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Elevated Levels of Platelet Microparticles Are Associated With Disease Activity in Rheumatoid Arthritis

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Objective. **Platelets are involved in various thrombotic events, often by means of platelet-derived microparticles (PMPs). It is likely that platelets are also involved in inflammation. Because inflammatory processes play a central role in rheumatoid arthritis (RA), we sought to determine whether PMPs are present in this disease.**

Methods. **This descriptive, cross-sectional study included 19 RA patients and 10 healthy controls. Nine of the patients had active RA (erythrocyte sedimentation rate [ESR] ≥28 mm/hour and/or C-reactive protein [CRP] level ≥28 mg/liter, ≥9 painful joints, and ≥6 swollen joints), and 10 had inactive disease (ESR ≤27 mm/hour, CRP ≤27 mg/liter, no tender joints, and no swollen joints). Platelet counts and PMP numbers were determined using cell counter and flow cytometry, respectively.**

Results. **Platelet counts in the 3 groups were similar. However, levels of PMPs in RA patients were significantly higher than those in healthy controls (median 616 versus 118 × 10^6/liter; $P = 0.005$). PMP levels were higher in patients with active RA than in those with inactive RA (median 2,104 versus 504 × 10^6/liter; $P > 0.05$). Moreover, PMP levels correlated with disease activity ($r = 0.67$, $P = 0.05$).**

Conclusion. **PMPs are associated with RA, and PMP levels are correlated with disease activity. Thus, platelets probably play a part in the inflammatory process of RA by means of PMPs. Given the importance of PMPs in cardiovascular diseases, this may be one reason for the enhanced cardiovascular morbidity and mortality in RA.**

Platelet-derived microparticles (PMPs) are small vesicles that are released from the plasma membrane upon platelet activation (1). Thus far, PMPs have been mainly associated with thrombotic events (2,3). However, several facts suggest that platelets and PMPs may also be involved in the inflammatory processes of rheumatoid disease.

First, secretory phospholipase A_2 ($sPLA_2$) activity is significantly increased in both the synovium and serum of rheumatoid arthritis (RA) patients compared with healthy controls and is also correlated with disease activity (4,5). Secretory PLA_2, which is derived predominantly from activated platelets rather than monocytes or polymorphonuclear cells (6), catalyzes synthesis of arachidonic acid from phospholipids. Subsequently, arachidonic acid is converted by cyclooxygenase-2 (COX-2) into prostaglandins, which are essential for development of inflammation (7). Second, activation of both COX-2 and $sPLA_2$ is linked to platelet-activating factor (PAF), which is synthesized by various cells, including platelets (8). PAF is present in the synovial fluid of RA patients, and administration of a PAF receptor antagonist significantly reduces inflammation (9). In healthy individuals, the PAF released from platelets is detected primarily in association with PMPs (10). Third, plasma levels of soluble P-selectin are elevated in RA patients compared with controls (11). Surface expression and secretion of P-selectin are induced by activation of platelets. Although it is currently unknown whether soluble P-selectin in plasma from RA patients is membrane-associated (i.e., exposed on microparticles) or present as a "free" protein, in vitro P-selectin can be exposed on PMP (12).

P-selectin is an important adhesion receptor that mediates adhesion of platelets and endothelial cells to

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monocytes and granulocytes, thereby triggering expression of tissue factor and interleukin-8 in vitro (13,14). Thus, $sPLA_2$, PAF, and P-selectin are all associated with RA as well as with platelet activation. Because in various clinical conditions platelet activation results in production of microparticles, PMPs may also play a role in the inflammatory processes of RA. In this context, it is important to know that PMPs cannot be induced only by shear stress (15) or aggregating agents (16) but also by the complement C5b–9 complex (17). It has been suggested that this alternate pathway of the complement system plays a role in rheumatic disease as well (18).

In summary, several observations support the theory that PMPs are involved in the inflammatory processes of rheumatic diseases. Until now, however, only 2 studies (one of which included only a single RA patient) dealt with this subject (19,20). The aim of the present study was to investigate the level of plasma PMPs in patients with RA compared with healthy controls and to study the relationship between PMP levels and disease activity in RA.

PATIENTS AND METHODS

Patients and controls. Consecutive patients at the outpatient Department of Rheumatology were selected for inclusion in this descriptive, cross-sectional study. All patients had RA based on American College of Rheumatology criteria (21). Ten laboratory technicians served as healthy controls. Patients had to be between the ages of 18 and 80 years. Exclusion criteria included use of anticoagulants and/or corticosteroids. Only those patients with either very active RA or inactive disease were included. Active RA was defined as erythrocyte sedimentation rate (ESR) $\geq$28 mm/hour and/or C-reactive protein (CRP) level $\geq$28 mg/liter, combined with $\geq$9 painful joints and $\geq$6 swollen joints. Criteria for inactive RA were ESR $\leq$27 mm/hour, CRP $\leq$27 mg/liter, and the absence of painful and swollen joints. For all RA patients, a disease activity score (DAS) comprising the ESR, the tender joint count (TJC; 28 joints), the swollen joint count (SJC; 28 joints), and the patient's assessment of global well-being (on a 100-mm visual analog scale [VAS]) was calculated using the following formula: $(0.56 \times [\sqrt{TJC}]) + (0.28 \times [\sqrt{SJC}]) + (0.70 \times \ln\text{-ESR}) + (0.014 \times VAS)$. This DAS 28-joint count has been shown to be valuable in RA patients with both early and late disease (22). A DAS >3.8 indicates active disease, and a DAS $\leq$2.4 indicates that RA is in remission. All patients gave informed consent, and the study was approved by the local ethics committee.

Laboratory methods. EDTA-anticoagulated blood was collected. Blood cells were removed by centrifugation at 1,550*g* for 20 minutes at room temperature. Aliquots of cell-free plasma (first snap frozen in liquid nitrogen) were stored at −70°C until the time of analysis.

Isolation of microparticles. Cell-free plasma (250 μl) was centrifuged at 17,570*g* for 30 minutes at 20°C, after which 225 μl of microparticle-free plasma was removed. Phosphate buffered saline (PBS; 225 μl), 154 mmoles/liter of NaCl, 1.4 mmoles/liter of phosphate, and 10.9 mmoles/liter of trisodium citrate (pH 7.4) was added to the microparticle pellet and the remaining plasma (25 μl). The microparticles were resuspended and centrifuged at 17,570*g* for 30 minutes at 20°C. Again, 225 μl of the microparticle-free supernatant was removed, and microparticles were resuspended in the remaining 25 μl. For flow cytometric analysis, the 25-μl microparticle suspension was diluted 4-fold with PBS/citrate, of which 5 μl per incubation was used.

Flow cytometric analysis. The samples were analyzed in a FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA). Forward scatter (FS) and side scatter (SS) were set at logarithmic gain. Earlier studies showed that upon addition of a calcium ionophore (A23187) to platelets, particles (PMP) are released that are smaller (lower FS) and less dense (lower SS) than platelets (23). These in vitro–generated microparticles can be easily stained with annexin V, and antibodies can be directed against proteins present on the platelet surface, such as glycoprotein (GP) IIIa (CD61). Annexin V is a protein that in the presence of calcium ions binds with high affinity and high specificity to negatively charged phospholipids. GPIIIa is part of the integrin GPIIb–IIIa complex and is the most abundant receptor on the platelet surface.

To exclude events caused by noise, the microparticles were labeled with annexin V in the absence of calcium ions and an IgG1 control antibody to set the fluorescence thresholds (24,25). Because >75% of the events generated in vitro bind both annexin V and CD61, we and other investigators assume that, by far, the majority of microparticles in vivo expose negatively charged phospholipids. For instance, Aupix et al used annexin V–coated enzyme-linked immunosorbent assay plates to capture cell-derived microparticles from plasma, which after washings were identified by cell-specific antibodies (26). The mixtures were incubated in the dark for 15 minutes at room temperature. Subsequently, 200 μl of PBS/calcium was added, and the suspensions were centrifuged at 17,570*g* for 30 minutes at 20°C. Finally, 200 μl of the microparticle-free supernatant was removed. The microparticles were resuspended with 300 μl of PBS/calcium buffer before flow cytometry. All samples were analyzed for 1 minute, during which time the flow cytometer analyzed ~150 μl of the microparticle suspension. To estimate the number of microparticles per liter of plasma, the number of microparticles (N) found in the upper right quadrant of the flow cytometry analysis was used in the following formula: $N/\text{liter} = N \times (100/5) \times (355/150) \times (10^6/250)$.

Statistical analysis. Nonparametric tests (Mann-Whitney) were used to compare the values of PMPs in the various groups. Because data were not normally distributed, Spearman's test was applied to correlate the number of PMPs with disease activity. All data were expressed as the median (range).

RESULTS

Nineteen patients (9 with active RA, 10 with inactive RA) and 10 healthy controls were included. The

Table 1. Characteristics of rheumatoid arthritis (RA) patients*

	Active RA (n = 9)	Inactive RA (n = 10)
Age, years	61.5 (43.8–78.5)	61.5 (47.3–72.0)
Male-to-female ratio	2:7	2:8
Disease duration, months	66 (3–160)	81 (2–186)
% rheumatoid factor–positive	89	70
DAS28	6.7 (5.1–7.5)	2.1 (1.3–2.4)
Erythrocyte sedimentation rate, mm/hour	72 (28–96)	15.5 (6–23)
C-reactive protein level, mg/liter	35 (5–152)	<10
No. of swollen joints	10 (7–15)	0
No. of painful joints	12 (9–19)	0
No. of DMARDs used		
Methotrexate	6†	0
Sulfasalazine	2†	3
Gold	0	2
Hydroxychloroquine	0	4
Leflunomide	1	0
None	1	1
% using NSAIDs	67	50

* Values are the median (range) unless otherwise indicated. DAS28 = disease activity score in 28 swollen and 28 painful joints; DMARDs = disease-modifying antirheumatic drugs; NSAIDs = nonsteroidal anti-inflammatory drugs.
† One patient used a combination of methotrexate and sulfasalazine.

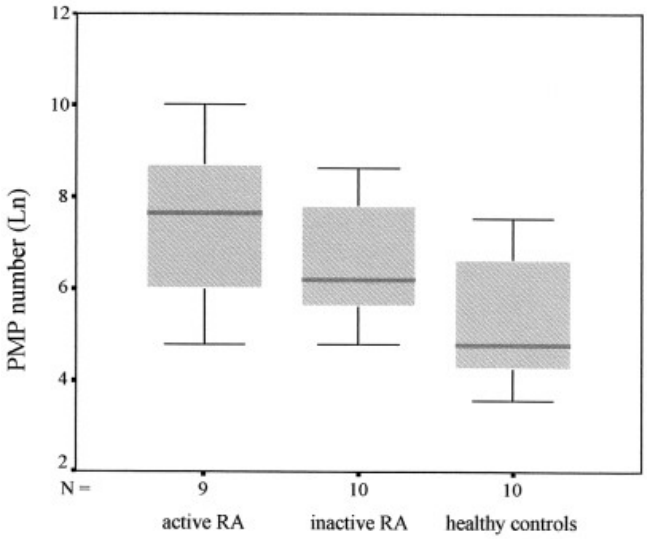

Figure 1. Number of platelet microparticles (PMPs). Horizontal lines show the medians; boxes show the interquartile ranges; vertical lines show the high and low values. RA = rheumatoid arthritis; Ln = natural logarithm.

median age of RA patients was 60.6 years (range 43.8–78.5), and the healthy controls were younger (median age 39.5 years, range 27.0–54.0). Between RA groups, there were no differences in age, sex distribution, disease duration, or presence of rheumatoid factor (Table 1).

Table 2 shows the laboratory results. Platelet counts were within the normal range in all 3 groups, although they were slightly higher in RA patients, especially those with active disease. The number of PMPs was higher in RA patients than in healthy controls ($P = 0.05$). Patients with active RA had more PMPs than did patients with inactive RA, but this difference was not statistically significant (Figure 1). Within the group of patients with active RA, the PMP count correlated with disease activity as measured by the DAS 28-joint count ($r = 0.67$, $P = 0.05$) (Figure 2). The PMP count did not correlate with either the CRP level or the ESR alone. The number of PMPs was higher (although the difference was not statistically significant) in patients who used nonsteroidal antiinflammatory drugs (NSAIDs) than in those who did not (median 1,879 [range 120–22,441] versus 398 [146–10,243] $\times 10^6$/liter; $P > 0.05$).

Table 2. Platelet microparticles (PMPs) in the study groups*

	Rheumatoid arthritis			Healthy controls (n = 10)
	Active (n = 9)	Inactive (n = 10)	Total (n = 19)	
Platelet count, $\times 10^6$/liter				
Median	260	245	251	237
Range	159–398	182–319	159–398	185–307
Mann-Whitney test	NS	NS	NS	
No. of PMPs, $\times 10^6$/liter				
Median	2,104	504	616	118
Range	120–22,441	121–5,562	120–22,441	35–1,881
Mann-Whitney test	$P = 0.010$ vs. controls; P NS vs. inactive	$P = 0.028$ vs. controls	$P = 0.005$ vs. controls	

* NS = not significant.

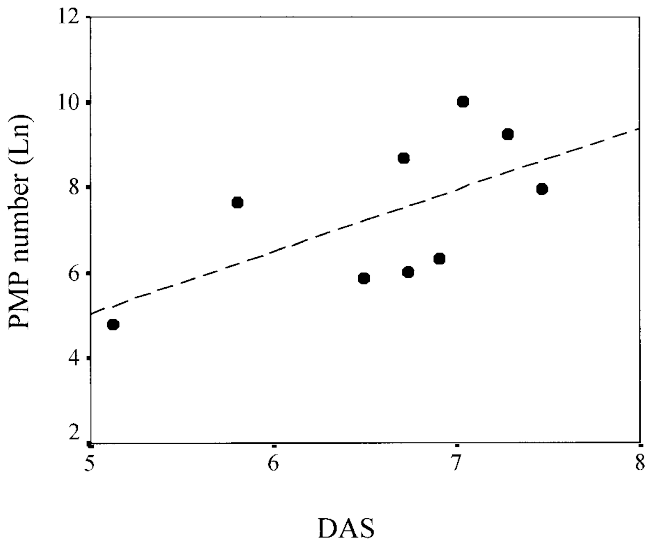

Figure 2. Correlation between platelet microparticles (PMPs) and disease activity score (DAS) in patients with active rheumatoid arthritis. Ln = natural logarithm.

DISCUSSION

This cross-sectional study demonstrates that PMP levels are higher in patients with RA than in healthy controls; moreover, the number of PMPs correlates with disease activity. These results confirm our hypothesis that PMPs are involved in the inflammatory processes of RA. Previous studies showed elevated numbers of PMPs in patients with symptoms of Raynaud's disease (one of whom had RA) and in patients with systemic lupus erythematosus (19,20). Other inflammatory conditions have also been associated with PMPs (25,27). The role of PMPs in inflammation is further supported by the observation that they can activate neutrophils (28,29). Moreover, PMP formation can be initiated by complement factors (30,31), which probably also play a role in rheumatic disease (18). This fact supports data from the present study.

Some remarks about the current study should be made. First, the patients and controls were not matched, resulting in a rather large difference in age between groups. Thus far, however, there is no indication that the number of PMPs increases with age. The only study that dealt with this issue demonstrated more PMPs in healthy neonates than in adults (32). Therefore, we postulate that the observed differences in PMP levels between RA patients and healthy controls result from disease activity rather than from higher age. Second, the RA patients in this study were allowed to use NSAIDs, which are known to interfere with platelet function. Because formation of PMPs is dependent on platelet activation, NSAID use may be expected to decrease the number of circulating PMPs. However, the present study demonstrates the opposite effect. Therefore, the actual numbers of PMPs in the patients using NSAIDs may be underestimated. In addition, it is possible that the influence of the inflammatory disease overrides the effect of NSAIDs on PMPs.

There was no significant difference in platelet levels between the 3 groups studied, although there was a trend toward higher platelet counts in patients with active RA than in patients with inactive RA and healthy controls. This contrasts with earlier studies that demonstrated elevated platelet counts in patients with active disease (33,34). Perhaps the number of subjects in the present study was too small to allow detection of significant differences. A higher number of PMPs per platelet rather than a higher platelet count, or differences in their production rate and/or clearance, may also be responsible for the higher PMP numbers in patients with active RA.

Our observation of elevated levels of PMPs in patients with RA can yield an interesting discussion concerning the possible role of PMPs in the development of cardiovascular disease. RA patients are known to have higher cardiovascular mortality compared with the general population, and there seems to be a correlation with the extent of inflammation (35). Elevated numbers of PMPs circulate in patients at risk for thromboembolic events. In vitro, PMPs are released from platelets in native blood under shear conditions comparable with those in stenosed arteries (36), and this release is further enhanced by plasma from patients with acute myocardial infarction (37). PMPs facilitate platelet adhesion to exposed subendothelial matrix, in vitro as well as in a rabbit model of arterial injury (38). PMPs also trigger coronary artery smooth muscle cell mitogenesis (39) and activate platelets and endothelial cells by transcellular activation of bioactive lipids such as arachidonic acid (39). This activation results in production of cytokines and tissue factor (29). PMPs promote leukocyte–leukocyte interaction under flow conditions (41) and act directly as procoagulant surface by exposure of negatively charged phospholipids to enable tenase and prothrombinase complex formation (17). Subsequently, the thrombin formed on the microparticle surface may promote not only clotting but also inflammation and the additional release of PMPs (12). Finally, high numbers of cell-derived microparticles are deposited in human atherosclerotic plaques (42). Thus, pa-

tients who have elevated numbers of circulating PMPs are prone to developing atherogenesis. PMPs might prove to be the missing link between active inflammation and thromboembolic processes in patients with RA.

In conclusion, PMPs are increased in patients with RA and are associated with disease activity. Their role in the inflammatory processes needs to be further elucidated, and their connection to cardiovascular disease deserves closer attention as well.

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