Shed Membrane Particles From T Lymphocytes Impair Endothelial Function and Regulate Endothelial Protein Expression

Sophie Martin, PhD*; Angela Tesse, PhD*; Bénédicte Hugel, PhD; M. Carmen Martínez, PhD; Olivier Morel, MD; Jean-Marie Freyssinet, PhD; Ramaroson Andriantsitohaina, PhD

Background—Microparticles (MPs) are membrane vesicles with procoagulant and proinflammatory properties released during cell activation. The present study was designed to dissect the effects evoked by T lymphocyte–derived MPs on vascular function.

Methods and Results—MPs were produced by treatment of the human lymphoid CEM T cell line with actinomycin D or phytohemagglutinin. Incubation of mouse aortic rings with 30 nmol/L MPs resulted in a time-dependent impairment of acetylcholine-induced relaxation of precontracted vessels, with a maximal reduction after 24 hours. MPs also impaired shear stress–induced dilatation of mouse small mesenteric arteries by affecting the nitric oxide (NO) and prostacyclin but not the endothelium-derived hyperpolarizing factor components of the response. However, neither alteration of calcium signaling in response to agonists nor reduction of cyclooxygenase-1 expression accounted for the impairment of the NO and prostacyclin components of the endothelial response. The effect of MPs was rather because of a decrease in expression of endothelial NO synthase and an overexpression of caveolin-1. Furthermore, lymphocyte-derived MPs from diabetic patients or in vivo circulating MPs from either diabetic or HIV-infected patients reduced endothelial NO synthase expression. Finally, the effects of MPs on endothelial cells were not driven through CD11a/CD18 adhesion molecules or the Fas/FasL pathway.

Conclusions—MPs from T cells induce endothelial dysfunction in both conductance and resistance arteries by alteration of NO and prostacyclin pathways. MPs regulate protein expression for endothelial NO synthase and caveolin-1. These data contribute to a better understanding of the deleterious effects of enhanced circulating MPs observed in disorders with cardiovascular or immune complications. (Circulation. 2004;109:1653-1659.)

Key Words: endothelium • lymphocytes • nitric oxide • prostaglandin • cardiovascular diseases
Surface Markers of MPs From Actinomycin D– or PHA-Treated T CEM Cells and From Both Actinomycin D–Activated Lymphocytes and Plasma From 2 Diabetic Patients

<table>
<thead>
<tr>
<th>Surface Markers</th>
<th>T CEM Cells</th>
<th>Diabetic Patients</th>
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<tr>
<td></td>
<td>Actinomycin D</td>
<td>PHA</td>
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<tr>
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<tr>
<td>GPIb</td>
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<td>CD31</td>
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Western Blotting

After treatments, cells or crushed tissue were homogenized and lysed. Protein (50 μg) was separated on SDS-PAGE. Blots were probed with anti-human endothelial NOS (eNOS), inducible NOS (iNOS) (Transduction Laboratories), caveolin-1 (BD Biosciences), COX-1, COX-2 (Cayman Chemical), Fas/APO-1 (Bender MedSystems), and CD31 (Caltag). Results were expressed as the percentage of protein staining versus basal expression.

Calcium Measurements

After loading with fura 2-AM, cells were washed and placed in a salt solution containing 1.8 mmol/L CaCl₂. Thrombin (10 U/mL) or histamine (10 μmol/L) was applied locally for 1 minute, and digital Ca²⁺ imaging on single cells was viewed on an inverted microscope (Nikon Diaphot). Results were expressed in nanomoles per liter.

Statistical Analysis

Data are represented as mean±SEM. In arterial experiments, n refers to the number of animals studied. Statistical analyses were performed by Student’s t test or ANOVA. Differences were considered at a value of P<0.05.

Results

Shed Membrane MPs Reduce Acetylcholine-Induced Endothelial Vasodilatation

Incubation of aortic rings with MPs induced a time-dependent reduction in acetylcholine-induced relaxation of precontracted vessels. Although no significant effect was observed after 4 hours of incubation, the relaxation at the highest concentration of acetylcholine was reduced by 12 or 24 hours of MP incubation (Figure 1A). Acetylcholine failed to produce relaxation in aorta without endothelium (not shown).

Because the effect of MPs was maximal at 24 hours of incubation, this exposure time was used in all the following experiments unless indicated.

Shed Membrane MPs Attenuate Shear Stress–Induced Dilatation

Under physiological conditions, mesenteric resistance arteries submitted to increasing flow released NO, prostaglandins, and EDHF and showed subsequent progressive dilatation. This effect was strongly attenuated in vessels incubated with MPs (Figure 1B). Even though the inhibitory effects of MPs on flow-induced vasodilatation were observed at high levels of shear stress, the experimental conditions used here allow us to demonstrate that MPs produced endothelial dysfunction in response to flow and also to chemical stimuli, as shown in Figure 1A.

Blockade of NO synthesis by L-NNA significantly reduced the flow-induced dilatation in both control and MP-treated vessels (Figure 1C). L-NNA was less efficient in MP-incubated arteries than in control vessels (Figure 1D). The largest differences were obtained at the application of the highest flow-induced shear stress.

The involvement of vasodilatory products from the COX pathway (ie, prostacyclin) was investigated by incubating vessels with L-NNA plus indomethacin. In such conditions, a further inhibition of flow-induced vasodilatation was observed in control vessels, whereas no reduction of flow-induced dilatation was observed in MP-treated vessels (Figure 1E). Thus, MP treatment completely abolished
the prostacyclin component of shear stress–induced vasodilatation.

The EDHF component of flow-induced dilatation sensitive to KCl depolarization was not significantly different in control and MP-treated arteries, suggesting that MP treatment did not affect the EDHF component.

**Effects of Shed Membrane MPs on Ca^{2+} Responses of HUVECs**

Cytosolic Ca^{2+} is the intracellular step for the production and release of endothelial factors. However, the increase in [Ca^{2+}]_{i} evoked by thrombin or histamine was not modified by incubation of HUVECs with MPs (Figure 2).

**Effect of MPs on the COX Pathway in Mouse Aorta and in HUVECs**

MP treatment did not modify COX-1 protein expression in either mouse aorta or HUVECs (Figure 3). Under the same conditions, no expression of COX-2 protein was observed in HUVECs (not shown).

**Shed Membrane MPs Decrease eNOS Expression in Mouse Aorta and in HUVECs**

Incubation of mouse aortic rings with MPs decreased eNOS protein levels as assessed by immunoblotting (Figure 4A). Although eNOS expression was not affected by short-time incubation (4 hours), an inhibition was observed after 12 or 24 hours of MP incubation in HUVECs (Figure 4B), showing that the MP effect was dependent on the time of exposure. MPs obtained from PHA-treated CEM cells also decreased eNOS expression in HUVECs (Figure 4C). Because neither COX-2 nor iNOS was detected, it is unlikely that the endothelial dysfunction observed here is a result of contamination of MPs by endotoxin.

**Effect of MPs on eNOS Is Not Related to an Alteration of Endothelial Integrity, LFA-1 Interaction, or Fas/FasL Pathway**

MPs did not affect endothelial CD31 expression (Figure 5A), indicating that its effect on eNOS expression was not related to an alteration of endothelial integrity.

To study whether LFA-1 (or CD11a/CD18) mediates the effect of shed MPs on eNOS, MPs were preincubated with neutralizing antibodies. Both anti-CD11a and anti-CD18
failed to inhibit the MP-induced reduction of eNOS expression (Figure 5B), suggesting that the MP effect was not related to LFA-1 interaction.

Incubation of HUVECs with MPs did not modify Fas expression (Figure 5C), suggesting that the interaction between Fas ligand (FasL), expressed by MPs, and Fas receptor, carried by endothelial cells, did not account for the effect of MPs on eNOS expression.

Shed Membrane MPs Induce Caveolin-1 Overexpression
MP treatment of HUVECs leads to a significant increase of caveolin-1 expression (Figure 6A). To verify whether the increase in caveolin-1 expression was not because of the transfer of this protein from MPs to endothelial cells, a blot of MPs was performed. These MPs did not express caveolin-1 (Figure 6B).

In Vivo Circulating MPs From Diabetic and Immune Patients and Lymphocyte-Derived MPs From Diabetic Patients Decrease eNOS Expression in HUVECs
MPs obtained from actinomycin D–treated lymphocytes isolated from diabetic patients and in vivo circulating MPs from either diabetic or HIV-infected patients all reduced the level of eNOS expression (Figure 7). These data suggest that...
circulating MPs, at a concentration at which they can be found in several pathophysiological situations, play a significant role in the development of endothelial dysfunction.

Discussion
The present study shows that T lymphocyte–derived MPs, at concentrations that can be reached in circulating blood in pathological disorders, produce endothelial dysfunction in conductance and small resistance arteries in response to agonist and shear stress, respectively. Of particular interest is that MP treatment reduces NO- and prostacyclin- but not EDHF-mediated dilatation. The MP effect resulted in the decrease in expression of eNOS and overexpression of caveolin-1. No change in transduction pathway linked to Ca²⁺ handling was observed, and MP effects were not mediated through its interaction with LFA-1 or Fas/FasL. Finally, reduced expression of COX-1 does not account for the impairment of the prostacyclin component of endothelial response. Most importantly, we provide evidence that lymphocyte-derived MPs from diabetic patients or in vivo circulating MPs from either diabetic or HIV-infected patients were able to impair eNOS expression.

Studies of the MP effects on cell function have been performed using MPs from platelets. Elevated levels of circulating endothelial MPs have also been detected. However, MPs are released not only from platelets or endothelial cells but also from various cell types including T cells. Thus, in diabetic patients, the amount of MPs from leukocytic origin is 3-fold higher than in controls. Here, we used 30 nmoL/L PS eq MPs because this concentration is frequently observed in several pathological conditions. Determination of MPs retained in the atherosclerotic plaque indicated that they were primarily of monocytic and lymphocytic origin. Even higher concentrations (≥60 nmoL/L PS eq) were measured in patients with myocardial infarction, and the patients who experienced documented recurrent myocardial ischemia were precisely those who had the highest levels. HIV-infected patients showed elevated levels of MPs bearing CD4 antigens. Hence, the use of MPs from T cells appears relevant in exploring their effects on endothelium, inasmuch as contact between lymphocytes and endothelial cells is a prerequisite for the recruitment of immune cells at the sites of inflammation. MPs from freshly isolated leukocytes behave as inflammatory mediators and initiate signal transduction in HUVECs. Here, MPs were obtained from apoptotic or activated T cells. MP characterization from T CEM lymphocytes and the present study indicate that they can disseminate procoagulant activity and also membrane antigens. Here, we show an impairment of endothelium-mediated relaxation in response to chemical and physical stimuli in MP-treated vessels, suggesting that MPs act directly on the endothelial layer. Endothelial dysfunction induced by MPs occurs both in conductance arteries, in which atherosclerotic plaque formation is often observed, and in small arteries, which participate in the regulation of peripheral resistance. The most relevant hypothesis to explain the reduced endothelial relaxation by MP treatment is an impairment of either the generation of or response to endothelial relaxant factors. With regard to NO, L-NNA reduced shear stress–induced vasodilatation in small arteries, but it was less efficient in MP-treated than in control vessels. Thus, MPs reduced flow-induced endothelial NO vasodilatation. The contribution of prostanoids has been assessed in small mesenteric arteries after blockade of NO synthesis to avoid cross talk between the 2 factors. In the presence of L-NNA, indomethacin produced a significant reduction of flow-induced dilatation in control but not in MP-treated vessels. Thus, MP treatment abolished the prostacyclin component of shear stress–induced vasodilatation. The latter effect of MPs is not associated with a change in the constitutive form of COX-1. Furthermore, no modification of COX-1 levels and no expression of the inflammatory isoform COX-2 were observed after exposition of HUVECs with MPs. The present results contrast with previous studies, which report an upregulation of COX-2 in cells treated with platelet MPs. These differential results might be related to the cell origin or the agents used for MP production. Finally, our data point out the implication of
EDHF in response to flow that was preserved by MP treatment.

The mechanisms of decreased endothelial NO vasodilatation might result either from a direct effect on the pathway leading to NO production or from diminution of eNOS activity expression, or both. An alteration of the cascade producing NO is unlikely, because MPs did not modify endothelial Ca\(^{2+}\) responses. Another possible explanation might be related to the interaction of eNOS with caveolin-1, which inhibits NO synthase activity.\(^{20}\) Here, MPs induced overexpression of caveolin-1 in HUVECs. Thus, MPs might enhance the association of eNOS with caveolin-1 and subsequently decrease translocation of the enzyme into the cytosol and its activation by Ca\(^{2+}/\mathrm{calmodulin}\).

Few studies have shown a link between caveolin-1 and eNOS expressions. However, an inverse correlation between the expression level of both proteins has been reported.\(^{21,22}\) These results and those from our study suggest that caveolin-1 could inhibit not only eNOS activity\(^{20}\) but also its expression. MPs might carry intracellular content from T lymphocytes that was able to activate endothelial membrane proteins coupled to intracellular pathways. The mechanisms of such transactivation might result from connections of MPs to the cytoskeletal apparatus or ligand transport involving caveolae.\(^{23,24}\) Thus, MPs might affect endothelial NO vasodilatation by altering eNOS expression. In this respect, mouse vessels and HUVECs treated with MPs displayed a dramatic reduction of eNOS expression. No expression of iNOS was observed, suggesting that MPs directly impair endothelial function, most likely through reduction of eNOS expression. The present study contrasts with the report showing that circulating MPs from patients with acute myocardial infarction cause endothelial dysfunction in rat aorta by affecting endothelial NO transduction pathway but not eNOS expression.\(^{25}\) The discrepancies might be related to differences in their cellular origins and mode of induction. Indeed, MPs from patients with myocardial infarction were rich in endothelial and platelet surface markers.\(^{5,23}\) MPs stemming from either actinomycin D- or PHA-stimulated T lymphocytes reduced eNOS expression in HUVECs even though they bear different surface markers, suggesting that the observed effect is specific to T cell MPs independently of the stimulus at the origin of their generation. Furthermore, lymphocyte-derived MPs from diabetic patients or in vivo circulating MPs from either diabetic or HIV-infected patients impaired eNOS expression. Circulating MPs from diabetic patients are heterogeneous but contain a proportion of T cell–derived MPs. Altogether, these results emphasize the critical role of T cell–derived MPs in promoting endothelial dysfunction in vivo even though MPs of different cell origins may participate in this phenomenon.

A direct interaction of membrane MPs with endothelial cell surface may account for MP-induced endothelial cell activation of intracellular pathways. The interaction between FasL and Fas receptor is unlikely, because Fas engagement elicits endothelial apoptosis,\(^{26}\) and no apoptosis was observed (not shown). In addition, MPs from PHA-treated T cells did not bear Fas or FasL but were able to reduce eNOS expression, as were MPs from actinomycin D–treated cells. Hence, the Fas/FasL signaling does not seem to account for the reduction of eNOS expression.

**Figure 6.** Shed membrane MPs induced caveolin-1 overexpression in HUVECs. Western blot revealing caveolin-1 expression (A) in HUVECs exposed to either control medium (C) or MPs and (B) in MPs from actinomycin D–treated T cells. C+, antibody-positive control. ***P<0.001. n=4.

**Figure 7.** Effect of shed membrane MPs from diabetic or HIV-infected patients on eNOS expression. Western blot revealing eNOS expression in HUVECs exposed to either control medium (C) or MPs from actinomycin D–treated lymphocytes isolated from diabetic patients (LD), from plasma of 1 diabetic patient (PD1), and of HIV-infected individuals (P HIV). ***P<0.001. n=3.
Leukocyte-derived MPs have been found to contain a functional adhesion complex, LFA-1, that enables interaction with adhesion molecules from epithelial cells.27 Here, the MP-induced effect on eNOS was unaffected by neutralizing antibodies, excluding the interaction through LFA-1. Our results and those of others19 show that MPs from lymphocytes affect endothelial function independently of CD11a/CD18 interaction.

In conclusion, the present results demonstrate that MPs from T cells impair endothelial function in response to agonist and to flow in both conductance and resistance arteries. They shed light on the alteration by MPs of the NO and prostacyclin, but not EDHF components of the endothelial vasodilatation. MPs affect endothelial NO vasodilatation by regulating the protein expression for eNOS and caveolin-1. These data contribute to a better comprehension of the deleterious effects of enhanced circulating MPs observed in cardiovascular or immune diseases.

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References

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