Circulating Endothelial Microparticles Are Associated with Vascular Dysfunction in Patients with End-Stage Renal Failure

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Endothelial dysfunction and arterial stiffness are major determinants of cardiovascular risk in patients with end-stage renal failure (ESRF). Microparticles are membrane fragments shed from damaged or activated cells. Because microparticles can affect endothelial cells, this study investigated the relationship between circulating microparticles and arterial dysfunction in patients with ESRF and identified the cellular origin of microparticles associated with these alterations. Flow cytometry analysis of platelet-free plasma from 44 patients with ESRF indicated that circulating levels of Annexin V+ microparticles were increased compared with 32 healthy subjects, as were levels of microparticles derived from endothelial cells (three-fold), platelets (16.5-fold), and erythrocytes (1.6-fold). However, when arterial function was evaluated noninvasively in patients with ESRF, only endothelial microparticle levels correlated highly with loss of flow-mediated dilation (r = −0.543; P = 0.004), increased aortic pulse wave velocity (r = 0.642, P < 0.0001), and increased common carotid artery augmentation index (r = 0.463, P = 0.0017), whereas platelet-derived, erythrocyte-derived, and Annexin V+ microparticle levels did not. In vitro, microparticles from patients with ESRF impaired endothelium-dependent relaxations and cyclic guanosine monophosphate generation, whereas microparticles from healthy subjects did not. Moreover, in vitro endothelial dysfunction correlated with endothelial-derived (r = 0.891; P = 0.003) but not platelet-derived microparticle concentrations. In fact, endothelial microparticles alone decreased endothelial nitric oxide release by 59 ± 7% (P = 0.025). This study suggests that circulating microparticles of endothelial origin are tightly associated with endothelial dysfunction and arterial dysfunction in ESRF.


Patients with end-stage renal failure (ESRF) have a high prevalence of cardiovascular complications (1,2). Vascular disease develops rapidly in uremic patients, involving endothelial dysfunction (3,4), a systemic disorder and a key variable in the pathogenesis of atherosclerosis and its complications (5). Moreover, arterial wall stiffening, which is partly influenced by nitric oxide (NO) and the endothelium (6,7), occurs frequently during ESRF and has been reported as an independent predictor of cardiovascular events (7–9).

Circulating microparticles (MP) are shed membrane vesicles resulting from apoptosis or activation of several cell types in response to various stimuli (10,11). We previously demonstrated that circulating MP that are isolated from patients with acute coronary syndromes directly induce endothelial dysfunction in vitro and hypothesized that this effect could be of clinical relevance (12). In this study, we sought to extend those earlier observations by investigating the possible relationships between circulating MP levels and in vitro arterial properties in patients with ESRF. In addition, we undertook to determine the cellular origin of the circulating MP associated with these vascular alterations.

Materials and Methods

We included 44 patients with ESRF from the Fleury-Mérogis hemodialysis center. Patients were eligible for inclusion when (1) they had had no clinical cardiovascular complication during the 6-mo period preceding entry and (2) they agreed to participate in the follow-up study, which was approved by our Institutional Review Board and adhered to the Declaration of Helsinki. Patients were dialyzed three times per week using high-permeability membranes AN69 and polysulfone. The duration of hemodialysis (HD) was individually tailored (4 to 6 h per session) to control body fluids and blood chemistries and to achieve a Kt/V >1.2 (1.46 ± 0.13). The dialysate was prepared with double osmosis ultrapure water and delivered by a system that included bicarbonate delivery, adjustable sodium concentration, and controlled ultrafiltration. Patients were regularly prescribed iron and erythropoietin. Plasma hemoglobin, high-sensitive-C-reactive protein, controlled ultrafiltration. Plasma hemoglobin, high-sensitive-C-reactive protein, LDL cholesterol, triglycerides, and albumin were measured before MP determination. The control population (n = 32; 53 ± 4 yr of age; 41% men) was recruited among healthy subjects in La Pitié-Salpêtrière Hospital, Paris.
MP Isolation

Circulating MP were isolated as described earlier (12) from 10 ml of venous citrated blood drawn from the fistula-free arm, 72 h after the last dialysis. Briefly, 4 ml of platelet-free plasma (PFP) was separated from whole blood (12) and further subjected to centrifugation at 20,500 \( \times g \) (45 min) to pellet MP for in vitro studies. MP pellets then were washed with DMEM (supplemented with 10 \( \mu \)g/ml polymyxin B, 100 UI of streptomycin, and 100 U/ml penicillin) and centrifuged again (20,500 \( \times g \) for 45 min). The majority of supernatant was extracted, and pellets were resuspended into the remaining 200 \( \mu \)l of supernatant. For each included patient, PFP, MP pellet, and supernatant were frozen and stored at \(-80^\circ C\) until subsequent use. Samples were frozen and thawed only once.

Cytofluorometry Analysis

Analyses were performed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Marseilles, France) by two independent examiners who were unaware of the subject status. For each patient, PFP, MP, pellet, and supernatant were diluted five-, 10-, and five-fold in PBS, respectively. One hundred microliters of these solutions was incubated with different fluorochrome-labeled antibodies or their respective isotypic immunoglobulins (Beckman Coulter). We used anti–CD31-phycoerythrin (PE; 20 \( \mu \)l/test), anti–CD41-PC5 (10 \( \mu \)l/test), anti–CD144-PE (20 \( \mu \)l/test), anti–CD235a-FITC (20 \( \mu \)l/test), anti–CD3-FITC (20 \( \mu \)l/test), anti–CD11b-PC5 (20 \( \mu \)l/test), anti–CD45-FITC (20 \( \mu \)l/test), and anti–CD66b-FITC (20 \( \mu \)l/test) antibodies obtained from Beckman Coulter. MP that expressed phosphatidylserine were labeled using fluorescein-conjugated Annexin V solution (20 \( \mu \)l/test; Roche Diagnostics, Mannheim, Germany) in the presence of CaCl2 (5 mM) according to the recommendation of the supplier. Diluted solutions and fluorescence antibodies were incubated at room temperature for 30 min with regular shaking. An equal volume of Flowcount calibrator beads (Beckman Coulter) then was added, and samples were analyzed.

Events with a 0.1- to 1-\( \mu \)m diameter were identified in forward scatter and side scatter intensity dot representation, gated as MP, and then plotted on one- or two-color fluorescence histograms. MP were defined as elements that had a size <1 \( \mu \)m and >0.1 \( \mu \)m and were

![Figure 1. Representative graphs of cytofluorometry analysis of circulating microparticles (MP) in platelet-free plasma from patients with end-stage renal failure (ESRF). (A) Circulating MP and calibrator beads (CAL; 10-\( \mu \)m diameter) are represented on a forward scatter/side scatter dot plot histogram. MP are defined as events, with a size of 0.1 to 1 \( \mu \)m, and are gated in the G window. (B and C) Size-selected events are plotted as a function of their fluorescence for specific Annexin V–FITC binding (FL1) on a one-color (B) or on FL1/forward scatter (C) histograms. Positive labeled events are included in gate H and considered as Annexin V+ MP.](image-url)
Figure 2. Circulating MP levels in patients with ESRF. Circulating levels of Annexin V+ binding MP (ANN V+ MP), platelet-derived MP (CD31+/CD41+ MP), red blood cell–derived (CD235a+ MP), endothelium-derived (CD31+/CD41− MP and CD144+ MP) were significantly increased in ESRF compared with control subjects. Results for endothelium-derived MP (EMP), platelet-derived MP (PMP), Annexin V, and CD235 MP are presented as medians and range (nonnormal distribution), whereas results for CD144 are given as means ± SD (normal distribution). *P < 0.001; +P = 0.01.

positively labeled by specific antibodies. Freezing MP did not affect MP forward scatter and side scatter distribution (data not shown). MP concentration was assessed by comparison with calibrator Flowcount beads (Beckman Coulter; 10-µm diameter) with a predetermined concentration. Sample analysis was stopped after the count of 20,000 events at medium flow-rate setting.

Several specific MP populations were defined, as previously reported elsewhere (13–15): Erythrocyte-derived MP (CD235a+), endothelium-derived MP (EMP; CD31+/CD41− MP), pan-leukocyte MP (CD45+), lymphocyte-derived MP (CD3+), granulocyte-derived MP (CD66b+), and granulocyte/monocyte-derived MP (CD11b+). Leuko-endothelial MP and platelet-derived MP were plotted on a two-color FL2 versus FL4 graph representing fluorescence for CD31/FL2 and CD41/FL4. Platelet-derived MP (PMP) were defined as MP positively labeled for both CD41 and CD31 antibodies, whereas CD31+/CD41− MP were considered as leuko-endothelial.

Arterial Hemodynamics

Common carotid artery (CCA) intima-media thickness, CCA and brachial artery (BA) diameters, and wall motion were measured with a high-resolution B-mode (7.5-MHz) echotracking system (Wall-Track, Maastricht, The Netherlands) (16,17). Vessel walls are identified automatically, and their displacement is tracked throughout the cardiac cycle. According to phase and amplitude, the radiofrequency signal over six cardiac cycles is digitized and stored in a large bank memory. The accuracy of the system is ±0.30 µm for diastolic diameter (Dd) and ±1.9 µm for stroke diameter change. Aortic pulse wave velocity (PWV) was determined by an automatic computer-assisted system (CompliorSp, Artech Medical, Pantin, France) over 15 cardiac cycles as described previously (17). CCA pulse pressure (PP) and CCA augmentation index were measured noninvasively by applation tonometry with a SphygmoCorPx (Atcor Medical Pty Ltd, Moreton-in-Marsh, UK), and diastolic and integrated mean brachial pressures were used to calibrate CCA pressure as described previously (16). CCA distensibility (CCAdi) was determined from the changes in CCA diameter during the systole (Ds) and diastole (Dd) and simultaneously measured changes in CCA pulse pressure (APP) according to the following formula: CCA distensibility = 2[(Ds − Dd)/Dd]/APP. The CCA incremental elastic modulus (Einc) was calculated as Einc = 3(1 + LCSA/IMCSA) × 1/CCAdi, where LCSA is the luminal cross-sectional area, and IMCSA is the intima-media cross-sectional area.

Flow-mediated dilation was evaluated in the BA in 23 patients. Briefly, the arm that was free of arteriovenous shunts was immobilized in a deflatable splint, an echoprobe was positioned with a stereotactic arm above the elbow, and the hand was introduced into a thermostrolled water bath. After 15 min, measurements were obtained first at 34°C and then at 44°C. BA dilation was expressed as percentage change in diameter from the baseline value. After 20 min of recovery, BA diameter was measured before and after sublingual application of glyceryne trinitrate (150 µg). Vessel walls are identified automatically, and their displacement is tracked throughout the cardiac cycle (16,18). The spontaneous variations in BA baseline diameter are 2.6 ± 0.4%.

In Vitro Effects of MP

Rings (3 to 4 mm in length) from Wistar rat thoracic aortas were incubated (24 h at 37°C) in sterile DMEM in the presence of MP from patients with ESRF (at their circulating concentration) or with an equal volume of MP supernatant (control condition), as described earlier (12). In some experiments, preparations were also exposed to MP from healthy subjects, but at a concentration of EMP that matched circulating levels in patients with ESRF. After 24 h of incubation, rat aortic rings were mounted in organ chambers to study acetylcholine-induced relaxation, endothelium-independent responses to the NO donor DEA-NONOate, and changes in cyclic guanosine monophosphate (cGMP) as described earlier (12). Relaxations are expressed as percentage inhibition of norepinephrine (3 to 5.10−7 M) contraction. cGMP levels were measured in duplicate by enzyme immunoassay (Amersham, Orsay, France) and expressed as fmol/µg protein in each sample. This was done in the presence of isobutyl-methyl-xanthine, under basal conditions or after acetylcholine stimulation (10−5 M; 150 s), in the presence or absence of L-NAME (10−4 M). In some experiments, magnetic panmouse IgG Dynabeads (Dynal, Villepinte, France) that were coated with CD41-, CD235a-, or CD45-purified mAb (Beckman Coulter) were used to selectively and successively remove platelet-, erythrocyte-, and leukocyte-derived MP from the circulating MP samples by 97, 65, and 99%, respectively. The remaining MP suspension then was spun down (20,500 × g for 45 min), and the amount of CD31+/CD41− MP was measured in the pellet by flow cytometry as described above. Pellets then were diluted in rat aortic ring incubation medium to reach the plasma concentration of EMP in patients with ESRF. Under these conditions, the final concentration of purified EMP averaged 1350 ± 340 particles/µl in the incubation medium. Aortic rings were exposed...
to MP for 24 h before acetylcholine stimulation. Care and use of laboratory animals conformed to European Community standards and were approved by our local ethics committee.

Statistical Analyses

Data are expressed as median and range or mean ± SEM according to the normality of distribution. The t test or Mann Whitney test for independent samples were used. ANOVA tests for repeated measures were used for in vitro analysis of MP effects on endothelium-dependent relaxations. Multiple comparisons on GMP levels were performed by one-way ANOVA followed by Bonferroni post hoc test. Statistical analysis was performed with SPSS 10.0 software for Windows (SPSS Software, Chicago, IL). Quantitative variables with nonnormal distribution (all MP, with the exception of CD144) were log-transformed to achieve normal distribution before correlations analysis. Stepwise multivariable regression was used to analyze associations between degree of endothelial dysfunction in vitro, in vivo, PWV, Einc, CCA intima-media thickness, PP, CCA distensibility, and levels of circulating MP (Annexin V+ MP and specific subgroups). Each significant predictor that was identified by this analysis was subsequently tested in a multivariable linear correlation. Differences were considered significant at *P* < 0.05.

Results

Baseline characteristics of patients and healthy subjects are given in Table 1. There was no significant difference between patient and healthy subject groups for age (*P* = 0.064) or gender ratio (*P* = 0.236).

Circulating MP Are Elevated in Patients with ESRF

Figure 1 depicts a representative graph of flow cytometry analysis and Annexin V labeling of circulating MP in PFP from patients with ESRF. The results regarding circulating MP measurements are given in Figure 2. Greater numbers of MP labeled with Annexin V (Annexin V+ MP; *P* < 0.001) were observed in patients with ESRF than in healthy subjects. Circulating levels of MP derived from platelets (CD31+/CD41+ PMP), red blood cells (CD235a+ erythrocyte-derived MP), and endothelial cells (CD144+ EMP) were also augmented in patients with ESRF (*P* < 0.001, *P* < 0.001, and *P* = 0.01, respectively) compared with healthy subjects, as well as MP of leucocyte-endothelial origin (CD31+/CD41−MP; *P* < 0.001). Additional labeling demonstrated the presence of only 3.2% CD45+ 1.4% CD3+, 2.2% CD11b+, and 1.7% CD66b+ MP in the leucocyte-endothelial MP population. Thus, CD31+/CD41−MP were considered to be of endothelial origin in subsequent analyses.

Circulating Endothelial MP Correlate with Impaired Vascular Function In Vivo

Values of the different arterial parameters recorded in the patients with ESRF are given in Table 2. No association was observed between the different indices of arterial stiffness and Annexin V+ MP from patients with ESRF (*n* = 44). However, we found robust and statically significant relationships between endothelial circulating MP (either CD144+ or CD31+/CD41−populations) and aortic pulse wave velocity (Table 2, Figure 3A), carotid augmentation index (Table 2, Figure 3B), carotid incremental elastic modulus, and distensibility (Table 2). Furthermore, multiple regression analysis demonstrated a persistent relationship between CD31+/CD41−EMP and CD144+ EMP and PWV and carotid augmentation index after adjustment for age and mean BP.
two major determinant of aortic stiffness (Tables 3 through 6). The loss of flow-induced dilation also correlated with plasma levels of CD144/H11001 or CD31/H11001/CD41/H11002 EMP (n=23 patients; Figure 4), yet this was not observed for glycerine trinitrate–induced dilation (P=0.101; Table 2). Conversely, no correlations were found between the other specific MP subpopulations and the arterial hemodynamic parameters (Table 2).

Circulating Endothelial MP Induce Endothelial NO Dysfunction

For experiments that investigated the effects of MP from patients with ESRF, the concentrations of CD31/H11001/CD41/H11002 EMP (1882±550 events/µl) and PMP (7787±2876 events/µl) in the incubation medium matched those measured in the plasma samples from the selected patients with ESRF (EMP=1796±401 events/µl, and PMP=7195±3605 events/µl; P=0.90). Exposure to MP from patients with ESRF did not alter norepinephrine maximal response (control 3384±369 mg; MP group 3128±356 mg; P=0.53) or its precontraction level before acetylcholine (control 81±3%; MP group 81±2%; P=0.47).

For experiments dealing with MP from healthy subjects, MP pellets were diluted in the incubation medium to reach a final concentration of CD31/H11001/CD41/H11002 MP not different from EMP circulating concentration in patients with ESRF (1904±589 events/µl).

Exposure of rat aortic rings to circulating levels of MP isolated from patients with ESRF but not from healthy subjects selectively impaired the relaxation to acetylcholine when compared with the supernatant group (P=0.008; Figure 5A) but did not affect the response to the NO donor DEA-NONOate (P=0.84; Figure 5B). Exposure to the NO synthase inhibitor L-NAME fully inhibited acetylcholine response in all preparations (Figure 5A). We also found a highly significant inverse relation between the EMP levels in aortic ring incubation media and the degree of relaxation to acetylcholine (r=0.891, P=0.0001).

Table 3. Correlation matrix section for aortic PWV

<table>
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<tr>
<th></th>
<th>Age</th>
<th>Mean BP</th>
<th>CD31+/CD41− MP</th>
<th>CD144+ MP</th>
<th>Aortic PWV</th>
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<tr>
<td>Age</td>
<td></td>
<td></td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>Mean BP</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>CD31+/CD41− MP</td>
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<td>r = 0.170</td>
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<td>0</td>
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<tr>
<td></td>
<td>P = 0.068</td>
<td>P = 0.271</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD144+ MP</td>
<td>r = 0.435</td>
<td>r = 0.178</td>
<td>r = 0.661</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P = 0.003</td>
<td>P = 0.248</td>
<td>P &lt; 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic PWV</td>
<td>r = 0.482</td>
<td>r = 0.115</td>
<td>r = 0.642</td>
<td>r = 0.682</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>P = 0.001</td>
<td>NS</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Multiple regression report: Aortic PWV as dependent variable

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<th>Independent Variable</th>
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<th>P</th>
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<tr>
<td>Intercept</td>
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<tr>
<td>Age</td>
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<tr>
<td>CD31+/CD41− MP</td>
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<td>0.016</td>
</tr>
<tr>
<td>CD144+ MP</td>
<td>2.426</td>
<td>0.020</td>
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</table>

\[ R^2 = 0.576; F \text{ ratio} = 12.92; P < 0.00001. \]

Figure 3. Correlations between circulating MP levels, aortic pulse wave velocity, and common carotid artery (CCA) augmentation index, which represents the effect of arterial wave reflection on pulse pressure amplitude in central arteries. Aortic pulse wave velocity (PWV; A) and CCA augmentation index (CCA Aix; B) are represented as a function of circulating MP. The increase of PWV and Aix were correlated with the number of either CD31+/CD41− or CD144− EMP, whereas these relationships did not exist with PMP or red blood cell–derived MP.
Basal levels of cGMP were lower in rings that were exposed to MP than in controls ($P = 0.01$). Acetylcholine transiently increased cGMP levels above basal values ($P = 0.017$) in control rat aortas (not exposed to MP but exposed to their supernatant), and this effect was inhibited by L-NAME ($P = 0.005$; Figure 5E).

Discussion

Previous studies (12,19,20), including an original work from our group (12), suggested a potential role of circulating MP in endothelial dysfunction, but no investigation had examined the relationship between circulating MP and in vivo vascular dysfunction thus far. In addition, the specific role of distinct populations of circulating MP was unknown.

We studied patients with ESRD because these patients are characterized by arterial stiffening, endothelial dysfunction, and a reduced NO production (3,4,21). Although traditional risk factors, such as age, hypertension, and dyslipidemia, are associated with alterations in endothelial function (5) and changes in arterial elasticity (7), they do not fully account for those modifications (22) and other explanations are required. In our study, we found that circulating MP levels are augmented in patients with ESRD, as previously reported for individuals with acute coronary syndrome (14), diabetes (23), severe hypertension (24), or preeclampsia (15). More important, we demonstrate here that the level of circulating endothelial MP in patients with ESRD inversely correlates with BA flow–induced dilation and shows robust positive correlation with indices of arterial stiffening. It is interesting that no such observations could be made for circulating platelet MP, erythrocyte-derived MP, or Annexin V+ MP. These results show for the first time a close correlation between a specific population of circulating MP, the endothelium-derived MP, and in vivo signs of vascular dysfunction and thus suggest a possible role for EMP in this disease process.

Because endothelial cells and NO are key regulators of vascular tone, we examined the direct effect of circulating MP from patients with ESRD on endothelial function and NO release in vitro. We observed that the overall pool of circulating MP—at a concentration that matched their plasma levels—specifically impairs endothelium-dependent relaxations to acetylcholine in the rat aorta, in accordance with our previous results with MP.
Circulating MP on endothelial cells could involve MP membrane and/or cytosolic components but obviously would require further investigations. Our results also show that the overall pool of circulating MP from patients with ESRF impair acetylcholine-induced NO release from the rat aorta, as indicated by the changes in cGMP levels. Most important, we demonstrate for the first time that circulating MP of endothelial origin exclusively correlate with in vivo endothelial dysfunction and that purified circulating MP impair acetylcholine-induced cGMP release in vitro, suggesting that circulating MP represent specific inhibitors of the endothelial NO pathway and contribute to alterations in arterial properties.

In patients with ESRF, one could speculate that the initial stimulus that leads to the generation of endothelial MP could result from the effect of endogenous lipopolysaccharide, advanced-glycation end products, oxidized LDL, cytokines, or other factors (26,27). Our results are important for the understanding of the pathophysiology of vascular dysfunction because they suggest that after an initial vascular damage, MP are an important biomarker of vascular injury and dysfunctional endothelial cells and are associated with functional changes of arterial system such as aortic stiffening and pronounced effect of arterial wave reflections. As a whole, this study identifies circulating endothelial MP as a potential new risk factor in the occurrence of cardiovascular events in patients with ESRD and provides evidence for using EMP as a surrogate marker of endothelial dysfunction in cardiovascular diseases.

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