Microparticles From Human Atherosclerotic Plaques Promote Endothelial ICAM-1–Dependent Monocyte Adhesion and Transendothelial Migration

Pierre-Emmanuel Rautou, Aurélie S. Leroyer, Bhama Ramkhelawon, Cécile Devue, Dominique Duflaut, Anne-Clémence Vion, Gilles Nalbone, Yves Castier, Guy Leseche, Stéphanie Lehoux, Alain Tedgui, Chantal M. Boulanger

Rationale and Objective: Membrane-shed submicron microparticles (MPs) released following cell activation or apoptosis accumulate in atherosclerotic plaques, where they stimulate endothelial proliferation and neovessel formation. The aim of the study was to assess whether or not MPs isolated from human atherosclerotic plaques contribute to increased endothelial adhesion molecules expression and monocyte recruitment.

Method and Results: Human umbilical vein and coronary artery endothelial cells were exposed to MPs isolated from endarterectomy specimens (n=62) and characterized by externalized phosphatidylserine. Endothelial exposure to plaque, but not circulating, MPs increased ICAM-1 levels in a concentration-dependent manner (3.4-fold increase) without affecting ICAM-1 mRNA levels. Plaque MPs harbored ICAM-1 and transferred this adhesion molecule to endothelial cell membrane in a phosphatidylserine-dependent manner. MP-borne ICAM-1 was functionally integrated into cell membrane as demonstrated by the increased ERK1/2 phosphorylation following ICAM-1 ligation. Plaque MPs stimulated endothelial monocyte adhesion both in culture and in isolated perfused mouse carotid. This effect was also observed under flow condition and was prevented by anti–LFA-1 and anti–ICAM-1 neutralizing antibodies. MPs isolated from symptomatic plaques were more potent in stimulating monocyte adhesion than MPs from asymptomatic patients. Plaque MPs did not affect the release of interleukin-6, interleukin-8, or MCP-1, nor the expression of VCAM-1 and E-selectin.

Conclusion: These results demonstrate that MPs isolated from human atherosclerotic plaques transfer ICAM-1 to endothelial cells to recruit inflammatory cells and suggest that plaque MPs promote atherosclerotic plaque progression. (Circ Res. 2011;108:335-343.)

Key Words: microparticle ■ ICAM-1 ■ adhesion ■ monocyte ■ microvesicle

Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of leukocytes, lipids, and fibrous tissue in the intima of arteries. In atherosclerotic plaques, endothelial cells express elevated amounts of adhesion molecules such as selectins (P-selectin and E-selectin) and intercellular (ICAM-1) and vascular (VCAM-1) adhesion molecules at their surface. Cytokines and chemokines are also secreted in excess by activated vascular cells in this context. These conditions favor the recruitment and the accumulation of monocytes and lymphocytes in the intima of vessels.

Human atherosclerotic plaques contain large amounts of microparticles (MPs), which are submicron membrane vesicles released following cell activation or apoptosis. MPs harbor at their surface most of the membrane-associated proteins of the cells they stem from and are characterized by the exposure of phosphatidylserine on their outer leaflet. MPs isolated from human atherosclerotic lesions are highly thrombogenic and originate from multiple cells, including macrophages, lymphocytes, erythrocytes, and smooth muscle and endothelial cells. MPs are no longer taken as innocent bystanders because several studies point out that MPs generated in vitro from cultured cells can affect several cellular functions, including inflammatory responses. However, the interpretation of such studies is difficult because lipid and protein fractions of MPs, and thus likely MPs effects, greatly vary depending on the stimulus initiating cell blebbing and MP release.
In view of these data, we tested the hypothesis that MPs isolated from human atherosclerotic plaques can regulate the inflammatory response of endothelial cells, increasing adhesion of monocytes to endothelium and transendothelial migration.

Methods

Isolation and Characterization of MPs From Human Endarterectomy Specimens and Plasma

This study, approved by our Hospital Review Board, included 62 patients undergoing carotid endarterectomy who gave their informed consent before inclusion (Table 1). MPs were isolated from human atherosclerotic plaques according to a previously described procedure, which does not generate MPs from healthy human arteries (see the Online Data Supplement, available at http://circres.ahajournals.org). All experiments were performed with pelleted MPs gently resuspended in fresh DMEM and compared with the effects either of DMEM alone or of the supernatant obtained after pelleting MPs from plaque homogenates and filtered successively on 0.22- and 0.1-μm membranes to completely eliminate potentially residual MPs. Plaque MPs content averaged 183 ± 72 annexin V positive (AnnV+) MPs per μg lesion (n=62). Flow cytometric analysis of MPs is described in the Online Data Supplement. Unless otherwise stated, experiments using plaque MPs were performed at the concentration of 3000 AnnV+ MPs/μL for duration of 24 hours.

Circulating MPs were isolated from platelet-free plasma obtained by successive centrifugations of venous blood drawn from the same patients, as reported earlier. Experiments on ICAM-1 expression were performed with pelleted MPs gently resuspended in fresh DMEM and compared with the effects of DMEM alone or of the supernatant obtained after pelleting MPs from plaque homogenates and filtered successively on 0.22- and 0.1-μm membranes to completely eliminate potentially residual MPs. Plaque MPs content averaged 183 ± 72 annexin V positive (AnnV+) MPs per μg lesion (n=62). Flow cytometric analysis of MPs is described in the Online Data Supplement. Unless otherwise stated, experiments using plaque MPs were performed at the concentration of 3000 AnnV+ MPs/μL for duration of 24 hours.

Assessment of Adhesion Molecule Expression

Expression of adhesion molecules on human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), and COS-7 cells was determined by flow cytometry. Anti-human E-selectin–phycoerythrin (1 μL/test), anti–ICAM-1–phycoerythrin-Cyanin5 (20 μL/test) and anti–VCAM-1–phycoerythrin (4 μL/test) antibodies, were all provided by BD Pharmingen (San Diego, CA). Details appear in the Online Data Supplement.

Non-specific protein synthesis inhibitors (cycloheximide, Sigma, St Louis, MO; chelerythrine chloride [LC Laboratories, Woburn, MA], and Ro 31-8220 [Calbiochem; Nottingham; UK]). Fusion of MPs with PMN, or their vehicle (24 hours), washed, and then exposed to anti-human P-selectin antibody at a concentration of 1 μg/mL (R&D Systems; Minneapolis, Minn), anti-human PSGL-1 antibody at a concentration of 50 μg/mL (R&D Systems; Minneapolis, MN), and their corresponding isotypic control (respectively IgG1, R&D Systems; Minneapolis, MN; and IgM; Sigma, Jerusalem, Israel).

Functional Integration of ICAM-1 in Target Cell Membrane

Confluent COS-7 cells plated in 6-well plates were incubated with MPs, or their vehicle (24 hours), washed, and then exposed to activating mouse anti–ICAM-1 antibody (AbD Serotec, Oxford, UK; 30 minutes) or its corresponding isotypic control. A sheep antimouse antibody (Amersham, GE healthcare) was used for cross-linking (15 minutes;18). COS-7 cells in DMEM supplemented or not with 10% FCS served as positive and negative controls, respectively. Proteins were separated by electrophoresis, transferred onto nitrocellulose membranes (Bio-Rad). Membranes were first exposed to anti–P-ERK (Santa Cruz Biotechnology), and then to secondary antibody. After initial immunodetection, membranes were stripped and reprobed with anti-GAPDH antibody (Chemicon, Millipore, Billerica, MA). Details appear in the Online Data Supplement.

Fluorescent Cell Preparation

Human polymorphonuclear (PMN) and U937 cells (details for isolation and culture are provided in the Online Data Supplement) were labeled with 0.5 μmol/L fluorescent dye (CellTracker Orange CMTCMR; Molecular Probes, Eugene, OR) as described previously. Briefly, the cells were incubated with the fluorescent dye for 30 minutes, centrifuged, washed, and then resuspended in fresh DMEM. After incubation, the samples were washed, then fixed in 4% paraformaldehyde, pH 7.4. The fluorescence intensity was measured by flow cytometry.
minutes, washed twice in RPMI and then resuspended in culture medium.

**Cell Adhesion Assay**

HUVECs plated in 96-well plates were exposed for 24 hours to DMEM, plaque, or plasma MPs or 20500g supernatant. After washout using plaque or plasma MPs or 20500g supernatant. After washout using plaque medium 1640, fluorescent U937 cells (5000/well) or phorbol-12-myristate-13-acetate (PMA)-activated human PMN cells (2000/well) were allowed to adhere to HUVECs for 30 minutes. After washout with a primary mouse antihuman ICAM-1 antibody (10 μg/mL; R&D Systems; Minneapolis, MN) for 1 hour before the U937 cell adhesion protocol, U937 cells were pretreated or not with anti–LFA-1 (lymphocyte function-associated antigen-1) antibody (10 μg/mL; Bender MedSystems, Vienna, Austria) or its corresponding isotypic control (IgG1) (10 μg/mL; R&D Systems; Minneapolis, MN).

**Cell Adhesion Assay Under Flow Condition**

Confluent HUVECs plated in a parallel plate chamber were exposed to plaque MPs or 20500g supernatant. After 24 hours, the parallel plate chamber was connected to a syringe pump and fluorescent U937 cells (555/L) were infused for 30 minutes at a controlled level of laminar low (1 dyn/cm²) or high (10 dyn/cm²) shear stress. During adhesion, the circuit was placed in a sterile 5% CO² incubator set at 37°C. After 2 washouts, adherent monocytic cells were counted under a fluorescence microscope (Zeiss Axiovert 200 inverted microscope).

**Organ Culture**

C57BL/6 mouse left and right carotid arteries were isolated, cannulated at both extremities as described previously. Plaque MPs (3000 AnnV⁺ MPs/μL) were injected within the arterial lumen. After 24 hours, fluorescent U937 cells were injected in the lumen and allowed to interact for 30 minutes (5×10⁶ cells/mL). Adherent cells were counted under a fluorescence microscope. More details appear in the Online Data Supplement.

**Transmigration Assays**

Confluent HUVECs from the upper chamber of a transmigration assay were exposed to plaque MPs or 20500g supernatant (see the Online Data Supplement). After 24 hours, the medium of the upper chamber was removed and replaced by a medium containing 10⁶ PMA-activated human PMN cells. After 24 hours, transmigrated cells in the lower chamber were numbered by flow cytometry analysis.

**Immunofluorescent and Scanning Electron Microscopy Analysis**

Details regarding immunofluorescent and electron microscopy analysis appear in the Online Data Supplement. To test the hypothesis of a transfer of ICAM-1 from MPs to endothelial cells, plaque MPs were labeled with a primary mouse antihuman ICAM-1 antibody (unlabeled), washed twice, and incubated with HUVECs (3000 AnnV⁺ MPs/μL, corresponding to 30 AnnV⁺ MPs/cell) for 24 hours. After washout, cells were incubated either with a fluorescent goat antimouse secondary antibody and analyzed under fluorescence microscope, or with gold-conjugated goat antimouse secondary antibody and analyzed with a scanning electron microscope.

To analyze the mechanism of interaction of MPs with endothelial cells, plaque MPs were incubated with a fluorescent dye, washed twice, and incubated with HUVECs (3000 AnnV⁺ MPs/μL). After 24 hours, HUVECs were incubated with primary mouse antihuman CD31 antibodies and then with donkey antimouse secondary antibodies conjugated to Cyanin5. After two more washouts with PBS, nuclei were stained with DAPI.

**Quantitative RT-PCR, Western Blotting, and ELISA**

Details appear in the Online Data Supplement.

**Statistics**

Data are presented as means±SEM. Wilcoxon and Mann-Whitney tests were used respectively for paired and independent samples. All tests were 2-sided and used a significance level of 0.05. Data handling and analysis were performed with SPSS 12.0 (SPSS Inc, Chicago, IL).

**Results**

Endothelial cell exposure to plaque MPs augmented the levels of ICAM-1 in a time- and concentration-dependent manner (Figure 1). However, unlike TNF-α, plaque MPs did not affect endothelial expression of interleukin-8 mRNA (Online Figure I), endothelial release of interleukin-6, -8, monocyte chemotactrant protein-1 (Table 2) and endothelial expression of VCAM-1 or E-selectin (Online Table I). The effect of plaque MPs on endothelial ICAM-1 levels was already detectable at 30 minutes and was as potent as that induced by TNF-α after 4 hours exposure (P=0.47; Figure 1B). After 24 hours of exposure, plaque MPs increased percentage of ICAM-1⁺ cells by 3.4-fold when compared with the effect of the vehicle (P<0.001; Figure 1B). This increase in ICAM-1⁺ cells tended to be more robust for MPs obtained from symptomatic than asymptomatic patients, but did not reach statistical significance (4.8±0.7 versus 3.9±0.9% of ICAM-1⁺ cells, n=25 and 24, respectively; P=0.19). A similar augmentation in ICAM-1 levels was observed for HAECs following exposure to plaque MPs (3.5±1.3 versus 0.9±0.1% of ICAM-1⁺ HAECs for MP and vehicle treated cells, respectively; n=6; P=0.03). The effect of plaque MPs on endothelial ICAM-1 was not observed with circulating MPs obtained from the same patients, indicating that the augmented level of ICAM-1 is specific of MPs isolated from human atherosclerotic plaques (Online Figure II).

We then sought to evaluate the molecular mechanisms leading to the increase in endothelial ICAM-1 levels following exposure to plaque MPs. Quantitative RT-PCR experiments revealed that, if anything, plaque MPs decreased endothelial ICAM-1 mRNA expression after 6 hours exposure, whereas TNF-α significantly increased endothelial ICAM-1 mRNA expression (Figure 2A). No effect of plaque MPs on ICAM-1 mRNA expression was also observed after 24 hours exposure (data not shown). The effect of plaque MPs on ICAM-1 levels was not affected by actinomycin D or cycloheximide, whereas these agents significantly reduced ICAM-1 expression induced by TNF-α (data not shown). We also investigated pathways known to regulate ICAM-1 synthesis in endothelial cells and observed that inhibitors for PI3-kinase (LY-294002), NFκB (pyrrolidine dithiocarbamate), MAPK (PD 98059), and PKC (staurosporine, chelerythrine, and Ro 31-8220) pathways did not modify endothelial levels of ICAM-1 following plaque MPs exposure (data not shown). Collectively, these data suggest that the effect of plaque MPs on endothelial ICAM-1 does not result from either increased mRNA expression or protein synthesis.

Given the rapid increase in ICAM-1 (already detectable at 30 minutes), and the lack of effect of plaque MPs on ICAM-1...
mRNA expression and protein synthesis, we tested the hypothesis that ICAM-1 transfers from MPs to endothelial cells. ELISA and Western blot analysis demonstrated the presence of substantial amount of ICAM-1 in plaque MPs (Figure 2B), but the absence of significant amounts of VCAM-1 or P-selectin (Online Table II). Unfortunately, ICAM-1 was only weakly detectable on plaque MPs using flow cytometry analysis (0.4±0.2% of AnnV+/H11001 MP; n=22), precluding to the determination of the cellular origin of ICAM-1+/H11001 MPs in the plaque. No significant difference in ICAM-1 level was detected between MPs from symptomatic and asymptomatic patients (327±231 and 245±235 ng/mL respectively; P=0.25; n=40). To test the hypothesis that ICAM-1 transfers to endothelial cells, plaque MPs were labeled with a mouse anti–ICAM-1 antibody, washed twice to remove unbound antibody, and exposed to endothelial cells. Control experiments were performed with MP washing supernatant. Addition 24 hours later of a fluorescent antimouse secondary antibody revealed that incubation with ICAM-1-tagged MPs resulted in an intense ICAM-1 staining on the endothelial surface, whereas supernatant had no effect (Figure 3A). In a second set of experiments, the secondary antibody to ICAM-1 was gold-conjugated and scanning electron microscopy analysis confirmed ICAM-1 presence at the endothelial surface (Figure 3B). Interestingly, the labeling was mostly observed in smooth areas of the endothelial cell surface. In a third set of experiments, MPs were labeled with the fluorescent dye CellTracker Green BODIPY to further assess the interaction between MPs and endothelial membrane. Confocal microscopy analysis demonstrated that CellTracker labeling colocalized with CD31 staining at the endothelial surface (Figure 3C). Furthermore, diffuse but significant CellTracker labeling was observed in the endothelial cytosol, attesting that some plaque MPs had fused with the endothelial membrane and delivered their fluorescent dye inside the cytoplasm (Figure 3C). Because plaque MPs are mostly of leukocyte origin,4 we investigated whether the MP-endothelial interaction depended on P-selectin and PSGL-1.20 PSGL-1 was detected by flow cytometry analysis on plaque MPs (14±12 PSGL-1+/H11001 MP; n=7). However, neutralizing antibodies against PSGL-1 and P-selectin (incubated with MPs and endothelial cells respectively) did not affect endothelial ICAM-1 levels on exposure to MPs (n=4; 24 hours; data not shown). As anionic phospholipids such as phosphatidylserine favor spontaneous fusion with cell membranes,21,22 we then examined the effect

Table 2. Effect of Plaque MPs on Cytokine Released by HUVECs

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>TNF-α (10 ng/mL)</th>
<th>DMEM</th>
<th>100 AnnV+ MPs/μL</th>
<th>1000 AnnV+ MPs/μL</th>
<th>3000 AnnV+ MPs/μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-6 (pg/mL)</td>
<td>1811±670</td>
<td>58±11</td>
<td>63±7</td>
<td>65±12</td>
<td>56±10</td>
</tr>
<tr>
<td>Interleukin-8 (pg/mL)</td>
<td>15 028±2 328</td>
<td>1458±269</td>
<td>1439±237</td>
<td>1478±237</td>
<td>1250±291</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>50 423±10148</td>
<td>17 351±3931</td>
<td>20 557±5859</td>
<td>16 128±4214</td>
<td>15 503±4201</td>
</tr>
</tbody>
</table>

Unlike TNF-α (positive control), plaque MPs did not enhance the release of interleukin-6, -8, or monocyte chemotactic protein-1 (MCP-1) following 24 hours of stimulation of HUVECs (n=8). Data are given as means±SEM.
of annexin V on ICAM-1 transfer to endothelial cells. 

Preincubation of plaque MPs with annexin V (in the presence of 5 mmol/L CaCl₂) decreased by 69±21% endothelial ICAM-1 level resulting from plaque MP exposure (n=6, P=0.03; Figure 3D). To test whether the mechanisms of ICAM-1 transfer were specific for endothelial cells, we exposed COS-7 cells, known not to express ICAM-1, with plaque MPs; this resulted in the presence of ICAM-1 antigenic reactivity at the surface of COS-7 cells (Figure 3E). We then examined whether or not MP-borne ICAM-1 was functionally integrated in the plasma membrane of COS-7 cells following exposure to plaque MPs (24 hours). An activating anti–ICAM-1 antibody increased ERK1/2 phosphorylation, a known downstream event in ICAM-1 mediated signal transduction in COS-7 cells exposed to plaque MPs, but not to supernatant (vehicle) (Figure 3F). Collectively, these data demonstrate that MP-borne ICAM-1 is transferred and functionally integrated in target cell membranes.

We next examined the potential functional consequences of the increased ICAM-1 levels attributable to plaque MPs. Exposure to plaque MPs (24 hours) enhanced in vitro adhesion of fluorescent U937 cells to HUVECs, when compared with DMEM or with MP vehicle-treated HUVECs (Figure 4A and 4B). Conversely, adhesion of fluorescent U937 cells to HUVECs was neither increased by circulating MPs at a concentration of 1000 AnnV⁺ MPs/μL (plasma MPs: 9.1±0.9; plasma supernatant: 8.1±0.4 cells/mm²; P=0.176; 24 hours; n=7) nor at a concentration of 3000 AnnV⁺ MPs/μL (plasma MP: 6.4±1.7; plasma supernatant: 7.2±1.8 cells/mm²; P=0.345; 24 hours; n=5). Plaque MPs also increased the endothelial adhesion of human peripheral PMN cells (2.8±0.6 PMN cells/mm²; n=30), as compared with DMEM (1.8±0.2 PMN cells/mm²; n=19; P=0.007) and with supernatant-treated HUVECs (1.75±0.2 PMN cells/mm²; n=30; P=0.002). The increase in endothelial adhesion of PMN cells was more pronounced for MPs obtained from symptomatic than asymptomatic patients (Figure 4C). The adhesion of U937 cells was decreased by 58±3% using an anti-LFA-1 neutralizing antibody, and by 72±2% when combining anti-LFA-1 with anti–ICAM-1 antibodies (Figure 4D). The effect of plaque MPs on monocyte adhesion to endothelial cells persisted under low shear (plaque MP: 4.1±0.3; supernatant: 3.5±0.3 cells/mm²; P=0.043; 3000 AnnV⁺ MPs/μL; 24 hours; n=5) and high shear stress conditions (plaque MP: 2.7±0.2; supernatant: 2.0±0.1 cells/mm²; P=0.018; 3000 AnnV⁺ MPs/μL; 24 hours; n=7). Monocyte adhesion was also evaluated ex vivo in mouse carotid arteries maintained in organ culture at 80 mm Hg intraluminal pressure and exposed intraluminally to either plaque MPs or vehicle under static conditions for 24 hours. The number of adherent U937 cells was increased by 1.7 fold in vessels exposed plaque MPs, as compared with control conditions (P=0.02; n=7) (Figure 4E). Exposure of U937 cells to anti-LFA-1 and carotid artery to anti–ICAM-1 neutralizing antibodies decreased U937 cells adhesion to carotid artery by 83±11% as compared with the corresponding isotypic IgGs controls (n=4, P=0.039) (Online Figure III). Thus, exposure of endothelial cells to plaque MPs favors monocytic cell adhesion both in vitro and ex vivo, under static and under flow conditions. Then, we sought to evaluate whether or not plaque MPs promote endothelial transmigration of PMN cells. Plaque MPs (24 hours) significantly increased PMN transmigration (n=16; P=0.013) and this effect was more robust for MPs isolated from symptomatic than asymptomatic patients (140±34 versus 107±19% of vehicle effect; n=8 each; P=0.04).

Discussion

The present study demonstrates that ICAM-1 can transfer from human plaque MPs to endothelial cells, leading to increased leukocyte adhesion and transmigration. MPs originating from plaques may thus contribute to the progression and development of human atherosclerotic lesions.

We observe here that exposure to MPs isolated from human atherosclerotic plaques rapidly increases ICAM-1 at the endothelial cell surface, despite the lack of endothelial

Figure 2. Plaque MPs harbor ICAM-1, but do not stimulate endothelial ICAM-1 mRNA expression. A, Quantitative RT-PCR analysis of endothelial ICAM-1 expression (6 hours; n=6). As compared with plaque supernatant, MPs decreased ICAM-1 mRNA expression, whereas TNF-α increased ICAM-1 mRNA expression. *P<0.05; **P<0.01. B, Presence of ICAM-1 in plaque MP pellet as compared with supernatant (ELISA assay; n=14; ***P<0.001). Inset, Representative immunoblot (of 3 experiments) demonstrating the presence of ICAM-1 in plaque MPs.
Figure 3. ICAM-1 transfer to target cells. **A**, Plaque MPs were labeled with an anti–ICAM-1 antibody, washed to remove unbound antibody, and incubated with HUVECs for 24 hours (3000 AnnV MPs/μL). Fluorescent secondary antibody was then applied, revealing an intense staining on the surface of endothelial cells incubated with MPs but not with washing supernatant. In the upper panels, nuclei are stained with DAPI (blue) (original magnification, × 40). These images are representative of 5 experiments. In the lower panels, confocal microscopy analysis confirmed the plasma membrane localization of the ICAM-1 staining (original magnification, × 40; representative of 3 experiments). In these experiments, plaque MPs, but not HUVECs, were incubated with the anti–ICAM-1 antibody. This explains why there is no staining for ICAM-1 on endothelial cell treated with “supernatant.” **B**, Plaque MPs were labeled with an anti–ICAM-1 antibody, washed to remove unbound antibody, and incubated with HUVECs for 24 hours (3000 AnnV MPs/μL). Gold–conjugated secondary antibody was then applied, revealing by electron microscopy an intense staining on the surface of endothelial cells incubated with MPs but not with washing supernatant. **C**, Plaque MPs were labeled with the fluorescent CellTracker Green BODIPY dye (green), washed to remove unbound dye, and incubated with HUVECs (3000 AnnV MPs/μL). After 24 hours, fluorescent MPs colocalized with CD31 staining (red, arrow) but cytoplasm was also green (arrowhead), suggesting that at least a part of MPs had fused with cell plasma membrane. In the top images, nuclei are stained with DAPI (blue) (original magnification, ×40). These images are representative of 9 experiments. In the bottom images, confocal microscopy analysis confirmed the presence of MPs on the plasma membranes, as demonstrated by the colocalization of CD31 (red) and Cell-tracker labeled MPs (green) (original magnification, ×40; resolution 1 μm). These images are representative of 3 experiments. **D**, Preincubation of plaque MPs with annexin V
ICAM-1 mRNA upregulation and that plaque MPs harbor measurable amounts of ICAM-1. As tagging MP ICAM-1 leads to the recovery of tagged ICAM-1 on endothelial cells, we hypothesized that ICAM-1 might be transferred from plaque MPs to endothelial membranes as the molecular link between exposure to MPs and increased monocyte adhesion. Such a transfer of protein from MPs to cells has been previously reported for instance for CXCR4 or CCR5.25–27 Such a transfer of protein from MPs to cells does not involve P-selectin and PSGL-1, but we cannot rule out the possibility that other ligand/receptor complexes could be involved. Furthermore, the increase in ICAM-1 levels observed in COS-7 cells, a cell line known not to express ICAM-1,23 following MP exposure argues against the contribution of specific endothelial docking receptors and strongly favors the membrane fusion hypothesis. Interestingly, the effect of MPs on endothelial ICAM-1 appears to be specific for MPs isolated from human plaque, for an equivalent concentration of MPs isolated from the blood of the symptomatic patients induced more adhesion of U937 cells (5000/well; n=7), whereas asymptomatic patients induced only moderate adhesion (2000 PMN cells/well; n=7). Data are given as means±SEM. *P<0.05.

Figure 4. Adhesion assays. A, Fluorescent U937 monocyctic cells (orange) adhering to HUVECs exposed for 24 hours to either plaque MPs or DMEM in 96-wells plates (original magnification, ×10). Nuclei are stained with DAPI (blue). Representative of 7 experiments. B, Comparison of HUVECs to plaque MPs (3000 AnnV MPs/L, for 24 hours) enhanced adhesion of U937 cells (5000/well; n=7). C, Plaque MPs (3000 AnnV MPs/L) from symptomatic patients induced more adhesion of PMN cells (2000 PMN cells/well) to HUVECs than those from asymptomatic patients (n=30; P=0.03). Data are given as the difference in adherent PMN cell number per well between control conditions (MPs vehicle) and plaque MPs exposed cells. D, Exposure of U937 cells to anti-LFA-1 neutralizing antibodies decreased U937 cell adhesion to HUVECs previously incubated with plaque MPs (3000 AnnV MPs/L; 24 hours; n=4). The combined exposure of U937 cells to anti-LFA-1 and HUVECs to anti-ICAM-1 antibodies further decreased this adhesion (3000 AnnV MPs/L, for 24 hours; n=4). Control experiments were performed with the respective IgG isotypes (IgG). Data are means±SEM. E, Quantification of U937 cell adhesion ex vivo. Mouse arteries were incubated at 80 mm Hg for 24 hours with either plaque MPs or the 20 500g supernatant. Fluorescent U937 cells were then injected into the intraluminal space and left to adhere for 30 minutes; adherent cells were counted after washout. Monocytic cell adhesion was enhanced in vessels exposed to plaque MPs (n=7). Data are given as means±SEM. *P<0.05.
same patients did not affect endothelial ICAM-1. Previous work from our group demonstrated that plaque MPs were of different cellular origins than their circulating counterparts and originated mostly from leukocytes. The cellular origin of plaque MPs harboring ICAM-1 remains unclear, but leukocyte-derived or endothelial MPs present in human plaques could harbor ICAM-1 expressed by cells they stem from. Furthermore, we observed that MP-borne ICAM-1 was functionally integrated in target cell membranes. This was associated with an augmented adhesion of U937 monocytes observed both in vitro and ex vivo and a stimulation of transendothelial migration of PMN cells. Anti-ICAM-1 and anti-LFA-1 neutralizing antibodies inhibited U937 cell accumulation, thus demonstrating the contribution of ICAM-1 to this process. Interestingly, plaque MPs from symptomatic patients induced greater monocytc cell adhesion and transmigration than MPs from asymptomatic patients, suggesting a functional difference between these MPs. Although not statistically significant, the apparent greater abundance of phosphatidylserine and of ICAM-1 (this study) in MPs isolated from symptomatic patients could contribute to an increased fusion and transfer of microparticle ICAM-1 to endothelial membrane, leading to a more robust functional effect than that of MPs from asymptomatic patients. However, the present study does not rule out the possibility that another adhesion mechanism could contribute the greater adhesion of monocyte to endothelial cells exposed to MP from symptomatic patients.

Several leukocyte adhesion molecules, including ICAM-1, VCAM-1 and E-selectin, are expressed on vascular endothelial cells in human atherosclerotic plaques. Furthermore, several studies have demonstrated the major implication of ICAM-1 in atherosclerosis through the regulation of monocyte recruitment into atherosclerosis-prone areas. Indeed, ICAM-1 expression is elevated in atherosclerosis-prone aortas. Treatment of Apoe−/− mice with anti–ICAM-1 antibodies reduced by 70% macrophage short-term homing into atherosclerotic lesions. In addition, the absence of ICAM-1, CD18 (the β-subunit of LFA-1), or both reduced aortic lesion size, suggesting that ICAM-1 together with CD18 participates in the regulation of monocyte homing. Together with the data presented here, these findings support the concept that plaque MPs, which accumulate in atherosclerotic lesions, may favor their development by enhancing the adhesion of monocytes and their transendothelial migration. However, the extent to which plaque MPs contribute to increased endothelial ICAM-1 expression in atherosclerotic lesions remains presently unknown as no general mechanism for MP formation has been demonstrated yet, thus precluding to the design of murine models of atherosclerosis where the formation of plaque MPs could be specifically prevented. Despite this limitation, the concomitant presence of MPs bearing ICAM-1 together with microvascular endothelial cells in advanced lesions let us speculate that plaque MPs could contribute in vivo to the increased ICAM-1 expression observed in atherosclerotic plaque. Indeed, given the existence within the plaque of abnormal microvessels characterized by disorganized branching and immature endothelial tubes with “leaky” imperfect linings, plaque MPs may diffuse within blood stream and thus transfer ICAM-1 to the endothelial cell surface in a “paracrine” manner. It should be highlighted that experiments of the present study were performed with MP concentrations lower than the estimated averaged MP abundance in lesions. Alternatively, given the high concentration in MPs in the plaque (200-fold more concentrated than in the plasma from the same patients), plaque MPs could increase systemic endothelial inflammation at the time of plaque rupture. They could also directly bind to Mac/LFA complexes in the circulation, therefore preventing leukocyte tethering and binding. However, both these potential effects in the circulation would likely be blunted by the high thrombogenic activity of plaque MPs.

In conclusion, our results demonstrate that MPs isolated from human atherosclerotic plaques transfer ICAM-1 onto nonactivated endothelial cells to facilitate monocyte adhesion and transendothelial migration. Therefore, accumulation of MPs in atherosclerotic lesions may contribute to atherosclerotic plaque progression by stimulating the recruitment of inflammatory cells in the lesion.

Acknowledgments

We acknowledge Bruno Esposito for excellent technical assistance, José Vilar for helpful discussions, Dr Brigitte Arbeille for electron microscopic analysis, Stéphanie Riou for advice regarding adhesion assays, and Emmanuelle Hoareau for Western blotting analysis.

Sources of Funding

This work was supported by the Institut National de la Santé et de la Recherche Médicale, an educational grant from Institut de Recherches Servier (Suresnes, France), the Leducq Foundation (LINK project), ANR-06-PHYSIO-038, Plan National de Recherche en Hépato-gastroentérologie 2007 (A07146SP), Société Nationale Française de Gastroentérologie, Association Française d’Études du Foie, and the European Vascular Genomics Network of Excellence (LSHM-CT-2003-503254; Brussels, B). P.E.R. was supported by the “poste d’accueil INSERM,” A.S.L. was supported by the “Ministère de la Recherche et de l’Enseignement Supérieur,” S.L. holds a Canada Research Chair in Cardiovascular Physiology, and C.M.B. was supported by “Contrat d’Interface Assistance Publique Hopitaux de Paris.”

Disclosures

None.

References


25. Rozmyslowicz T, Majka M, Kijowski J, Murphy SL, Conover DO, Poncz M, Ratajczak J, Gaulton GN, Ratajczak MZ. Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. *AIDS.* 2003;17:33–42.


Microparticles From Human Atherosclerotic Plaques Promote Endothelial ICAM-1–Dependent Monocyte Adhesion and Transendothelial Migration

Pierre-Emmanuel Rautou, Aurélie S. Leroyer, Bhama Ramkhelawon, Cécile Devue, Dominique Duflaut, Anne-Clémence Vion, Gilles Nalbone, Yves Castier, Guy Leseche, Stéphanie Lehoux, Alain Tedgui and Chantal M. Boulanger

_Circ Res._ 2011;108:335-343; originally published online December 16, 2010; doi: 10.1161/CIRCRESAHA.110.237420

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/108/3/335

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/12/16/CIRCRESAHA.110.237420.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL FOR:

**Microparticles From Human Atherosclerotic Plaques Promote Endothelial ICAM-1-dependent Monocyte Adhesion and Transendothelial Migration.**

Pierre-Emmanuel RAUTOU, Aurélie S LEROYER, Bhama RAMKHELAWON, Cécile DEVUE, Anne-Clémence VION, Dominique DUFLAUT, Gilles NALBONE, Yves CASTIER, Guy LESECHE, Stéphanie LEHOUX, Alain TEDGUI, Chantal M BOULANGER.

**DETAILED METHODS**

**MPs isolation from human endarterectomy specimens**

MPs were isolated from human atherosclerotic plaques according to a previously described procedure, which has been shown not to generate MPs from healthy human arteries. MPs were characterized by flow cytometry using Annexin V labeling and their presence was confirmed by electron microscopy.

Surgical samples were rapidly rinsed in cold sterile Dulbecco's modified eagle medium (DMEM; Gibco BRL) supplemented with antibiotics (100 IU/mL Streptomycin, 100 IU/mL Penicillin and 10 µg/mL Polymyxin B; Sigma; St Louis, MO) and atherosclerotic lesions were separated from the apparently healthy vessel wall. In order to suspend MPs, plaques were then thoroughly minced using fine scissors in a volume of fresh DMEM, supplemented with antibiotics and filtered on 0.22 µm membrane, corresponding to 10 fold the respective weight of each lesion (100 mg tissue in 1 mL medium). The resulting preparations were centrifuged first at 400 g (15 min) and then at 12500 g (5 min) to remove cells and cell debris. The resulting supernatant (called "plaque homogenate") was subsequently used for flow cytometry experiments and further centrifuged at 20500 g for 150 minutes at 4°C to pellet MPs. All experiments were performed with pelleted MPs gently resuspended in fresh DMEM and compared to the effects either of DMEM alone or of the supernatant obtained after pelleting MPs from plaque homogenates and filtered successively on 0.22 µm and 0.1 µm membranes to completely eliminate MPs. The absence of effect of the procedure on healthy tissue was confirmed using freshly isolated rat aorta (n=4), for which 3±1 AnnV+ MPs/µg tissue were retrieved, as compared with 183 ± 17 AnnV+ MPs/ µg lesion (n = 62) obtained from human atherosclerotic lesions in this study, and in accordance with previous reports.

Plaque MP preparations were assayed for endotoxin contamination using Limulus lysate assay (QCL-1000, Cambrex, Lonza, Walkersville, MD): final endotoxin contamination was in all cases below detection limit, so that 3000 AnnV+ MPs/µL contained less than 5.2 ± 2.0 endotoxin units/mL (mean ± SEM).

**Circulating MP isolation**

Circulating MPs were isolated from platelet-free plasma obtained by successive centrifugations of venous blood drawn from the same patients, as reported earlier. Briefly, citrated venous blood (5 mL) was centrifuged at 400 g for 15 min and then at 12500 g for 5 min at room temperature. In order to obtain, after dilution in endothelial cell medium, concentrations in MPs similar to those present in the plasma of the patients, circulating MPs were concentrated from platelet-free plasma using centrifugation at 20500 g for 150 minutes (+4°C) and resuspended in a volume of plasma corresponding to 1/10 of the initial volume of plasma remaining above the MP pellet.
Flow cytometry analysis of MPs
MPs were analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Villepinte, France) as previously described. A known amount of Flowcount calibrator beads (Beckman Coulter, Fullerton, CA; 20 µL) was added to each sample just before performing flow cytometry analysis. Regions corresponding to MPs were identified in forward light scatter (FSC) and side-angle light scatter (SSC) intensity dot plot representation set at logarithmic gain. MP gate was defined, using calibration beads (Megamix, Biocytex, France and 0.1µm beads, Invitrogen, Eugene, OR), as events with a 0.1-1µm diameter and then plotted on a fluorescence/FSC fluorescence dot plot to determine MP counts positively labeled by specific antibodies. MP concentration was assessed by comparison to Flowcount calibrator beads. As previously described, we incubated 10 µL of plaque homogenate with anti-human P-Selectin glycoprotein ligand-1 (PSGL-1) phycoerythrin (20 µL/test) or anti-ICAM-1-phycoerythrin-Cyanin5 (20 µL/test) antibodies (BD Pharmingen; San Diego; CA) or their corresponding isotype-matched immunoglobulin G (IgG) controls at room temperature for 30 minutes in the dark. Presence of phosphatidylserine at the surface of plaque or plasma MPs was assessed using fluoroisothiocyanate-conjugated Annexin V (Roche Diagnostics, Mannheim, Germany) diluted in appropriate buffer (140 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4) (Roche Diagnostics, Mannheim, Germany) in the presence or in the absence of CaCl$_2$ (5 mmol/L), as a negative control.

Assessment of adhesion molecule expression
Expression of adhesion molecules on endothelial cell was determined by flow cytometry. Human umbilical vein endothelial cells (HUVEC), human coronary artery endothelial cells (HCAEC) and COS-7 cells were plated in 96-well plates and incubated at 37°C in a 5% CO$_2$ incubator. HUVECs (7 different donors) and HCAECs (1 donor) were obtained from Promocell (Heidelberg, Germany) and cultured respectively in endothelial cell basal medium supplemented with serum and growth factors and in endothelial cell growth medium (Heidelberg, Germany). All experiments were performed between passage 2 and 6. COS-7 cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% fetal calf serum (Gibco, Scotland, UK). When cells reached sub-confluence, they were stimulated with either MPs (20500 g pellet resuspended in fresh DMEM for plaque MPs or in filtered plasma supernatant for plasma MPs), their vehicle (20500 g supernatants), DMEM (as a negative control) or tumor necrosis factor-α (TNF-α) (Calbiochem, Nottingham, UK; 10 ng/mL, as a positive control) for 30 minutes, 4, 12, 24 and 48 hours. Thereafter, cell supernatant was replaced by phosphate buffer saline (PBS). Cells were mechanically scraped and incubated with the following fluorochrome-labelled antibodies or their corresponding isotype-matched IgG controls at room temperature for 30 minutes in the dark: anti-human E-Selectin-phycoerythrin (1 µL/test), anti-ICAM-1-phycoerythrin-Cyanin5 (20 µL/test) and anti-VCAM-1-phycoerythrin (4 µL/test) antibodies, all provided by BD Pharmingen (San Diego, CA). HUVECs, HCAEC and COS-7 cells were analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Villepinte, France) and numbered by comparison to Flowcount Calibrator beads of known concentration.

Fluorescent Cell Preparation
Peripheral venous blood (15–20 mL) was obtained from healthy volunteers by venopuncture and collected into heparinized, sterile, pyrogen-free tubes (BD Pharmingen, Grenoble, France). Blood was diluted 1:1 in PBS and monocytes were isolated by density-gradient centrifugation over endotoxin-free Ficoll–Paque (human Pancoll, Pan Biotech GmbH, Aidenbach, Germany). The cell layer containing polymorphonuclear (PMN) cells was collected and washed twice in PBS. The PMN cells were preactivated with phorbol-12-myristate-13-acetate (PMA; 0.1 µM; Sigma; St Louis, MO) for 30 minutes.
Cells of the human monocytic cell line U937 were cultivated in RPMI medium 1640 (Gibco BRL) containing antibiotics (100 IU/mL Streptomycin and 100 IU/mL Penicillin) and supplemented with 10% fetal calf serum.

**Organ Culture**

C57BL/6 mice (17 weeks, Charles River, France) left and right carotid arteries were isolated, cannulated at both extremities, and immersed in an organ culture bath filled with DMEM supplemented with 10% fetal calf serum and antibiotics (100 IU/mL Streptomycin, 100 IU/mL Penicillin and 10 µg/mL Polymyxin B) as described previously. Each arterial segment was connected to a closed perfusion circuit consisting of a 3-port reservoir and a pressure chamber allowing for the application of a controlled intraluminal hydrostatic pressure. Organ culture of carotid segments was performed under sterile conditions in an incubator set at 5% CO₂ and at 37°C. Vessels were exposed to a physiological pressure (80 mm Hg) for 24 hours. To avoid the potentially confounding effect of shear forces, no flow was applied. Plaque MPs (3000 AnnV+ MPs/µL) were injected within the arterial lumen. After 24 hours, fluorescent U937 cells were injected in the lumen of cultured vessels by the distal end, and allowed to interact for 30 minutes (5×10⁶ cells/mL). After washout, vessels were fixed in 4% paraformaldehyde for 15 minutes. Adherent cells were counted under a fluorescence microscope (Zeiss Axio Imager Z1). In some experiments, the lumen of cultured vessels were exposed to neutralizing antibody targeting ICAM-1 or its corresponding isotypic control (IgG₁; 10 µg/mL; R&D Systems; Minneapolis, MN) for 1 hour before the U937 cells adhesion protocol. U937 cells were pretreated or not with anti-lymphocyte function associated antigen-1 (LFA-1) antibody (10 µg/mL; Bender MedSystems, Vienna, Austria) or its corresponding isotypic control (IgG₁) (10 µg/mL; R&D Systems; Minneapolis, MN).

**Transmigration assays**

HUVECs were seeded onto uncoated, low-density (8×10⁵/cm²), 3.0 µm pore cell culture inserts which were placed in matching 24-well plates (BD Biosciences, Franklin Lakes, NJ) and cultured at 37°C in a 5% CO₂ incubator. Upper and lower chambers contained 200 µL and 700 µL supplemented endothelial cell basal medium, respectively. After 7 days, confluent HUVECs from the upper chamber were exposed to plaque MPs or 20500g supernatant. After 24 hours, the medium of the upper chamber was removed and replaced by 200 µL endothelial cell basal medium containing 10⁶ PMA-activated human PMN cells. After 24 hours, transmigrated cells in the lower chamber were labeled with anti-human LFA-1-phycoerythrin (20 µL/test) antibodies or their corresponding isotype-matched IgG controls (BD Pharmingen; San Diego, CA) and numbered by flow cytometry analysis (FACSCanto II flow cytometer; BD Biosciences, San Jose, CA) by comparison to Flowcount Calibrator beads of known concentration.

**Immunofluorescent analysis and scanning electron microscopy**

In order to test the hypothesis of a transfer of ICAM-1 from MPs to endothelial cells, plaque MPs were incubated for 1 hour with a primary mouse anti-human ICAM-1 antibody (10 µg/mL; R&D Systems; Minneapolis, MN). Unbound antibody was removed by washing plaque MPs twice: MPs were resuspended in DMEM and further centrifuged at 20500 g for 150 minutes at 4°C to pellet MPs. MPs or supernatant of the second centrifugation (used as negative control) were incubated with HUVECs (3000 AnnV+ MPs/µL, corresponding to 30 AnnV+ MPs/cell) for 24 hours. In a first set of experiments, after washout with prewarmed PBS, HUVECs were fixed in 4% paraformaldehyde for 15 minutes. After one more washout with PBS and incubation with 5% bovine serum albumin, (Sigma; St Louis, MO) for 30 minutes, the cells were incubated for 1 hour at room temperature with a 1:400 dilution of goat anti-mouse secondary antibody conjugated to Alexa 488 (Invitrogen, Eugene, OR). After two more washouts, nuclei were
then stained with DAPI (blue). Immunofluorescence was detected using a 40× oil-immersion objective on a Zeiss Axio Imager Z1 microscope. Confocal microscopy was also performed (Bio-Rad MRC 600 confocal head with a Nikon microscope).

In another set of experiments, after washout with prewarmed PBS, HUVECs were pre-fixed in 2% paraformaldehyde for 10 minutes. The cells were then incubated for 1 hour at 37°C with a 1:30 dilution of gold-conjugated goat anti-mouse secondary antibody (BBInternational, UK) diluted in PBS with 0.1% bovine serum albumin. The cells were then fixed with 85 mmol/L phosphate buffer pH 7.2 containing 4% paraformaldehyde and 1% glutaraldehyde. The preparations were post-fixed in 0.1% osmium tetroxide, dehydrated in graded ethanol baths, dried to critical point using hexamethyldisilizane, and coated by platine sputtering. They were examined with a Zeiss Gemini (Carl Zeiss, Le Pecq, France) scanning electron microscope.

In order to analyze the mechanism of interaction of MPs with endothelial cells, plaque MPs were incubated for 30 minutes with 20 µM fluorescent dye (CellTracker Green BODIPY; Molecular Probes; Eugene, OR) at 37°C, washed twice by resuspending MPs in DMEM and centrifugating at 20500 g for 15 minutes at 4°C to pellet MPs. The amount of CellTracker+ MPs corresponded to 62 ± 8% of AnnV+ MPs (n = 3). MPs or supernatant of the second centrifugation (used as negative control) were incubated with HUVECs (3000 AnnV+ MPs/µL). CellTracker Green BODIPY passes freely through cell membranes, but once inside the cytoplasm it is transformed into cell-impermeant reaction product 4,5. After 24 hours, HUVECs were washed with prewarmed PBS, fixed in 4% paraformaldehyde for 15 minutes. After one more washout with PBS and exposure to 5% bovine serum albumin for 30 minutes, the cells were incubated for 1 hour at room temperature with primary mouse anti-human CD31 antibodies (1:50 dilution; BD Pharmeding; San Diego, CA). After two washouts with PBS, the cells were incubated for 1 hour at room temperature with a 1:200 dilution of donkey anti-mouse secondary antibodies conjugated to Cyanin5 (Jackson ImmunoResearch Laboratories, West Grove, PA). After two more washouts with PBS, nuclei were stained with DAPI and analyzed under fluorescence (Zeiss Axio Imager Z1 microscope) and confocal (Bio-Rad MRC 600 confocal head with a Nikon microscope) microscopes.

Quantitative RT-PCR

RT-PCR was performed to quantify ICAM-1 and IL-8 mRNA levels using the RNeasy micro protocol (Qiagen) to isolate total RNA from HUVECs exposed to MPs for 6 hours for IL-8 and 6 and 24 hours for ICAM-1. One microgram of RNA was mixed with random primers and reverse transcribed according to the first-strand method (Supershift, Invitrogen). cDNA thus obtained was amplified by PCR under the following conditions: 30 seconds at 94°C, 30 seconds at 57.6°C, 30 seconds at 72°C, for 40 cycle s. PCR primers used were as follows: ICAM-1 sense, 5'-GCC CGA GCT CAA GTG TCT AAA GGA-3'; ICAM-1 antisense, 3'-TA GTA GTG ACA CCA TCG TCG GCG-5'; IL-8 sense 5’ CAAGA GCCAGGAAGAAACCA 3’ IL-8 antisense 5’ GTCCACTCTCAATCCTCTCAC 3’; GAPDH sense, 5’-GAA GAT GGT GAT GGG ATT TC-3'; HPRT sense, 5’ GGGGGCTATAAATTCTTTGCTGACCT 3'; HPRT antisense, 5’ TCGTGCGGATCTTTTTCAGC 3’. The same cDNA samples were used for GAPDH, and ICAM-1 and for IL-8 and HPRT amplification. PCR amplification resulted in 485192 bp fragments originated from ICAM-1 mRNA, 225 bp from IL-8 mRNA, 226 bp from GAPDH mRNA and 303 bp from HPRT mRNA. For quantification, the number of PCR cycles was chosen within the linear exponential phase with respect to the amount of cDNA template and the PCR performed.

ELISA assay

The medium of HUVECs exposed to plaque MPs for 12, 24 and 48 hours were removed and centrifuged at 500g for 15 min in order to eliminate cells debris. Interleukin-6, 8 and
monocyte chemoattractant protein-1 release were quantified with respectively Human interleukin-6, -8 and monocyte chemoattractant protein-1 ELISA kit provided by BD Pharmingen (San Diego, CA) according to the manufacturer’s instructions. ICAM-1, VCAM-1 and P-Selectin exposed on MPs was also quantified by ELISA with Human sICAM-1 and sICAM-1 ELISA kit provided by BD Pharmingen (San Diego, CA) and Human soluble P-Selectin/CD62P Immunoassay (R&D Systems; Minneapolis, MN).

Assessment of the functional integration of ICAM-1
Confluent COS-7 cells plated in 6-well plates were incubated with MPs, or their vehicle (20500 g supernatant). After 24 hours, COS-7 cells were washed once, and then exposed for 30 minutes to activating mouse anti-human ICAM-1 antibody (clone 6.5B5; 5 µg/mL; AbDSerotec, Oxford, UK) or its corresponding isotypic control (IgG1; 5 µg/mL; R&D Systems; Minneapolis, MN). Cells were then washed twice and a sheep anti-mouse secondary antibody (Amersham, GE healthcare) was used for cross-linking at 1:1000 for 15 minutes. As a negative control, confluent COS-7 cells were incubated with DMEM supplemented with 10% fetal calf serum (Gibco, Scotland, UK). As a positive control, confluent COS-7 cells were incubated for 24 hours with DMEM without fetal calf serum, and then were exposed to 10% fetal calf serum for remaining 30 minutes (Gibco, Scotland, UK). Finally, COS-7 cells were washed twice with cold PBS and scraped off in 50 µL of RIPA buffer (150mM NaCl, 50mM TrisHCl pH7.4, 2mM EDTA, 0.5% sodium deoxycholate, 0.2% Sodium dodecyl sulfate, 2mM activated orthovanadate, complete protease inhibitor cocktail tablet and complete phosphatase inhibitor cocktail tablet (Roche, Neuilly-sur-seine, France)). Lysates were sonicated and protein content was quantified using the Lowry (Bio-Rad) protein assay. As detailed below, p-ERK level was then evaluated by western blot analysis.

Western blotting
Detection of ICAM-1 on MPs pellet and 20500g supernatant was also performed by western blotting as previously described. The same quantities of proteins (determined using the Bio-Rad protein determination assay) were submitted to electrophoresis on a sodium dodecyl sulfate–polycrylamide gel (NuPAGE 10% acrylamide; Invitrogen). The anti-ICAM-1 antibody utilized (H-108) was from Santa-Cruz. Detection of p-ERK in COS-7 cells was performed as follows. Lysates were mixed with the reducing sample buffer for electrophoresis and subsequent transferred onto nitrocellulose membranes (Bio-Rad). Equal loading (25µg) was verified using Ponceau red solution. Membranes were incubated with anti-P-ERK (1:1000, Santa Cruz, CA). After secondary antibody incubation (1:3000; Amersham, GE healthcare, UK), immunodetection proceeded using an enhanced chemiluminescence kit (Immun-Star WesternC kit, Bio-Rad, Hercules, CA) and bands were revealed using the Las-4000 imaging system and Image Gauge software (Fuji Film, Tokyo, Japan). After initial immunodetection, membranes were stripped of antibodies and reprobed with anti-GAPDH antibody (1:5000; Chemicon, Millipore, Billerica, MA).
**Supplemental Figure I.** Quantitative RT-PCR analysis of endothelial interleukin-8 mRNA expression (6 hours; n = 6). Contrary to TNF-α, plaque MPs (3000 AnnV+MPs/µL) had no effect on interleukin-8 mRNA expression as compared both to plaque supernatant and to culture medium (DMEM). **p<0.01.**
**Supplemental Figure II.** 24 hours exposure to plaque MPs induced greater ICAM-1 expression on HUVECs than that of circulating MPs from the same patients (n = 6; 1000 AnnV+ MPs/µL, which is in the range of the concentration observed in the plasma of these patients¹). Data are mean ± SEM. * p<0.05.
Supplemental Figure III. Quantification of U937 cell adhesion *ex vivo*. Mouse arteries were incubated at 80 mmHg for 24 hours with plaque MPs (3000 AnnV+ MPs/μL; n = 4). Fluorescent U937 cells were then injected into the intraluminal space and left to adhere for 30 minutes; adherent cells were counted after washout. Exposure of U937 cells to anti-LFA-1 neutralizing antibodies and of carotid artery lumen to anti-ICAM-1 neutralizing antibodies decreased adhesion of U937 cells to carotid artery. Control experiments were performed with the respective IgG isotypes (IgG). Data are mean ± SEM. * p<0.05.
SUPPLEMENTAL TABLES

Supplemental Table I. Exposure of HUVECs to plaque MPs did neither augment endothelial expression of VCAM-1 nor of E-selectin (flow cytometry analysis; n = 4; 24 hours).

<table>
<thead>
<tr>
<th>MP concentration (MPs/µL)</th>
<th>TNF-α (10 ng/mL)</th>
<th>DMEM</th>
<th>100 AnnV+ MPs/µL</th>
<th>1000 AnnV+ MPs/µL</th>
<th>3000 AnnV+ MPs/µL</th>
<th>6000 AnnV+ MPs/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of VCAM-1 + HUVECs</td>
<td>10.2 ± 2.1</td>
<td>0.3 ± 0.3</td>
<td>0.3 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>% of E-selectin + HUVECs</td>
<td>14.2 ± 2.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM.

Supplemental Table II. Presence of VCAM-1 and P-Selectin on plaque MPs determined by ELISA in MP pellet and 20500 g supernatant (n = 14).

<table>
<thead>
<tr>
<th></th>
<th>MP pellet</th>
<th>20500 g supernatant</th>
<th>n =</th>
<th>p =</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1 (ng/mL)</td>
<td>7.47 ± 2.16</td>
<td>5.28 ± 1.5</td>
<td>5</td>
<td>0.225</td>
</tr>
<tr>
<td>P-selectin (ng/mL)</td>
<td>2.33 ± 0.35</td>
<td>1.86 ± 0.22</td>
<td>7</td>
<td>0.149</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM.
SUPPLEMENTAL REFERENCES


