-RA patients.