Cellular Origins and Thrombogenic Activity of Microparticles Isolated From Human Atherosclerotic Plaques

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Objectives	In this study, we evaluated the cellular origins and thrombogenic potential of microparticles.
Background	Human atherosclerotic plaques contain submicron vesicles (microparticles) released during cell activation or apoptosis.
Methods	Microparticles were purified from plaques and platelet-free plasma from 26 patients undergoing carotid endarterectomy. Flow cytometry analysis revealed the presence of large amounts of microparticles in plaques but not in healthy vessels.
Results	Most plaque microparticles originated from leukocytes, of which 29 ± 5% were macrophages, 15 ± 3% lymphocytes, and 8 ± 1% granulocytes. Plaques microparticles also derived from erythrocytes (27 ± 4%), smooth muscle (13 ± 4%) and endothelial cells (8 ± 2%), but not from platelets. Plaques from asymptomatic and symptomatic patients showed no differences in microparticle origins. Microparticles were at least 200-fold more concentrated in plaque than in plasma. Plasma microparticles were primarily platelet-derived in contrast with those of plaque and showed no smooth muscle cell origin. Both plaque and plasma microparticles exposed tissue factor and generated thrombin, but this activity was twice as high in microparticles isolated from plaques, reflecting the thrombogenic contribution of the individual classes of microparticles.
Conclusions	These results demonstrate that microparticles are more abundant and more thrombogenic in human atherosclerotic plaques than in plasma. The different cellular origins of plaque and plasma microparticles might explain the increased thrombogenic activity of plaque microparticles. (J Am Coll Cardiol 2007;49:772–7) © 2007 by the American College of Cardiology Foundation

Atherosclerosis is a chronic inflammatory disease of the vessel wall resulting from the interaction between modified lipoproteins, monocytes-macrophages, lymphocytes, and vascular cells (1). The development and progression of atherosclerotic plaques are associated with apoptotic cell death (2). However, the local abundance of oxidized lipids and their antibodies affects apoptotic cell recognition by macrophages, leading to the saturation of phagocytes with indigestible material (1,3) and local accumulation of cell remnants. Indeed, membrane vesicles (microparticles [MPs]) shed by activated and apoptotic cells have been identified within the human atherosclerotic plaque (4).

Microparticles, mostly of platelet origin, also circulate in the blood, and their levels increase in patients with high atherothrombotic risk (5). We previously reported that plaque MPs bear tissue factor (TF) activity and expose phosphatidylserine, a major determinant of their procoagulant activity and clearance (6,7). We also observed that plaque MPs express lymphocyte and monocyte-macrophage markers but no platelet markers (7). However, no information is available regarding the presence of MPs originating from other cell types and their relative importance. In this study, we determined the cellular origins of MPs in both atherosclerotic plaque and blood from patients undergoing endarterectomy and investigate their respective procoagulant activity.

Materials and Methods

Patients. This study, approved by the hospital ethical committee, included 26 patients undergoing carotid endarterectomy who gave their informed consent (Table 1).

Microparticle isolation. Surgical samples were rinsed in sterile phosphate-buffered saline and human plaques (758 ± 100 mg) were isolated, minced in 0.2 μm filtered Dulbecco's modified Eagles medium (volume corresponding to the lesion weight), and centrifuged at 400 g (15 min) and 12,500 g (5 min). The 12,500 g supernatant (plaque homogenate) was analyzed by flow cytometry, and MPs were pelleted at 20,500 g (150 min). A similar protocol was used with the vessel wall adjacent to the lesion (n = 4) and with human internal mammary arteries (n = 3). Platelet-free plasma (PFP) was obtained from internal jugular vein and carotid artery blood (8). Platelet-free plasma, homogenates, and MP pellets were stored at −80°C.

Specific cell-derived MPs were obtained after induction of apoptosis in human umbilical vein endothelial, human vascular smooth muscle cells (SMCs) (Technoclone, Vienna, Austria) and the monocytic line U937 cells, using tumor necrosis factor-α (200 U/ml) and actinomycin-D (200 ng/ml). Microparticles also were prepared from sedimented erythrocytes stimulated with A23187 (5 mmol/l; 30 min). Platelet MPs were generated from a platelet pellet obtained from platelet-rich plasma, and stimulated with A23198 (5 μmol/l; 30 min).

Electron microscopy. Microparticles were fixed with glutaraldehyde and osmium teroxide, dehydrated, and embedded in Epon-Araldite. Sections contrasted with uranyl acetate and lead citrate were examined with a Philips CM50 microscope (Philips, Eindhoven, the Netherlands).

Cellular origin of plaque and plasma microparticles. Analyses were performed using selective fluorochrome-labeled antibodies or their corresponding isotype-matched immunoglobulin G (IgG). Anti-CD4-phycoerythrin was provided by BD-Biosciences Pharmingen (San Jose, California); anti-CD31-phycoerythrin, anti-CD41-phycoerythrin-Cyanin5, anti-CD62P-fuoroisothiocyanate, anti-CD66b-fluoroisothiocyanate, anti-CD144-phycoerythrin, and anti-CD235a-fluoroisothiocyanate were obtained from Beckman-Coulter (Villepinte, France); and anti-CD14-phycoerythrin was from Caltag Laboratories (Burlingame,

California). Microparticles expressing phosphatidylserine were labeled using fluoroisothiocyanate-conjugated AnnexinV (Roche Diagnostics, Paris, France) with or without $CaCl_2$ (5 mM). Smooth muscle cell actin (SMA) was assayed after paraformaldehyde fixation (2%), saponin permeabilization (0.1%), exposure to rabbit anti-SMA (Labvision Ltd., New Market, Suffolk, England), and AlexaFluor 555 donkey anti-rabbit IgGs (Molecular Probes, Inc., Eugene, Oregon). Tissue factor–positive MPs were identified using anti-human TF antibody (American Diagnostica, Stamford, Connecticut) and Alexa-fluor-488 goat anti-mouse IgG (Molecular Probes).

Microparticles were analyzed on a Beckman-Coulter EPICS XL flow cytometer in the presence of Flowcount calibrator beads. Microparticle gate was defined as events with a 0.1- to 1-μm diameter and examined on a fluorescence/forward light scatter plot.

Thrombin generation assay. Microparticles (10^3 to 10^5) were mixed with fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC (1 mmol/l; Bachem, Bubendorf, Switzerland) in the presence of calcium (Technothrombin Technoclone, Vienna, Austria). Thrombin formation was initiated by normal human MP-free plasma and monitored using a Microplate Fluorescence Reader (Wallac/PerkinElmer, Turku, Finland). The rate of increase in fluorescence intensity (fluorescence units [FU]) was calculated every min (ΔFU/min), and converted to thrombin concentrations (nmol/l) using a reference curve obtained with purified thrombin.

Statistical analysis. Microparticle levels were expressed as median and range and analyzed using nonparametric Mann-Whitney U tests. Thrombin-generating activity (TGA) data were expressed as mean ± SEM, according to the distribution normality, and analyzed by analysis of variance followed by Bonferonni post-test (StatView4.51, SAS Institute, Cary, North Carolina). Differences were significant at $p < 0.05$.
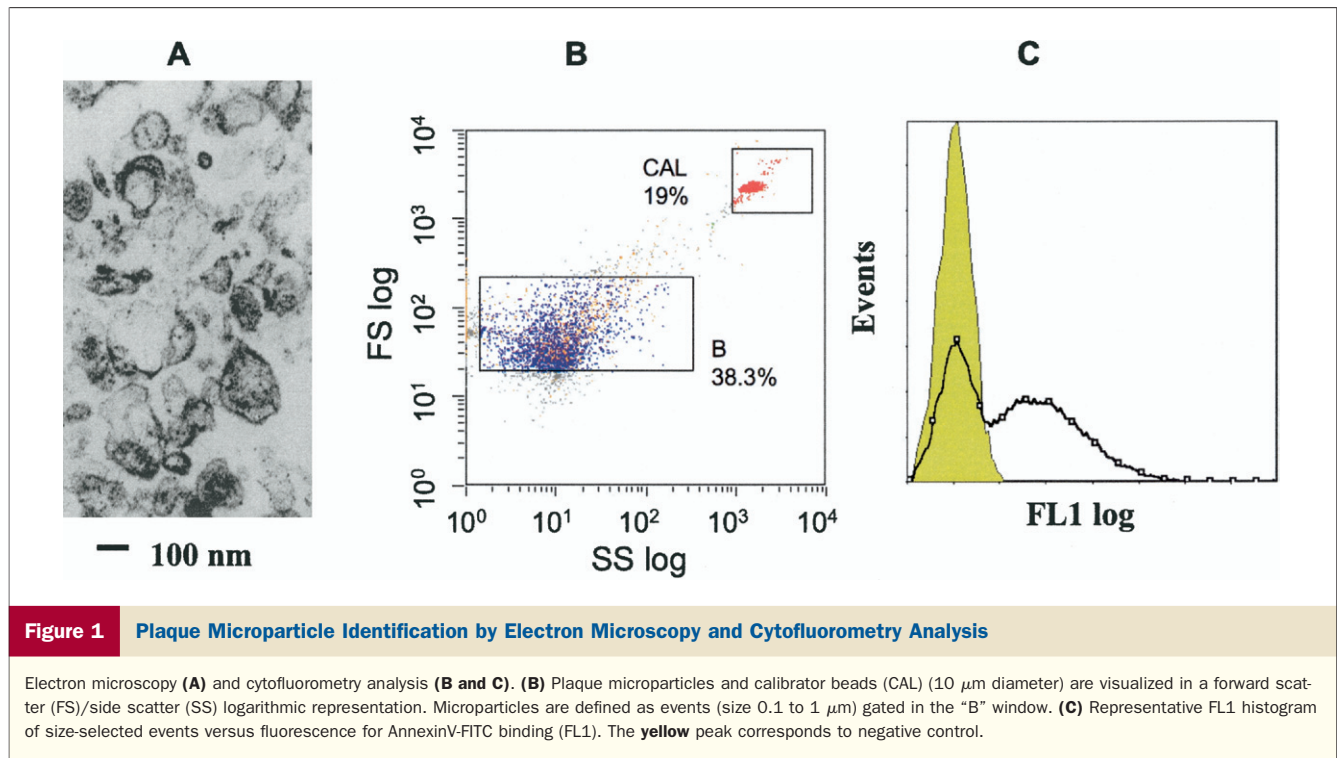
Abbreviations and Acronyms

IgG = immunoglobulin G

MPs = microparticles

PFP = platelet-free plasma

RBC = red blood cell

SMA = smooth muscle cell actin

SMC = smooth muscle cell

TF = tissue factor

TGA = thrombin-generating activity

Table 1	Patients' Baseline Characteristics (n = 26)
Age (yrs)	74 ± 2
Male (%)	81
BMI (kg/m^2)	25.0 ± 0.5
Symptomatic plaques (%) (stroke, transient ischemic attacks)	50
Clinical features (%)	
Diabetes	23
Hypertension	65
Smoking	31
Hypercholestorelomy	58
Treatments (%)	
Antiaggregants	100
Statins	60
Angiotensin-converting enzyme inhibitors	36
Beta-blockers	20

BMI = body mass index.

Results

Cellular origin of human atherosclerotic plaque MPs.
Microparticles were detected in human plaque homogenates by calcium-dependent AnnexinV labeling and electron microscopy analysis (Figs. 1A to 1C). Large amounts of AnnexinV-positive MPs were found in plaques and almost none in human mammary arteries, indicating that microparticles were not generated by the isolation procedure (Mann-Whitney U test, $p < 0.0001$) (Fig. 2).

Figure 1 Plaque Microparticle Identification by Electron Microscopy and Cytofluorometry Analysis

Electron microscopy **(A)** and cytofluorometry analysis **(B and C)**. **(B)** Plaque microparticles and calibrator beads (CAL) (10 μm diameter) are visualized in a forward scatter (FS)/side scatter (SS) logarithmic representation. Microparticles are defined as events (size 0.1 to 1 μm) gated in the "B" window. **(C)** Representative FL1 histogram of size-selected events versus fluorescence for AnnexinV-FITC binding (FL1). The **yellow** peak corresponds to negative control.

Human atherosclerotic plaques contained detectable levels of MPs derived from lymphocytes (CD4+), macrophages (CD14+), granulocytes (CD66b+), endothelial cells (CD144+), erythrocytes (CD235a+), and SMC (SMA+) (Fig. 3). The majority of plaque MPs originated from leukocytes (52 ± 9%), of which 29 ± 5% were from macrophages, 15 ± 3% from lymphocytes, and 8 ± 1% from granulocytes (Fig. 4). No CD31+/CD41+ or CD62P+/CD144-MPs were detected, indicating that platelet-derived MPs were below detectable levels in human plaques. Microparticle levels and cellular origin were unchanged between symptomatic and asymptomatic plaques (Table 2).

Circulating MPs from endarterectomized patients. Comparable levels of AnnexinV-positive MPs were detected in venous and arterial PFPs (1,196 ± 267 per μl vs. 1,881 ± 562 per μl, respectively; p = 0.55), whereas plaques contained 323 ± 63 AnnexinV-positive MP/μg. Because plaques were homogenized in a volume corresponding to the lesion weight, we can assume that 1 mg of plaque corresponds to 1 μl of plasma, indicating that AnnexinV-positive MPs were at least 200-fold more abundant in lesions than in plasma.

Most circulating MPs were derived from platelets and erythrocytes (Fig. 3). Microparticle subtypes were similar

Figure 2 AnnexinV-Positive MPs

AnnexinV-positive macroparticle (MP) levels in plaque compared with the arterial wall adjacent to the lesion (n = 4) and to microparticles isolated from human mammary arteries (n = 3) using the same isolation procedure. *p < 0.0001 (Mann-Whitney *U* test).
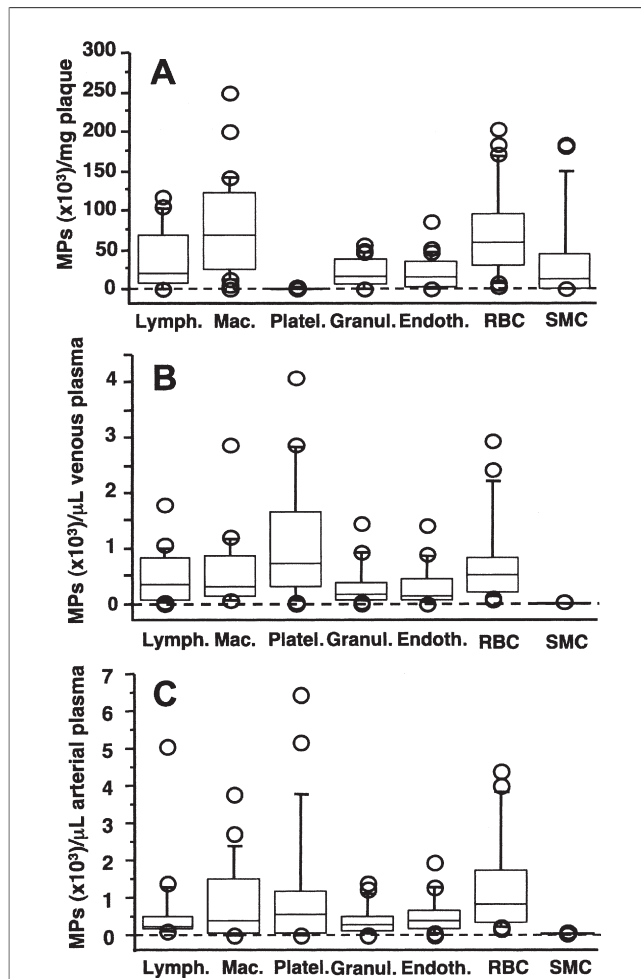
Figure 3   **Cellular Origin of Plaque MPs**

Microparticle (MP) distribution and cellular origins in human plaque **(A)**, venous plasma **(B)**, and arterial plasma **(C)** (n = 26). Data are given as median **(horizontal bar)**, 25th and 75th percentile **(boxes)**, and 90th percentile **(error bar)**. Endoth. = endothelial cell origin; Granul. = granulocyte origin; Lymph. = lymphocyte origin; Mac. = macrophage origin; Platel. = platelet origin; RBC = red blood cell origin; SMC = smooth muscle cell origin.

endothelial-, smooth muscle- and red blood cell-derived MPs but was significantly lower for monocyte and platelet origins (analysis of variance, $p < 0.001$; Bonferonni 10 comparisons) (Fig. 5D).

Discussion

Microparticles present in human atherosclerotic lesions trigger thrombus formation at the time of plaque rupture when they get in contact with plasma coagulation factors (7,9). This study depicts for the first time the cellular origins of MPs present in human atherosclerotic lesions and demonstrates that plaque MPs mainly originate from macrophages, erythrocytes, and SMCs. In this regard, their cellular origins are distinct from their plasma counterpart because the latter are derived mainly from platelets, indicating that circulating MPs are unlikely to result from ruptured plaques. We also demonstrate that MPs are more abundant and more thrombogenic in plaque than in plasma, a feature resulting from their differences in cellular origin.

We describe here a procedure to isolate MPs from human atherosclerotic plaques, where their presence has been documented earlier (4,7). Most of these MPs were derived from activated leukocytes, a hallmark of inflammation, and from erythrocytes, revealing the occurrence of intraplaque hemorrhages. Although erythrocytes are enucleated and lack mitochondria, they can undergo a rapid self-destruction process resembling apoptosis (10). Platelet MPs are absent from atherosclerotic lesions (7), possibly because of selective removal of platelets by phagocytes (11). Atherosclerotic plaques also contained MPs derived from SMCs, in agreement with the
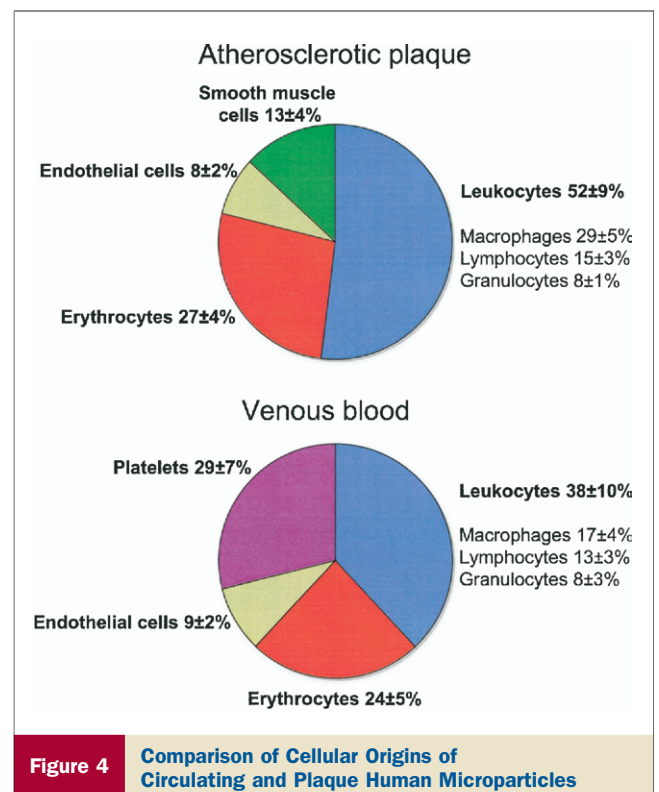
between venous and arterial samples (Fig. 3). However, the plasma MP population was very different from that found in atherosclerotic lesions because plaque contained MPs derived from SMCs but not from platelets, whereas plasma MPs were platelet-derived but not SMC-derived (Fig. 4).

Prothrombogenic activity. Tissue factor expression was comparable between plaque (58 ± 2%) and circulating MPs (68 ± 20%) and between symptomatic and asymptomatic plaques (not shown). Plaque MP TGA was approximately twice that of circulating MPs (Bonferonni $p = 0.006$, 3 comparisons) (Figs. 5A to 5C) but was unchanged between symptomatic (167 ± 63 nmol/l) and asymptomatic patients (234 ± 68 nmol/l), as was arterial and venous microparticle TGA. When TGA was measured using isolated cell-derived MPs, the thrombin peak was highest for

Figure 4   **Comparison of Cellular Origins of Circulating and Plaque Human Microparticles**

Table 2	Comparison in MP Cellular Origins Between Asymptomatic and Symptomatic Plaques		
	Asymptomatic	Symptomatic	p Value
Patients (n)	13	13	
Lymphocyte (CD4+) MPs	14,654 (0–97,584)	56,836 (0–118,324)	0.119
Macrophage (CD14+) MPs	37,678 (0–249,800)	91,249 (13,160–199,996)	0.191
Granulocyte (CD66b+) MPs	16,360 (0–41,890)	21,198 (2,090–58,028)	0.174
Endothelial (CD144+) MPs	9,977 (0–53,271)	27,105 (0–85,050)	0.106
Erythrocyte (CD235a+) MPs	60,510 (3,657–170,035)	57,097 (3,951–201,600)	0.555
SMC (SMA+) MPs	15,374 (0–119,559)	0 (0–184,250)	0.659

Median values and range are expressed as numbers per microgram lesion.

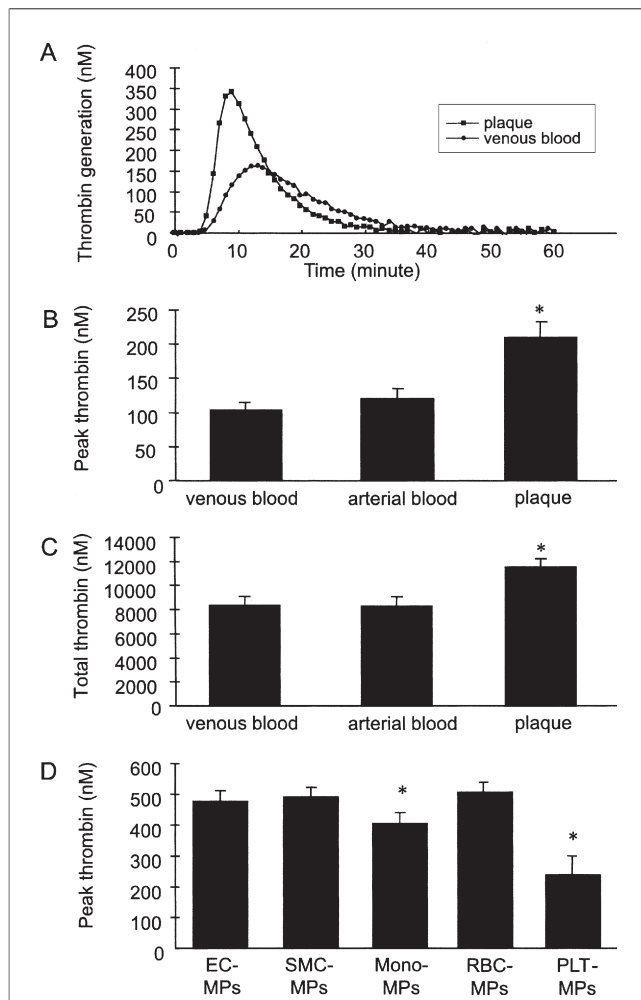MP = microparticle; SMA = smooth muscle cell actin; SMC = smooth muscle cell.

Figure 5   **Plasma and Plaque MP Thrombin-Generating Activity**

(A) Representative trace of thrombin generation by plasma and plaque microparticles (MPs) isolated from the same patient. (B) Peak thrombin generation by venous, arterial, and plaque microparticles (n = 18, 15, and 20, respectively). (C) Total thrombin generated (area under curve) for the same samples as in panel B. (D) Peak thrombin generated by 10^5 cell-derived microparticles. *$p < 0.05$ (analysis of variance followed by Bonferonni post-test; 3 comparisons in panels B and C; 10 in panel D). EC = endothelial cell–derived; PLT = platelet-derived; RBC = red blood cell–derived; SMC = smooth muscle cell–derived.

presence of apoptotic SMCs and SMC-derived remnants in human lesions (4,12). Finally, human plaques also contained endothelial MPs, possibly originating from apoptotic luminal cells (13) or from activated and/or injured endothelial cells of intraplaque microvessels, resulting in microvessel leakiness and intraplaque hemorrhage.

Although there was a trend toward greater numbers of MPs of different cellular origins in symptomatic plaque, this trend did not reach statistical significance. However, we cannot exclude the possibility that MPs might have been released at the time of rupture, leading to an underestimation of MP abundance before this event. Unlike plaque MPs, plasma MPs from patients undergoing endarterectomy mainly were derived from platelets, erythrocytes, and monocytes, which is in agreement with previous reports (5,8). Thus, the present study clearly demonstrates that most circulating MPs do not result from ruptured plaques and that they must be generated within the blood compartment or at the vessel-wall interface.

Plaque MPs were more thrombogenic than their circulating counterparts possibly because plaques contain highly thrombogenic SMC-derived MPs, whereas circulating MPs, which were derived mainly from platelet but not from SMC, have lower thrombin-generating potential. Indeed, purified endothelial, SMC, and erythrocyte MPs displayed peak thrombin generation significantly higher than monocyte and platelet-derived MPs. However, the protrombogenic activity of plaque microparticles may be limited in vivo by the fact that only a fraction of these MPs would participate in thrombosis in the event of plaque rupture. Obviously, this point will require further study.

Conclusions. This first report characterizing the cellular origins of human plaque MPs demonstrates that they derive mainly from macrophages, erythrocytes, and SMCs and that MPs are more concentrated and more thrombogenic in human atherosclerotic plaque than in the corresponding plasma in part because they include highly thrombogenic SMC-derived MPs.

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