Cell-derived microparticles: composition and function
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CHAPTER 13

Summary
Cell-derived microparticles are small vesicles of 0.1 – 1 μm, released from various cell types upon activation or apoptosis by a budding process of the surface membrane. In vivo, the numbers, cellular origin, composition and function of the microparticles vary depending on the body fluid analyzed, as well as the pathophysiological state of the individual. In the past decades, an exponentially increasing number of studies have aimed at analyzing the mechanisms behind the formation of microparticles, as well as their composition and function in various disease states. However, many issues have as yet remained unresolved. It has not been previously investigated, for example, whether lipids are sorted selectively into the microparticles, and whether different stimuli result in different lipid compositions of the microparticles released from the same cell type. Also, regarding the function of microparticles, those released in vivo in the human circulation have not previously been tested regarding their thrombogenic properties in in vivo models. Concerning their inflammatory properties, their ability to activate complement has only been established in vitro, and not yet in vivo.

The objective of the studies described in this thesis was therefore to further investigate the unknown aspects of the composition of cell-derived microparticles as well as of their role in coagulation and inflammation (in particular, complement activation) in various clinical conditions.

Section I

In Chapter 2, the methods employed in our laboratory for blood collection and the initial processing and storage of samples for microparticle analyses were described, as well as our methods used for isolation and flow cytometric analysis of microparticles. Blood collection without prolonged use of a tourniquet, and a large diameter needle are used to avoid cell activation or damage. The anticoagulant used is mostly citrate, and after separation of the microparticle-containing plasma from the cells by centrifugation, samples are snap-frozen in liquid nitrogen and stored at −80°C until analysis, when they are thawed on melting ice, to ensure the best possible preservation of microparticle structure and function. For flow cytometric analysis, microparticles are isolated by differential centrifugation, labeled fluorescently with antibodies and/or annexin V, and measured. Appropriate controls are essential as well as careful titration of all the probes used for labeling.

Section II

In the studies presented in this section, the composition and procoagulant properties of microparticles of various origin were investigated.

In Chapter 3, the phospholipid composition and cholesterol content of platelet-derived microparticles obtained with various stimuli was compared with that of isolated platelet membrane fractions (plasma-, granule- and intracellular membranes), and the exposure of activation markers on platelets and platelet-derived microparticles was determined. Regarding their phospholipid content, the membranes of the microparticles differed
significantly from the intracellular membranes of platelets, and were intermediate compared with platelet plasma- and granule membranes, suggesting that they were a composite of the latter two. This indicated that fusion of the platelet plasma- and granule membranes occurs at least partly prior to the release of microparticles. This was also in line with the exposure on microparticles of antigens present exclusively in the granule membranes of resting platelets. The phospholipid composition of the microparticles also showed subtle but significant differences depending on the platelet agonist. These differences correlated with the exposure of P-selectin on the microparticles in the case of the physiological stimuli collagen, thrombin, or a combination of the two, but not in the case of calcium ionophore A23187. This suggests that the calcium ionophore A23187 gives rise to microparticles that are qualitatively different from those obtained with physiological stimuli. Furthermore, microparticles tended to have a higher cholesterol content when compared with all three platelet membrane fractions, suggesting a possible enrichment of lipid rafts in the microparticles, which should be further investigated.

In Chapter 4, the thrombogenicity of cell-derived microparticles isolated from human blood was investigated in an in vivo model, and the role of tissue factor (TF) exposed on the microparticles was determined herein. Microparticles isolated from pericardial blood of patients undergoing cardiac surgery with cardiopulmonary bypass (CPB) were highly thrombogenic in a venous stasis thrombosis model in rats, whereas microparticles isolated from venous blood of healthy individuals were not. Microparticle-exposed TF antigen levels were higher in pericardial compared with healthy individual samples, and the thrombogenicity of pericardial microparticles could be completely abolished with an inhibitory antibody against human TF, while a control antibody had no effect. This demonstrated that human cell-derived microparticles promote thrombus formation in vivo in a TF-dependent manner, and that the phospholipid surface they provide is by itself not sufficient to trigger thrombus formation in this in vivo model.

In Chapter 5, the phospholipid composition of endothelial cell-derived microparticles released from resting or interleukin (IL)-1α-stimulated cells was analyzed. Furthermore, the thrombogenicity of these microparticles and the role of TF herein was determined in the in vivo thrombosis model also employed in Chapter 4. Microparticles released from IL-1α-stimulated cells contained higher amounts of the aminophospholipids phosphatidylserine and phosphatidylethanolamine, as well as more TF antigen compared with microparticles released from non-stimulated cells. The microparticles released from stimulated cells were strongly thrombogenic in the venous stasis thrombosis model in rats, while those released from non-stimulated cells were not. The thrombogenicity of microparticles released from stimulated cells was again dependent on TF, as it could be abolished by an antibody against TF.

In Chapter 6, the relationship between the activation status of the platelets, assessed by the analysis of the exposure of the activation markers P-selectin and CD63 on their surface, and the numbers and P-selectin and CD63 exposure of the microparticles released
from them upon *in vitro* stimulation was determined. Platelets were stimulated with the physiological agonist thrombin receptor activating peptide (TRAP), or with the calcium ionophore A23187. Both activators caused a dramatic increase in the P-selectin and CD63 exposure of the platelets, compared with non-activated platelets. The numbers of microparticles released did not differ from non-activated platelets upon stimulation with TRAP, but were significantly higher upon stimulation with A23187. However, high percentages of the microparticles released exposed platelet activation markers in the case of both TRAP and A23187, while microparticles released from non-activated platelets did not expose these markers. These experiments showed that the percentages of platelet-derived microparticles exposing P-selectin and CD63 reflect the platelet activation status better than the total numbers of platelet-derived microparticles.

Subsequently, the concentrations of platelet-derived microparticles circulating *in vivo* and the percentages exposing P-selectin or CD63 were determined in young and older healthy individuals, and patients suffering from cardiovascular diseases. The total concentrations of platelet-derived microparticles were comparable in all groups, however, higher percentages of P-selectin but not CD63 exposing platelet-derived microparticles were found in older compared with younger healthy individuals. Furthermore, higher percentages of both P-selectin and CD63 exposing platelet-derived microparticles were found in patients with myocardial infarction and only higher percentages of CD63 but not P-selectin exposing platelet-derived microparticles were found in patients with peripheral arterial disease compared with their controls, which was the older group of healthy individuals.

In Chapter 7, markers of coagulation, platelet and endothelial cell activation, fibrinolysis and the acute phase response were measured, as well as the numbers and cellular origin of microparticles in patients with Fabry disease. In male patients, levels of soluble TF (sTF, non-microparticle-bound TF) and β-thromboglobulin (a platelet-specific activation marker, secreted from platelet α-granules upon activation) were elevated compared to sex- and age-matched controls. In female patients, thrombin-antithrombin complexes, reflecting coagulation activation, as well as β-thromboglobulin, platelet factor-4 (also a platelet-specific activation marker, secreted from platelet α-granules upon activation), CD63 exposing platelet-derived microparticles, and IL-6 were elevated. sTF levels showed a strong correlation with renal function and disease severity in both males and females. Overall, the detected abnormalities were minimal, and could at least partly be attributed to renal impairment. However, it must also be borne in mind that more than half of the patients were on enzyme replacement therapy, and the untreated patients had a milder disease.

**Section III**

In the studies described in this section, the complement activating properties of microparticles were investigated in various clinical conditions.
First, in **Chapter 8**, a brief overview of the complement system was given, and evidence supporting the pathogenic role of the complement system in rheumatoid arthritis (RA) was reviewed. Increased levels of complement activation products had been found in the circulation and synovial fluid of the patients, along with decreased levels of native complement components in synovial fluid. Furthermore, deposition of activated complement components in synovial tissue and cartilage had been demonstrated. As activators of the complement system, immune complexes, C-reactive protein (CRP), and certain immunoglobulin (Ig)G glycoforms had been identified. Furthermore, an association between complement activation and inflammatory responses in the diseased joints or in individual cell types found in RA joints had been shown, and studies on animal models of the disease had also provided evidence that complement activation plays a pathogenic role in RA. At the end of this chapter, an overview was presented of the therapeutic agents under development to influence the complement system in RA.

Subsequently, in **Chapter 9**, microparticles from synovial fluid and plasma of RA patients and from plasma of healthy individuals were analyzed for bound complement components and complement activator molecules. Microparticles with bound complement components C1q, C4 and/or C3 were abundant in synovial fluid of RA patients, whereas in plasma of the patients and healthy individuals their levels were low. The same was true for the fluid phase complement activation products C4b/c and C3b/c. The presence of C1q, and especially of C4 and C3 (which bind covalently to the surfaces on which they are activated) bound to the microparticle surface suggested that classical pathway activation had occurred on that surface, and these results support the role of microparticles in the low grade complement activation in plasma of patients and healthy individuals, and in the increased complement activation in synovial fluid of RA patients *in vivo*. Regarding activator molecules of the classical pathway such as CRP, serum amyloid P component (SAP), IgM and IgG, an association was found in synovial fluid of RA patients between IgM and IgG bound to the microparticle surface and activation of the classical pathway on that surface, whereas in plasma of the patients and healthy individuals such an association was present between bound CRP and classical pathway activation.

In **Chapter 10**, similar analyses of microparticles isolated from pericardial and systemic blood of patients undergoing cardiac surgery with CPB were conducted. Pericardial blood contained high levels of microparticles with bound complement components, whereas systemic blood at the beginning of surgery contained low levels of such microparticles, in line with fluid phase complement activation products C4b/c and C3b/c. In pericardial blood microparticle-bound SAP and IgM, while in systemic blood at the beginning of surgery CRP was associated with complement activation on the microparticle surface.

Subsequently, it was investigated whether retransfusion of pericardial microparticles had any effect on systemic complement activation. Levels of fluid phase complement activation products as well as levels of microparticles binding the various complement
components and complement activator molecules in systemic blood at the end of surgery were compared in retransfused and not retransfused patients, but no differences were found, indicating that retransfusion of pericardial blood does not contribute to systemic complement activation.

In Chapter 11, the role of microparticles in complement activation in patients with myocardial infarction was investigated. No differences were found between patients and healthy individuals regarding the levels of microparticles with bound complement components. Concentrations of the fluid phase complement activation product C3b/c were slightly elevated in the patients. In accordance with previous studies, in healthy individuals microparticle-bound CRP was associated with complement activation on the microparticle surface. However, in the patients with myocardial infarction, in spite of elevated levels of fluid phase CRP as well as elevated levels of microparticles with bound CRP, not microparticle-bound CRP but IgG was associated with complement activation on the microparticle surface.

Finally, in Chapter 12, the role of microparticles in complement activation in preeclamptic and healthy pregnant women was analyzed. In preeclamptic compared with healthy pregnant women, no differences were found regarding the concentrations of microparticles with bound complement components. The same was true for fluid phase complement activation products. In healthy nonpregnant and pregnant women, CRP was associated with complement activation on the microparticle surface, and in healthy pregnant women IgM and IgG also contributed. In preeclamptic women, again in spite of elevated levels of fluid phase CRP as well as elevated levels of microparticles with bound CRP, microparticle-bound SAP and IgG but not CRP seemed to contribute to C1q binding, yet without a clear association to further complement activation on the microparticle surface. This (and the absence of CRP-induced complement activation on the microparticles in myocardial infarction patients) might be a result of the activity of complement inhibitors, and requires further studies.

In conclusion, the studies presented in this thesis further elucidate the composition of cell-derived microparticles, especially regarding their lipid content upon release in response to various stimuli from platelets and endothelial cells, and regarding the usefulness of the exposure of activation markers on platelet-derived microparticles in determining platelet activation status. Furthermore, these studies provide proof of the TF-dependent thrombogenicity of microparticles in vivo, and suggest that microparticles can contribute to complement activation not only in vitro but also in vivo, by a mechanism that differs in various clinical conditions.