Circulating Microparticles in Patients with Benign and Malignant Ovarian Tumors

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Abstract. *Background: Microparticles are known to be increased in various malignancies. In this prospective study, microparticle levels were evaluated in patients with benign and malignant ovarian lesions. Patients and Methods: Microparticles from platelets/megakaryocytes, activated platelets and endothelial cells, tissue factor exposing microparticles and D-dimer values were examined in patients with newly diagnosed ovarian lesions before surgery, and were correlated with tumor histology. Results: Higher counts of CD63-positive microparticles were detected in patients with ovarian cancer [mean=276×10^6 (range: 64-948)/l; n=12] as compared to patients with benign ovarian tumors [146×10^6 (45-390)/l; n=21; p=0.014]. D-dimer values were also increased in patients with cancer [860 (180-4500) ng/l versus 280 (170-2720) ng/l; p=0.001]. Conclusion: Elevated levels of CD63-positive microparticles and D-dimer reflect the procoagulant phenotype of these patients. However, for the discrimination between benign and malignant ovarian tumors, measuring preoperative levels of microparticles does not seem to be helpful.*

Ovarian cancer is the fifth leading cause of cancer deaths in women (1). Lacking specific warning symptoms, it is frequently diagnosed at an advanced stage. As yet, no screening test is available apart from ultrasound, which, however, is not always capable to discriminating between benign and malignant tumors, especially when dealing with small lesions. The only established plasma tumor marker for ovarian cancer is cancer antigen 125 (CA-125). Nevertheless, 50 % of patients with ovarian cancer detected at an early stage do not have increased CA-125 levels (2) and, likewise, this marker can also be elevated under benign conditions such as leiomyomatoma, endometriosis, ovarian cysts, and pregnancy (3). In recent years, more and more data have been accumulated with regard to the increased levels of circulating microparticles in patients with various types of malignancies including gastric (4), lung (5), colorectal (6), and breast cancer (7). Data concerning changes of microparticle levels in patients with ovarian cancer, however, are still lacking. Microparticles can be released from nearly every cell type following activation or apoptosis (8). Most information has been accumulated by measuring microparticles in plasma samples as a reflection of the microparticles that are circulating in the blood. In healthy humans microparticles are mainly derived from platelets/megakaryocytes (PMPs) and from endothelial cells (EMPs) (9, 10). The microparticle levels, the cellular origin, the composition and the function have been described for various disorders such as diabetes mellitus (11), hypertension (12), and heparin-induced thrombocytopenia (13). In particular the composition and function of PMPs have been intensively investigated (14, 15). Besides their role in the transport and transfer of bioactive molecules, the activation of other cells, and their contribution toward inflammatory processes (16, 17), PMPs are also major players in coagulation as they are highly thrombogenic (18), by providing a phospholipid surface with binding sites for several coagulation factors (19, 20). From this point of view, PMPs support coagulation and thus provide a link between the platelet-based haemostatic system and the plasmatic coagulation *in vivo*. Patients with Glanzman's disease (21) or Scott syndrome (22) have reduced counts of PMPs leading to bleeding disorders. On the other hand, patients with elevated levels of PMPs suffer from diseases such as cerebral

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infarction (23), acute coronary syndrome (24), sepsis (25) and venous thrombosis (26) due to a hypercoagulant status. In the latter cases, EMPs are also often increased, reflecting injuries of the vessel wall. Irregular neo-angiogenesis in cancer and expression of procoagulant factors on tumor cells are probably responsible for similar changes of microparticle concentrations in cancer patients as mentioned above. Our research group found a different microparticle pattern in women with breast cancer as compared to those with benign breast lesions (27). In this prospective case control study we investigated the levels of PMPs, EMPs, and of tissue factor (TF)-bearing microparticles in women with ovarian tumors as compared to women with benign ovarian lesions. Our aim was to investigate whether the microparticles' pattern might help to differentiate between benign and malignant lesions.

Patients and Methods

Study population and trial design. Patients with newly diagnosed ovarian tumors of unknown histology were enrolled in this prospective case control study. Those taking oral contraceptives, hormone replacement therapy, treatment with aspirin, or low-molecular weight heparin (LMWH) were excluded, as were those with a history of previous cancer, smoking , hypertension, diabetes or other chronic disorders (especially HIV or hepatitis). Patients were screened and recruited from the Department of Obstetrics and Gynecology, Ludwig Maximilians University, Grosshadern Clinic, between November 2007 and July 2009. The blood counts, and number of microparticles, and the D-dimers were measured in every patient prior to surgery. microparticle concentrations were then correlated with the final tumor entity. The Human Investigation Review Board of the Ludwig Maximilian University Munich approved the study. Signed written informed consent was obtained from all women, allowing for the analysis of all clinical and laboratory data mentioned in this article.

Blood sampling and isolation of microparticles. Blood samples were taken by puncture of the antecubital vein without tourniquet through a 20-gauge needle. For the isolation of microparticles, blood samples were centrifuged at 1550 ×*g* for 20 minutes within 15 minutes after sampling, and were stabilized in liquid nitrogen for 15 minutes. Samples were stored at –80˚C until assayed. Samples were defrosted carefully over a period of approximately one hour. After centrifugation of 250 μl plasma/endothelial cell culture supernatant at 18,890 ×*g* for 30 minutes, 225 μl of plasma/supernatant were removed. The microparticles were resuspended after the addition of 225 μl phosphate-buffered saline containing citrate (0.32% trisodium citrate) and were centrifuged again for 30 minutes at 18,890 ×*g*. After the removal of the supernatant (225 ml), 75 μl of PBS/citrate buffer was added, and the microparticle pellet was resuspended. Five microlitres of the microparticle suspension was diluted in PBS containing of 35 μl CaCl2 (2.5 mmol/l) or, alternatively, of 35 μl citrate for annexin V-negative controls. Finally, 5 μl of annexin V were added plus 5 μl of each cell-specific monoclonal antibody or isotype-matched control antibodies and were incubated in the dark at room temperature for 15 minutes. The reaction was stopped with 900 μl of phosphate-buffered saline containing calcium (2.5 mmol/l).

Reagents. EMPs were identified by double-staining with phycoerythrin (PE)-labeled annexin V (Immuno Quality Products, Groningen, the Netherlands) and a fluorescein isothiocyanate (FITC)-labeled anti-CD144 (Acris, Hiddenhausen, Germany). For analyzing PMPs, a staining with FITC-labeled annexin V (Immuno Quality Products) and a PE-labeled anti-CD61 antibody (BD Biosciences, Heidelberg, Germany), or a PE-labeled anti-CD63 antibody (Immunotech, Marseille, France) was used. For measuring TF-bearing microparticles FITC-annexin V were combined with a PE-labeled anti-CD142 antibody from BD Biosciences. The PE-labeled IgG control antibody was obtained from Immuno Quality Products. All antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mmol/l NaCl, 1.4 mmol/l phosphate, pH 7.4). Final dilutions (v:v) were: 1:100 for annexin V-FITC, 1:200 for annexin V-PE, 1:20 anti-CD144-FITC antibody, 1:100 for the anti-CD61-PE antibody, 1:20 for the anti-CD63-PE antibody, 1:10 for the anti-CD142-PE antibody, 1:100 for the IgG-FITC antibody, and 1:100 for the IgG-PE antibody.

Analysis of microparticles. The samples were analysed in a FACScan flow cytometer (BD Biosciences) running the Cell Quest Software (BD Biosciences, San Jose, CA, USA). FACScan was run on high pressure. Forward scatter (FSC), sideward scatter (SSC), and fluorescence channels were set at logarithmic gain. All samples were analyzed for one minute. The volume analysed in one minute (V; approximately 50-80 μl) was measured daily before the analysis by taking measurements of the weight of a sample (aqua destilata) before and after the analysis. The number of microparticles/μl of plasma was determined by using the following formula: microparticles/l=N × (100 μl/5 μl) × (950 μl/V) × 10^6/250 μl) according to Berckmans *et al*. (28) (N=absolute number of microparticles determined by FACS analysis; 100 μl=total volume of washed microparticle suspension; 5 μl=pellet used for the analysis; 950 μl=total volume before the analysis (pellet plus antibodies and buffer); 250 μl=original volume of the sample before the isolation of microparticles).

Statistical methods. Results are given as median values with interquartile ranges, unless stated otherwise. Differences between the groups were analyzed by the non-parametric Mann-Whitney test. All statistical tests were two-tailed. *p*-Values <0.05 were regarded as statistically significant. Data were analyzed with the SPSS software for Windows, release 18.0 (SPSS, Chicacgo, IL, USA).

Results

Study population. During the screening period, 62 women were presented with newly diagnosed ovarian tumor of unknown dignity. Thirty three women fulfilled the inclusion criteria of our study and were subjected to laparotomy. All patients were Caucasian. Complete removal of the ovarian tumor and histological analysis were possible in every patient. A benign ovarian tumor was found in 21 women, ovarian cancer was diagnosed in 12 women (FIGO 1 n=2, FIGO 2 n=3, FIGO 3 n=6, and FIGO 4 n=1). The median age of the women with benign ovarian tumors (n=21) was 50 (range=21-84) years, and younger than the patients with a diagnosis of malignancy (n=12) [61 (range=47-76) years; p=0.049].

Table I. *Blood counts, D-dimer levels, and microparticle concentrations in patients with benign versus levels of patients with malignant ovarian tumors. Numbers indicate median values (interquartile range).*

	Ovarian tumor with benign histology	Ovarian tumor with malignant histology
Leukocytes ($\times 10^9$/l)	7.6 (5.7-8.6)	8.2 (7.5-10.7)
Hemoglobin (g/dl)	14.0 (13.4-14.4)	14.0 (13.0-15.1)
Platelets ($\times 10^9$/l)	286 (256-338)	326 (217-402)
D-Dimer (ng/l)	280 (185-505)	860 (610-2570)*
Total microparticles ($\times 10^6$/l)	4771 (3580-7139)	4838 (4266-6421)
CD61 + microparticles ($\times 10^6$/l)	4231 (3000-6218)	3882 (3014-5648)
CD61 +/CD63 + microparticles ($\times 10^6$/l)	146 (78-208)	276 (152-518)*
TF-bearing microparticles ($\times 10^6$/l)	198 (161-330)	211 (194-230)
CD144 + microparticles ($\times 10^6$/l)	1579 (1233-2141)	1444 (1110-2167)

*$p<0.05$.

Microparticle analysis. The levels of total annexin V-binding microparticles did not differ between patients with benign ovarian tumors and those with malignant ovarian tumors. Similarly, no differences were found between the numbers of PMPs (CD61) or EMPs (CD144), nor in the plasma levels of TF-bearing microparticles. In contrast, the fraction of microparticles originating from activated platelets, *i.e.* exhibiting the activation marker CD63, was significantly elevated in women with ovarian cancer (7.12%) compared to women with a benign tumor (3.45%; $p=0.014$), Table I.

Blood count and D-dimer. No differences were found in median hemoglobin levels, nor in leucokytes or platelet counts between the investigated collectives. The median D-dimer level, however, was elevated in the group with malignant disease as compared to the group with benign disorders ($p=0.001$) (Table I).

Discussion

In this prospective case control study, the levels of circulating microparticles were evaluated as possible tumor markers in patients with benign and malignant ovarian lesions. However, preoperatively, the total levels of microparticles were independent of the postoperative histology. Most microparticles bare CD61, and the levels of these microparticles were comparable for both patient groups. CD61-bearing microparticles present in human blood have long been believed to originate solely from platelets, and for this reason they were called platelet derived microparticles, even though Cramer *et al*. showed that cultured megakaryocytes seem to release not only platelets but also microparticles (29). As megakaryocytes eject platelets directly into the blood stream through cytoplasmic processes, penetrating the sinus wall into the sinus lumen (30), this may also result in the concurrent release of megakaryocyte-derived microparticles directly into the circulation. Unfortunately in daily practice, it is not possible to distinguish between microparticles released from platelets and megakaryocytes because they share many common antigens such as glycoprotein IIb-IIIa and glyoprotein Ib, *i.e.* the platelet receptors for fibrinogen (CD41/CD61) and von Willebrand factor (CD42). Flaumenhaft *et al*. showed that PMPs without activation markers originate largely from megakaryocytes in mice (31). Data from our own research group indicate that this is also true in humans: killing of megakaryocytes by total-body irradiation in patients undergoing stem cell transplantation leads to a faster decline of CD61-bearing microparticles than of microparticles bearing platelet activation markers such as CD63 or P-selectin (CD62p) (32). In our opinion, most CD61-positive microparticles are likely to originate from megakaryocytes. Only a small fraction of CD61-positive microparticles (fewer than 5%) in healthy humans seem to be released from platelets (10). The release of these real PMPs is mainly triggered by platelet activation, *e.g.* by high shear stress around a vessel stenosis (33, 34) or by thrombin receptor agonists (35). During the progress of cell activation, the intracellular calcium levels increase, thus triggering or facilitating microparticle release. Following this consideration, it is obvious that the real PMPs carry platelet activation markers such as CD63. In our investigation, however, the fraction of CD63-positive microparticles was twice as high in patients with ovarian cancer compared to the control group. This result is in line with our finding that increased levels of microparticles bearing platelet activation markers, were detected in patients with breast cancer as compared to patients with benign breast tumors (27). The enhanced fraction of CD63-positive microparticles in patients with malignancies indicates a higher activation level of platelets, compared to that observed in patients with benign tumors. This hypothesis has been supported by Caine *et al*. (36) who found an enhanced rate of activated platelets in patients with advanced cancer. The hypercoagulable status of patients with ovarian cancer is indicated by increased D-dimer levels, which were also reported by other investigators and are shown to correlate with the FIGO stage (37-39).

Similarly, we found elevated plasma levels of prothrombin fragments 1+2, a marker of coagulation activation *in vivo*, in malignant breast tumors compared to benign ones (27). In general, an increase in the D-dimer levels is the result of activated coagulation and of fibrin degradation system, which can be found in most malignancies (40). TF may be perhaps the most important trigger of the plasmatic coagulation system concerning *in vivo* activation. For a long time, TF was thought to be expressed only in the extravascular compartment and at high levels in some organs such as the brain, the heart or the kidney, and especially in adventitial fibroblasts of the vessel walls (41, 42). Accordingly, TF was believed to be released into the circulation and to react with factor VII/IIa only after injury. However, this old dogma has been changed owing to the discovery of very low levels of TF in the blood of healthy humans – socalled blood-born TF (43). TF can be expressed, *e.g.* during sepsis, by monocytes and endothelial cells, which in turn can release TF-bearing microparticles (44). Increased levels of microparticles bearing TF are present in the blood of patients with cardiovascular diseases or diabetes (45). Moreover, increased TF plasma levels are frequently found in patients with malignancies (46). Aberrant TF on a cancer cell surface is one source (47), another is endothelial cells within the tumor vasculature (48), even if this finding is not universally accepted (49). Some of this cancer-specific TFs seem to be bound to vesicles/microparticles derived from cancer cells (50). Such tumor-derived TF-bearing microparticles have been shown to exert procoagulant activity and are associated with venous thromboembolism (51, 52). Regarding our study, an overexpression of TF can be detected in the majority of ovarian cancer cases – especially in patients which manifest thrombotic events (53). Additional TF expression on ovarian cancer cells can be induced by hypoxia, as shown in in-vitro experiments (54). In our study, however, no increased plasma levels of TF-positive microparticles were found in association with malignant ovarian tumors. This may be the result of the selection bias of patients whose cancer tissue expressed either weak or negative TF activity, a possibility supported by the fact that none of the patients enrolled in the study had developed a venous thrombosis. Another hypothetical explanation could be that ovarian cancer cells release their microparticles mainly into the peritoneal space and not in the blood circulation. To investigate this hypothesis, our ongoing research will focus on the microparticle pattern in ascites of patients with malignant ovarian tumors. We characterized microparticles derived from vascular endothelium (VE) using VE-cadherin (CD 144), which is thought to play an important role in endothelial cell biology by controlling the cohesion and in organization of the intercellular junctions (55). Endothelium injury raises the level of circulating CD144-bearing EMPs as shown in patients with atherosclerosis, especially those with plaque instability (56, 57). In our study, EMP levels did not differ between patients with and those without ovarian cancer. This result is in accordance with the finding that preoperative plasma levels of the VE-activation marker E-selectin also do not differ between patients with benign and those with malignant ovarian masses (58). Summing up, patients with ovarian cancer feature higher preoperative plasma levels of D-dimer and microparticles from activated platelets than do those with benign ovarian tumors. Both markers may reflect the hypercoagulable state in patients with ovarian malignancies and is probably associated with the known increased risk for thrombotic events. On the other hand, measuring preoperative levels of microparticles does not discriminate between benign and malignant ovarian tumors, and therefore is not helpful for diagnosis.

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