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CD40 Ligand+ Microparticles From Human Atherosclerotic Plaques Stimulate Endothelial Proliferation and Angiogenesis

A Potential Mechanism for Intraplaque Neovascularization

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Objectives Our goal was to demonstrate that microparticles (MPs) are the endogenous signal leading to neovessel formation through CD40 ligation in human atherosclerotic plaques.

Background Vulnerable atherosclerotic plaques prone to rupture are characterized by an increased number of vasa vasorum and frequent intraplaque hemorrhage. Although inflammatory cytokines, growth factors, or CD40/CD40 ligand (CD40L) are possible candidates, the mechanism of atherosclerotic plaque neovascularization remains unknown. Atherosclerotic plaques contain large amounts of membrane-shed submicron MPs released after cell activation or apoptosis.

Methods Microparticles were isolated from endarterectomy specimens surgically obtained from 26 patients and characterized by phosphatidylserine exposure and specific markers of cellular origin.

Results Plaque MPs increased both endothelial proliferation assessed by ^{3}H-thymidine incorporation and cell number and stimulated in vivo angiogenesis in Matrigel (BD Biosciences, San Diego, California) assays performed in wild-type and BalbC/Nude mice, whereas circulating MPs had no effect. Microparticles from symptomatic patients expressed more CD40L and were more potent in inducing endothelial proliferation, when compared with asymptomatic plaque MPs. Most of CD40L+ MPs (93%) isolated from human plaques were of macrophage origin. Microparticle-induced endothelial proliferation was impaired by CD40L or CD40-neutralizing antibodies and abolished after endothelial CD40-ribonucleic acid silencing. In addition, the proangiogenic effect of plaque MPs was abolished in Matrigel assays performed in the presence of CD40L-neutralizing antibodies or in CD40-deficient mice.

Conclusions These results demonstrate that MPs isolated from human atherosclerotic lesions express CD40L, stimulate endothelial cell proliferation after CD40 ligation, and promote in vivo angiogenesis. Therefore, MPs could represent a major determinant of intraplaque neovascularization and plaque vulnerability. (J Am Coll Cardiol 2008;52:1302–11) © 2008 by the American College of Cardiology Foundation

Atherosclerosis is an inflammatory disease of the vascular wall, leading to lipid accumulation, macrophage infiltration, and subsequent focal thickening of the intimal layer of arteries. Often silent and slow progressing, it becomes life threatening when the lesion ruptures, leading to local thrombus formation, arterial occlusion, and tissue ischemia. Vulnerable atherosclerotic plaques prone to rupture are characterized by an enlarged necrotic core containing apoptotic macrophages, an increased number of vasa vasorum, and more frequent intraplaque hemorrhage (1). The network of leaky neovessels is the source of in-

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traplaque hemorrhage, providing erythrocyte-derived free cholesterol within the lipid core and excessive macrophage infiltration, therefore promoting the transition from stable to unstable plaque (1). Endothelial proliferation indexes are increased in neovessels from human endarterectomy specimens (2), and intimal neovascularization is likely arising from the adventitia where there are numerous pre-existing vasa vasorum (3,4). Although angiogenesis is regulated by the combined action of various cytokines and growth factors released by infiltrating inflammatory cells, the exact mechanism of intraplaque neovascularization remains unknown (1). Ligation of CD40 is a possible candidate, as CD40 and CD40 ligand (CD40L) are highly expressed in human atherosclerotic plaques, and CD40 ligation results in the expression of several angiogenic factors (5–9). In addition, interruption of CD40 signaling improves plaque stability (10,11). However, the contribution of the CD40-CD40L pathway to plaque neovascularization remains speculative (1).

Advanced human atherosclerotic plaques contain large amounts of microparticles (MPs), which are submicron membrane vesicles released after cell activation or apoptosis (12–14). Microparticles harbor at their surface most of the membrane-associated proteins of the cells they stem from and are characterized by the loss of plasma membrane asymmetry and exposure of phosphatidylserine on their outer leaflet (15,16). Microparticles isolated from human atherosclerotic lesions are highly thrombogenic and originate from multiple cells, including macrophages, lymphocytes, erythrocytes, and smooth muscle and endothelial cells (13,14). Microparticles are no longer considered innocent bystanders since several recent studies point out that in vitro–generated MPs can affect several cellular functions, including inflammatory and angiogenic responses (15,16).

Therefore, we tested the hypothesis that MPs are the endogenous signal leading to neovessel formation through ligation of CD40 in human atherosclerotic plaques. The present results demonstrate that MPs isolated from human atherosclerotic lesions expressed CD40L, which stimulates endothelial cell proliferation and promotes in vivo neovessel formation after CD40 ligation.

Table 1 Patients' Baseline Characteristics (n = 26)

Age (yrs)	73 ± 2
Male subjects	85%
Symptomatic plaques	50%
Stroke	28%
Transient ischemic attack	16%
Amaurosis	6%
Clinical features	
Diabetes	35%
Hypertension	69%
Smoking	54%
Dyslipidemia	65%
Treatments	
Antiaggregants	100%
Statins	88%
Angiotensin-converting enzyme inhibitors	50%
Beta-blockers	35%

Data are expressed as mean ± SEM (age) or frequency in percentage.

Abbreviations and Acronyms

AnnV = Annexin V
CD40L = CD40 ligand
COX = cyclooxygenase
DMEM = Dulbecco's modified eagle medium
ELISA = enzyme-linked immunoadsorbent assay
FCS = fetal calf serum
FITC = fluoroisothiocyanate
HUVEC = human umbilical vein endothelial cell
IL = interleukin
MP = microparticle
PC5 = phycoerythrin-cyanin 5
PE = phycoerythrin
siRNA = small interfering ribonucleic acid
VEGF = vascular endothelial growth factor

Methods

MP isolation from human endarterectomy specimens. This study, which was approved by our hospital review board, included 26 patients undergoing carotid endarterectomy who gave their informed consent before inclusion in the study (Table 1). Microparticles were isolated from human atherosclerotic plaques according to a previously described procedure, which does not generate MPs from healthy human arteries (14).

Briefly, surgical samples were rapidly rinsed in cold sterile phosphate-buffered saline solution supplemented with 100 U/ml streptomycin and 100 U/ml penicillin, and atherosclerotic lesions were separated from the apparently healthy vessel wall. Plaques were then thoroughly minced using fine scissors in a volume of fresh Dulbecco's modified eagle medium (DMEM) (supplemented with 10 μg/ml polymyxin B, 100 U streptomycin, and 100 U/ml penicillin and filtered on a 0.22-μm membrane) corresponding to 10-fold the respective weight of each lesion in order to suspend MPs. The resulting preparations were centrifuged first at 400 *g* (15 min) and then at 12,500 *g* (5 min) to remove cells and cell debris. The resulting supernatants were subsequently used for flow cytometry experiments. Part of this so-called plaque homogenate was further centrifuged at 20,500 *g* for 150 min at 4°C to pellet MPs. Pellets were gently suspended in a volume of fresh DMEM in order to obtain a concentration of MPs near the concentration of the plaque. All experiments on endothelial proliferation were performed with pelleted MPs resuspended in DMEM (14) and compared with the effects of DMEM alone (vehicle). The proliferative effect of plaque MPs was also compared with that of 0.2- and 0.1-μm sterile filtered 20,500 *g* supernatant obtained after pelleting MPs from plaque homogenates.

Circulating MP isolation. Circulating MPs were isolated from platelet-free plasma obtained by successive centrifugations of venous citrated blood drawn from the same patients, as reported earlier (17). Briefly, citrated venous blood (5 ml)

was centrifuged at 500 *g* for 15 min and then at 13,000 *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 20,500 *g* for 150 min (+4°C) and resuspended in a volume of plasma remaining above the MPs pellet corresponding to one-tenth of the initial volume of plasma. Experiments on endothelial proliferation were performed with pelleted circulating MPs and compared with the effect of platelet-free plasma devoid of MPs (obtained after 20,500 *g* centrifugation and 0.2 then 0.1 μm sterile filtration; vehicle).

Flow cytometry analysis. For each patient, analyses were performed on the plaque homogenate (12,500 *g* supernatant) and on platelet-free plasma, as previously described (14,17). We incubated 10 μl of plaque homogenate with different fluorochrome-labeled antibodies or their corresponding isotype-matched immunoglobulin (Ig)G control specimens at room temperature for 30 min in the dark. Antihuman CD154 (CD40L)-phycoerythrin (PE) (20 μl/test), anti–CD4-PE (20 μl/test), anti–CD41-phycoerythrin-cyanin 5 (PC5) (10 μl/test), anti–CD62P-fluoroisothiocyanate (FITC), anti–CD66b-FITC, anti–CD144-PE, and anti–CD235a-FITC were provided by Beckman Coulter (Villepinte, France). Antihuman CD14-PE conjugated and its isotype-matched IgG control were provided by Caltag Laboratories (Burlingame, California). To determine the cellular origin of CD40L+ MPs, in some experiments we used antihuman CD154-FITC or isotype (BD Pharmingen, San Jose, California) for colabeling with PE-conjugated antibodies. The antibody against CD40 was provided by R&D Systems (Lille, France). As anti-CD40 was unconjugated, we used the polyclonal rabbit antimouse IgG-PC5 (Dakocytomation, Glostrup, Denmark) as a secondary antibody. The presence of phosphatidylserine at the surface of plaque MPs was assessed using FITC-conjugated Annexin V (AnnV) (Roche Diagnostics, Paris, France) diluted in appropriate buffer (Roche Diagnostics) in the presence or in the absence of $CaCl_2$ (5 mmol/l final concentration), as a negative control.

Microparticles were analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter) as previously described (14,17). A known amount of Flowcount calibrator beads (20 μl, Beckman Coulter) was added to each sample just before performing flow cytometry analysis. Regions corresponding to MPs were identified in forward light scatter and side-angle light scatter intensity dot plot representation set at logarithmic gain. An MP gate was defined as an event with a 0.1- to 1-μm diameter and then plotted on a fluorescence/forward light scatter fluorescence dot plot to determine positively labeled MPs by specific antibodies. Microparticle concentration was assessed by comparison with Flowcount calibrator beads (Beckman Coulter).

Assessment of cell proliferation. Endothelial cell proliferation was determined by ^{3}H-thymidine incorporation. Human umbilical vein endothelial cells (HUVECs) were plated in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere. The HUVECs (7 different donors) were obtained from Promocell (Heidelberg, Germany) and cultured on endothelial cell basal medium with a supplement pack (Promocell). All experiments were performed between passages 2 and 6. When cells reached 70% of confluence, they were rendered quiescent by replacing fetal calf serum (FCS) 10% by 0.1% bovine serum albumin for 24 h. Then they were stimulated with either MPs (20,500 *g* pellet resuspended in fresh DMEM for plaque MPs or in filtered plasma supernatant for plasma MPs), MPs' vehicle (20,500 *g* supernatant), or FCS (as a positive control) during 48 h. ^{3}H-thymidine incorporation (performed in triplicate for each data point) was determined after addition of 1 μCi of ^{3}H-labeled thymidine per well for an additional 16 h at 37°C. Radioactivity was counted by scintillation spectroscopy (TopCount NXT, PerkinElmer, Downers Grove, Illinois). Neutralizing antibodies used in this assay were provided by R&D Systems (Minneapolis, Minnesota). We assessed antihuman CD40 (clone 82111), antihuman CD40L (clone 40804), antihuman vascular endothelial growth factor (VEGF)-R1 (clone 49560), antihuman VEGF-R2 (clone 89115), and their corresponding isotypic control (IgG_{2B}, clone 20116, IgG1, clone 11711) at the final concentration of 5 μg/ml. Pilot experiments revealed that higher concentrations of the isotypic control significantly impair endothelial proliferation. Inhibitors NS-398 and LY-294006 were used at the concentration of 1 μmol/l; wortmanin was used at the concentrations of 0.1, 1, and 10 μmol/l. Endothelial cell proliferation was also assessed by flow cytometry numbering HUVECs by comparison with Flowcount calibrator beads of known concentration (Beckman Coulter, EPICS XL cytometer).

Enzyme-linked immunoadsorbent assay (ELISA). A medium of HUVECs exposed to plaque MPs for 48 h were removed and centrifuged at 500 *g* for 15 min in order to eliminate cell debris and then at 20,500 *g* for 45 min in order to eliminate MPs. Interleukin (IL)-8 release was quantified with a human IL-8 ELISA kit provided by BD Biosciences (San Diego, California) according to the manufacturer's instructions. CD40L exposed by MPs was also quantified in ELISA with the Human sCD40L Quantikine Kit provided by R&D Systems. VEGF release after 24-h exposure of HUVECs to MPs was quantified in MP-free medium (obtained after 20,500 *g* centrifugation, followed by 0.1-μm filtration) using antihuman VEGF provided by R&D Systems according to the manufacturer's instructions. Control experiments evaluated VEGF concentration in the medium containing the highest concentration of MPs, but not exposed to HUVECs.

Ribonucleic acid (RNA)-mediated silencing of CD40 in HUVECs. The RNA interference treatment was performed on subconfluent HUVECs plated in 96-well plates in serum and antibiotic-free medium. Transfection of small interfering ribonucleic acid (siRNA) (75 nmol/l

final concentration, ON-TARGETplusSMARTpool, cat. L-008101-0 and siCONTROL Non-Targeting siRNA#2, cat. D-001210-02-05) was performed by means of the DharmaFECT-1 transfection reagent (Dharmacon, Inc., Chicago, Illinois), according to the manufacturer's instructions. Transfection efficacy was assessed by flow cytometry analysis of CD40 expression on HUVECs after 72 h.

Matrigel assay. Ten-week-old BalbC/Nude or C57Bl/6 (Charles River, L'arbresle, France) and 10-week-old $CD40^{-/-}$-deficient female mice (10) (Department of Pathology, University of Maastricht, Maastricht, the Netherlands) received in their back a 0.5-ml subcutaneous injection of Matrigel containing either 3×10^6 plaque MPs (6,000 MPs/μl final concentration) or the equivalent volume of plaque MP supernatant, according to previously described protocol (18). After the injection, the Matrigel rapidly formed a subcutaneous plug that was left for 7 days. On day 7, mice were euthanized, and the skin of the mouse was easily pulled back to expose the Matrigel. Plugs were then removed and fixed with 4% formaldehyde at 4°C for 12 h, embedded in paraffin, sectioned, and stained with Masson Trichrome. Three successive sections of 5 μm, at the top, middle, and bottom of each plug, were then examined by an investigator unaware of the status of the material (×10, ×20, and ×40 magnification, Olympus BH-2, Leica, Paris, France). For each localization (top, middle, bottom) in the plug, 3 sections were analyzed, which led to an average of 9 acquisitions for each mouse. Neovessel formation was measured by means of 3 scores. Score 0 characterized the absence of cells in Matrigel section, score 1 corresponded to the presence of endothelial cells, and score 2 was defined by the presence of erythrocytes into the Matrigel plug. The presence of endothelial cells was confirmed using antimouse CD31-FITC (5 μg/ml, BD Biosciences) or FITC-labeled GSL-1-isolectin B4 (25 μg/ml, Vector Lab, Paris, France). Erythrocytes present in Matrigel plugs were identified using antimouse PE-labeled TER-119 antibody (2 μg/ml, BD Biosciences).

Statistical analysis. Data are presented as mean ± SEM, except for the cellular origin of plaque MPs shown in Table 1. Wilcoxon tests for nonparametric paired samples and Mann-Whitney *U* tests for independent samples were used. Differences were considered significant with a value of $p < 0.05$.

Results

Plaque MPs induce in vitro endothelial cell proliferation. Endothelial cell proliferation, evaluated by ^{3}H-thymidine incorporation in subconfluent quiescent HUVECs, was markedly elevated after exposure to increasing concentrations of plaque MPs (50 to 20,000 AnnV+ MPs/μl) for 48 h, whereas MPs' vehicle (20,500 *g* supernatant) had no effect on proliferation (n = 12) (Fig. 1A). Exposure to plaque MPs (6,000 AnnV+ MPs/μl; 48 h) led to a 2.5 ± 0.5-fold increase in endothelial cell number when compared with that of the vehicle's effect (n = 5) (Fig. 1B). This proliferative effect was also observed with human aortic endothelial cells (data not shown). The maximal ^{3}H-thymidine incorporation induced by plaque MPs at the concentration of 10,000 AnnV+ MPs/μl (3.8 ± 1.0-fold increase) was not different from that of 10% FCS (used as a positive control; p = 0.44; n = 8). In experiments performed at the concentration of 6,000 AnnV+ MPs/μl, we observed that the proliferative effect was more potent for MPs obtained from symptomatic than asymptomatic patients (100 ± 7% vs. 67 ± 13% of the effect of FCS, n = 6 each; p = 0.03) (Fig. 1C). Further experiments were performed at the suboptimal concentration of 6,000 AnnV+ MPs/μl.

We also investigated the potential proliferative effects of circulating MPs isolated from the same patients on endothelial cells. This effect was investigated for a concentration of 1,000 AnnV+ MPs/μl, which is within the range of MP plasma concentrations in these patients (14). There was no statistical difference in ^{3}H-thymidine incorporation between circulating MPs when compared with that in the vehicle (platelet-free plasma devoid of MPs; p = 0.46; n = 6). Moreover, when comparing similar concentrations of MPs (1,000 AnnV+ MPs/μl) obtained either from the plaque or the blood of the same patient, we observed that ^{3}H-thymidine incorporation induced by plaque MPs was greater than that of plasma MPs (3.6 ± 0.9-fold increase; n = 6; p = 0.03). These results suggest that the proliferative effect of MPs is specific to MPs isolated from atherosclerotic plaque.

Mechanism of plaque MP-induced endothelial cell proliferation. Because plaque MPs were mostly of leukocyte origin (14), we investigated the possible contribution of nicotinamide adenine dinucleotide phosphate oxidase-dependent reactive oxygen species to MP-induced ^{3}H-thymidine incorporation in endothelial cells. Apocynin (1 μmol/l) had no significant effect (n = 6; MPs = 128 ± 27% of FCS; MPs + apocynin = 108 ± 25% of FCS; p = 0.13), and Western blot analysis of plaque MPs using anti–p22-, p47-, p67-, and gp91-phox antibodies demonstrated that plaque MPs only express detectable amounts of the membrane subunits p22- and gp91-phox but not the cytosolic subunits p67- and p47-phox of nicotinamide adenine dinucleotide phosphate oxidase (n = 5, data not shown).

The possible contribution of CD40/CD40L to plaque MP-induced endothelial proliferation was then investigated. Endothelial cells expressed CD40, but not CD40L, and exposure to plaque MPs dose-dependently stimulated endothelial CD40 expression (Figs. 2A and 2B). Although MPs' abundance and cellular origin were not different between asymptomatic and symptomatic patients (n = 13 each; p > 0.05) (Table 2) as reported earlier (14), plaque from symptomatic patients expressed more CD40L+ MPs than those from asymptomatic patients (n = 13 each; p = 0.01) (Fig. 2D). The ELISA assay confirmed the presence

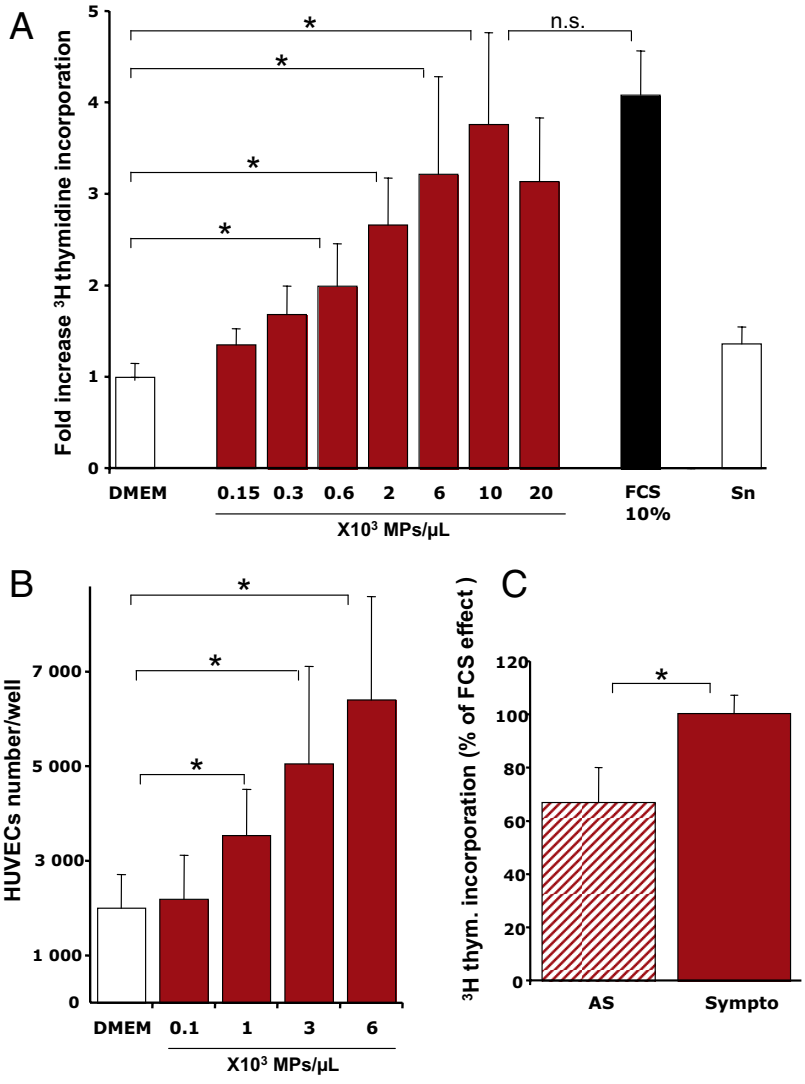

Figure 1 Plaque MPs Induce In Vitro Endothelial Cell Proliferation

(A) Plaque microparticles (MPs) induce a dose-dependent increase in ^{3}H-thymidine incorporation in human umbilical vein endothelial cells (HUVECs), whereas both MPs' vehicle (Dulbecco's modified eagle medium [DMEM]) and the 20,500 *g* supernatant (Sn) obtained after pelleting MPs from plaque homogenate did not. **(B)** Plaque MPs induce a dose-dependent increase in endothelial cell number (n = 6). **(C)** Plaque MPs (6,000 Annexin V+ MPs/μl) from symptomatic (Sympto) patients induce more proliferation than those from asymptomatic (AS) patients (n = 6). FCS = fetal calf serum.

of CD40L on MPs, while it was undetectable on the 20,500 *g* supernatant obtained after pelleting MPs from plaque homogenates (data not shown). Plaque MPs did not harbor CD40, and endothelial cells, used between passages 2 and 6, did not express CD40L. However, CD40L expression was measurable in HUVECs after tumor necrosis factor-alpha stimulation (Figs. 2A and 2B). We observed that 93 ± 4% of CD40L+ MPs are also positive for CD14, demonstrating that CD40L+ MPs are mostly of macrophage origin in the human plaque. We could also exclude that CD40L+ MPs derived from platelets, as we did not detect platelet MPs in human atherosclerotic plaques (absence of CD41+, CD42+, and CD62+CD144-labeling of plaque MPs) (14). Circulating MPs obtained from the same patients also expressed CD40L, but at much lower levels than those found in plaque homogenates (plasma: 959 ± 266 CD40L+ MPs/μl; plaque homogenates: 96,252 ± 19,565 CD40L+ MPs/μl; $p < 0.001$; n = 22). Unlike plaque MPs, no difference of CD40L expression was observed in circulating MPs when comparing plasmas from symptomatic and asymptomatic patients ($p = 0.87$).

Then, we examined the possible contribution of CD40 ligation in MP-induced endothelial proliferation using neutralizing antibodies and their corresponding isotypic IgGs as controls. Exposure of plaque MPs to an anti-CD40L neutralizing antibody decreased by 48 ± 17% MP-induced ^{3}H-thymidine incorporation (n = 12; $p = 0.003$) (Fig. 3A). Exposure of HUVECs to an anti-

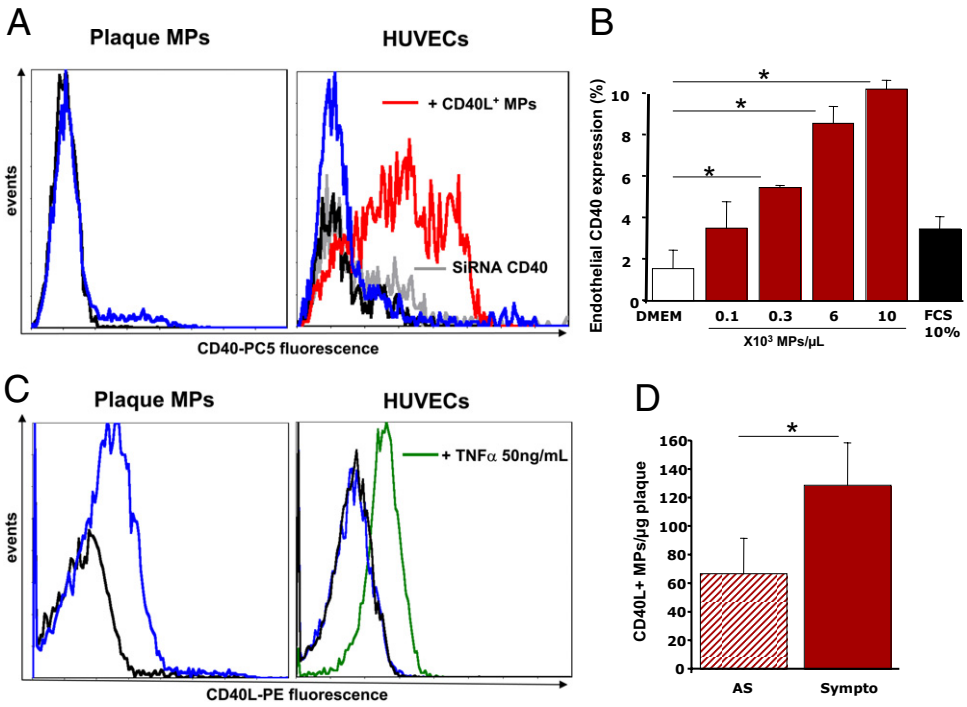

Figure 2 **HUVECs Exposed to Plaque MPs Express CD40 and Plaque MPs Are CD40L+**

(A) Representative flow cytometry fluorescence histogram showing the absence of CD40 on plaque MPs **(left)** and its presence on endothelial cells under basal conditions and after exposure to plaque MPs **(right)**. The **black line** corresponds to the negative control with an isotypic antibody, and the **blue line** reflects CD40-phycoerythrin-cyanin 5 (PC5) labeling. Endothelial CD40 expression is augmented when HUVECs are exposed to plaque MPs, as shown by the **red line (right)**. CD40 silencing of HUVECS with small interfering ribonucleic acid (siRNA) abolished PC5 labeling **(gray line, right)**. **(B)** Endothelial CD40 expression is augmented when HUVECs are exposed to increasing numbers of plaque MPs (n = 3). **(C)** Representative flow cytometry fluorescence histogram showing the presence of CD40L on plaque MPs **(left)** and its absence on endothelial cells under basal conditions or after exposure to plaque MPs **(right)**. The **black line** corresponds to negative control and the **blue line** to CD40L-phycoerythrin (PE)–positive MPs. Tumor necrosis factor (TNF)-stimulated HUVECS express CD40L **(green line)**, whereas HUVECs exposed to MPs do not **(blue line, right)**. **(D)** Plaque MPs from Sympto patients express more CD40L than those from AS patients (n = 13 each). Abbreviations as in Figure 1.

CD40–neutralizing antibody decreased it by 32 ± 2%, and the combination of these 2 neutralizing antibodies decreased it by 63 ± 16% (n = 6 each, p = 0.04 and 0.04, respectively) (Fig. 3A). Higher concentrations of neutralizing antibodies were also tested. Unfortunately, these results are difficult to interpret as the corresponding isotypes significantly modified endothelial proliferation on their own (data not shown).

Therefore, we investigated endothelial proliferation after exposure to plaque MPs using siRNA-CD40–transfected HUVECs. CD40-silenced HUVECs no longer expressed CD40 as demonstrated by flow cytometry analysis (Fig. 2A); however, ^{3}H-thymidine incorporation induced by FCS (10%) was comparable in control and siCD40-transfected HUVECs (p = 0.99) (Fig. 3B). Human recombinant CD40L, which was used at the concentration of 6 μg/ml because of its less potent biologically active monomeric form, increased ^{3}H-thymidine incorporation on control-transfected HUVECs (p = 0.04), whereas it had no effect on siRNA-CD40–transfected HUVECs (p = 0.51; n = 3)

Table 2 **Cellular Origins of MPs Isolated From Human Atherosclerotic Plaque Between Asymptomatic and Symptomatic Patients**

	Asymptomatic Patients (n = 13)	Symptomatic Patients (n = 13)	p Value
Lymphocyte (CD4+) MPs	19,155 (1,613–83,685)	39,681 (4,230–141,614)	0.249
Macrophage (CD14+) MPs	36,649 (16,939–158,645)	80,049 (5,613–179,378)	0.999
Granulocyte (CD66b+) MPs	16,853 (7,583–69,714)	23,626 (1,669–152,879)	0.870
Endothelial (CD144+) MPs	24,979 (9,382–117,783)	46,572 (2,557–123,085)	0.778
Erythrocyte (CD235a+) MPs	70,974 (0–292,836)	125,331 (6,443–359,774)	0.415
SMC (SMA+) MPs	40,518 (20,523–68,561)	54,941 (17,325–240,584)	0.463

Median values and ranges are expressed as n/mg lesion.
MP = microparticle; SMC = smooth muscle cell.

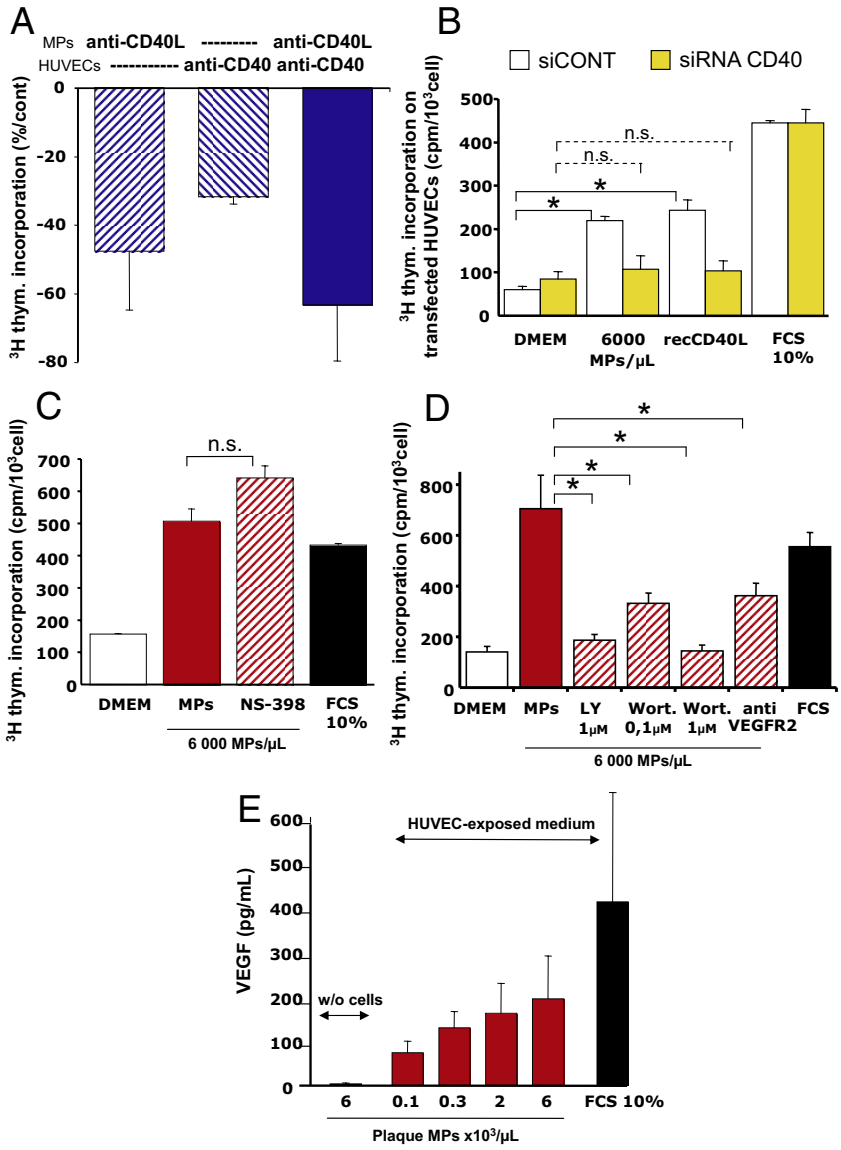

Figure 3 **Plaque MP-Induced Endothelial Cell Proliferation Is Mainly Dependent on CD40L Signaling**

(A) Exposure of MPs to an anti-CD40L or exposure of HUVECs to an anti-CD40 or the combination of these 2 neutralizing antibodies decreases the endothelial cell ^{3}H-thymidine (thym.) incorporation (n = 12). Control experiments **(dotted line)** were performed with the respective immunoglobulin G isotypes. **(B)** Endothelial proliferation induced by either plaque MPs or recombinant CD40L are abolished in CD40-silenced HUVECs (n = 3). **(C)** The inhibitor of cyclooxygenase 2, NS-398 (10^{-6} mol/l), has no effect on plaque MP-induced endothelial proliferation (n = 6). **(D)** Plaque MP-induced proliferation is impaired by either the PI3-kinase/Akt inhibitors (LY294002, 10^{-6} mol/l and wortmanin, 10^{-6} mol/l) or an antivascular endothelial growth factor (VEGF)-R2 blocking antibody (5 µg/ml, n = 3); the corresponding immunoglobulin G isotype did not significantly affect MP-induced proliferation. **(E)** Exposure of HUVECs to increasing concentrations of MPs **(red bars)** or FCS **(black bar)** for 24 h stimulated VEGF release in medium; VEGF levels were also determined in the medium containing the highest concentration of MPs, but not exposed to HUVEC (n = 6). %/cont = percent of control experiments performed with the respective immunoglobulin G isotypes; LY = LY-294002; recCD40L = recombinant CD40 ligand; si = small interfering; w/o = without cells; wort = wortmanin; other abbreviations as in Figure 1.

(Fig. 3B). Similar to recombinant CD40L, plaque MPs no longer increased ^{3}H-thymidine incorporation in CD40-silenced HUVECs when compared with that in the vehicle, while their effect on endothelial proliferation remained in siCONT-transfected HUVECs (p = 0.53 and p = 0.04, respectively) (Fig. 3B).

Previously published studies indicate that in vitro proliferative effects of CD40 ligation on endothelial cells might involve VEGF, IL-8, cyclooxygenase (COX)-2 expression, or activation of the PI3-kinase/Akt pathway (7–9,11,19,20). Exposure of HUVECs to plaque MPs (48 h) did not affect IL-8 release (vehicle: 1.9 ± 0.4 pg IL-8/µl; 3,000 MPs/µl: 1.8 ± 0.4 pg IL-8/µl, n = 5, p = 0.81; 6,000 MPs/µl: 1.2 ± 0.3 pg IL-8/µl, n = 4, p = 0.22), whereas experiments performed in parallel indicated a robust increase in IL-8 release after cytokine exposure (tumor necro-

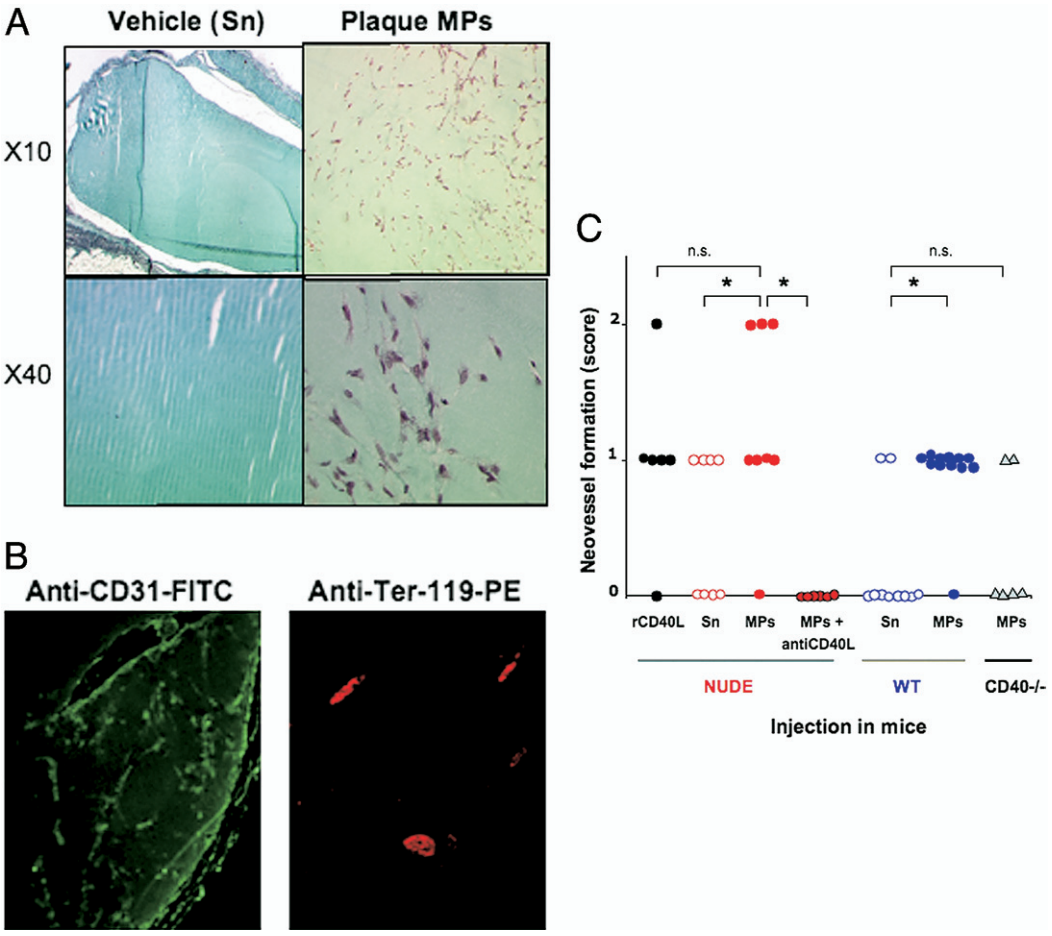

Figure 4 **Plaque MPs Induce In Vivo CD40L-Dependent Neovessel Formation**

(A) Matrigel sections stained with Masson Trichrome (**green** = collagen, **red** = cells) show infiltration of endothelial cells and red blood cells in presence of plaque MPs. **(B)** CD31 staining of Matrigel sections indicates the presence of endothelial cells; similar results were obtained with isolectin B4 staining (not shown). Antimouse Ter-119 staining was used to identify red blood cells in Matrigel. **(C)** Plaque MPs induce neovessel formation in Matrigel plugs from BalbC/Nude and wild-type (WT) mice, whereas MPs supernatant (Sn) has no effect. Microparticles effect on neovessel formation was abolished either in the presence of neutralizing antibody CD40L or when experiments were performed in $CD40^{-/-}$ mice. FITC = fluoroisothiocyanate; other abbreviations as in Figure 1.

sis factor-alpha 10 ng/ml: 18.1 ± 4.1 pg IL-8/μl). The preferential inhibitor of COX-2, NS-398 (10^{-6} mol/l), did not affect plaque-MP-induced thymidine incorporation (n = 6; 1,017 ± 95 cpm vs. 1,286 ± 137 cpm with NS-398; p = 0.17) (Fig. 3C). However, endothelial proliferation was impaired by either Wortmanin (1 μmol/l) (n = 5; 6,876 ± 1,841 cpm vs. 1,526 ± 633 cpm; p = 0.04) or LY294002 (1 μmol/l) (n = 7; 1,444 ± 304 cpm vs. 392 ± 116 cpm; p = 0.02), both inhibitors of PI3-kinase/Akt. An anti–VEGF-R2 blocking antibody also significantly reduced endothelial cell proliferation (n = 3; −20% inhibition; p = 0.04) (n = 6; p = 0.11) (Fig. 3D). Moreover, exposure of HUVECs to increasing concentrations of plaque MPs augmented VEGF release, whereas levels of VEGF were almost undetectable in plaque MPs (Fig. 3E).

Plaque MPs induced in vivo angiogenesis. In wild-type mice, plaque MPs (n = 12) induced significantly more angiogenesis when compared with that seen in vehicle (MPs supernatant; n = 10; p = 0.001).

In order to rule out a potential role of immune- and inflammation-induced angiogenesis, a Matrigel assay was performed in BalbC/Nude mice. In these mice, plaque MPs (n = 8) also induced significantly more angiogenesis in subcutaneous Matrigel plugs, when compared with that in vehicle (MPs supernatant; n = 8; p = 0.04). In some sections, plaque MPs caused erythrocyte infiltration in addition to endothelial cell infiltration in Matrigel (score 2), whereas the supernatant of these MPs never did (Fig. 4A). Endothelial cells were identified in Matrigel plugs using either FITC-labeled–isolectin B4 or antimouse CD31-FITC staining, whereas the presence of erythrocytes was evidenced using antimouse Ter 119-PE (Fig. 4B). Preincubation of MPs with a neutralizing antibody against CD40L abolished the proliferative effect of plaque MPs in

these mice (n = 6; p = 0.003). The proangiogenic effect of MPs was similar to that of recombinant CD40L in this model (n = 6; p = 0.47) (Fig. 4C).

In order to confirm the role of CD40 ligation, the Matrigel assay was also performed in $CD40^{-/-}$ mice; the in vivo proliferative effect was abolished in these mice (n = 6; p = 0.007 when compared with the effect of MPs in wild-type mice). Moreover, effects of plaque MPs in CD40-deficient mice were not different from that of vehicle (MPs supernatant; p = 0.89) (Fig. 4C). These results demonstrate that CD40 signaling mediates the ability of plaque MPs to induce formation of neovessels in mice.

Discussion

The present study demonstrates for the first time that shed-membrane MPs isolated from human atherosclerotic lesions stimulate endothelial cell proliferation and promote in vivo neovessel formation after CD40 ligation. The endothelial proliferative effect of plaque MPs was more pronounced when MPs were isolated from symptomatic patients compared with that seen in asymptomatic patients, and this finding was associated with an increased number of CD40L+ MPs in these patients. Therefore, accumulation of MPs in atherosclerotic lesions may represent an endogenous signal for atherosclerotic plaque neovascularization and vulnerability.

Shed-membrane vesicles have been observed in situ in human atherosclerotic plaque where they colocalize with markers of cellular apoptosis (12,13). Although very little is known regarding the mechanisms leading to their formation, they may result from cell plasma membrane budding after inflammation of the vessel wall leading to activation or apoptosis of cells from the vascular wall, as indicated by their multiple cellular origins (14,16). Accumulation of oxidized lipids and apoptotic cells in atherosclerotic lesions likely slows down efficient MP phagocytosis and removal by macrophages. Plaque MPs are highly thrombogenic and, therefore, contribute to thrombus formation when the lesion ruptures (13,14,21). However, apart from their role in thrombus formation, no other biological effect has been reported so far for MPs isolated from human atherosclerotic plaques. In order to investigate the possible biological activities of plaque MPs, we recently reported a method to isolate MPs from human atherosclerotic lesions, and we demonstrated that the procedure did not generate MPs from healthy human arteries (14).

In vitro studies indicate that MPs can no longer be taken simply as a marker of cell activation or apoptosis; several reports demonstrate that shed-membrane MPs can disseminate information to neighboring or remote cells (15,16). For instance, MPs of different cellular origin stimulate proinflammatory responses in endothelial cells (22–24). In addition, endothelial-, platelet-, and tumor-cell–derived MPs are capable of either promoting or inhibiting angiogenesis through reactive oxygen species, metalloproteinases, and growth factors such as VEGF or sphingomyelin (25–28). However, these studies were performed using in vitro–generated MPs, which may not reflect the biological activity of MPs found in vivo. Indeed, studies analyzing MP composition demonstrate that both lipid and protein fractions vary greatly depending on the stimulus initiating their vesiculation (29–31). We demonstrate here that MPs isolated from human atherosclerotic plaques strongly stimulate endothelial cell proliferation to the same level as FCS, reflecting their high proliferative effect. This effect is specific for MPs isolated from human plaque, because an equivalent concentration of MPs isolated from the blood of the same patients did not affect endothelial proliferation. Furthermore, this effect of plaque MPs on endothelial proliferation was not only observed in vitro but was also confirmed in vivo by the formation of neovessels in Matrigel assays in mice. Moreover, an important finding is that MPs isolated from symptomatic plaques were more potent than those from asymptomatic plaques. Taken all together, these findings support the concept that in atherosclerotic lesions, MPs could determine neovascularization and may favor plaque instability.

Although angiogenesis is regulated by the combined action of various cytokines and growth factors released by infiltrating inflammatory cells, the exact mechanism of neovessel formation in atherosclerotic plaque remains unknown (1). We examined the possible role of CD40 ligation in this process. Indeed, both CD40 and CD40L are highly expressed in human atherosclerotic plaques, and CD40 ligation stimulates endothelial proliferation and angiogenesis (5–9). Furthermore, in vivo blockade of CD40 signaling improves plaque stability (10,11). We demonstrate in the present study a crucial role of CD40 ligation in the proliferative effect of plaque MPs both in vitro and in vivo. We show that CD40L is bound to macrophage-derived MPs isolated from human atherosclerotic plaques, and even more so to MPs obtained from symptomatic patients, and that CD40L interaction with endothelial CD40 mediates the proliferative effect of MPs isolated from human plaque. Furthermore, we demonstrate here that in vivo neovessel formation induced by plaque MPs requires the presence of functional CD40 in mice. Several intracellular pathways could mediate the proliferative effect of CD40 ligation on endothelial cells under the present experimental conditions (5–9). The lack of effect of a COX-type 2 preferential inhibitor rules out the possible contribution of COX-2 in MP-induced proliferation (19). Furthermore, exposure of endothelial cells to plaque MPs did not affect the release of IL-8, which is another possible mediator of CD40 ligation proliferative effects on endothelial cells (20). However, the inhibitory effect of the PI3-kinase/Akt inhibitor and that of the VEGF-R2–neutralizing antibodies supports a role for VEGF and the PI3-kinase/Akt in the endothelial proliferative effect of plaque MPs through ligation of CD40. These findings are in agreement with previous studies performed with recombinant CD40L (7–9).

Conclusions

The present study reveals MPs as potential endogenous triggers of neovascularization and growth of atherosclerotic plaque. In addition, we demonstrated in advanced and complex symptomatic human atherosclerotic plaque that their deletery proangiogenic effect is augmented and could carry on the vicious circle of plaque instability: more apoptosis generating more MPs, contributing to neovascularization, and favoring intraplaque hemorrhage, lesion growth, and macrophage apoptosis.

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REFERENCES

1. Virmani R, Kolodgie FD, Burke AP, et al. Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. Arterioscler Thromb Vasc Biol 2005;25:2054–61.
2. O'Brien ER, Garvin MR, Dev R, et al. Angiogenesis in human coronary atherosclerotic plaques. Am J Pathol 1994;145:883–94.
3. Barger AC, Beeuwkes R 3rd, Lainey LL, Silverman KJ. Hypothesis: vasa vasorum and neovascularization of human coronary arteries. A possible role in the pathophysiology of atherosclerosis. N Engl J Med 1984;310:175–7.
4. Kwon HM, Sangiorgi G, Ritman EL, et al. Enhanced coronary vasa vasorum neovascularization in experimental hypercholesterolemia. J Clin Invest 1998;101:1551–6.
5. Mach F, Schonbeck U, Fabunmi RP, et al. T lymphocytes induce endothelial cell matrix metalloproteinase expression by a CD40L-dependent mechanism: implications for tubule formation. Am J Pathol 1999;154:229–38.
6. Mach F, Schonbeck U, Sukhova GK, et al. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. Proc Natl Acad Sci U S A 1997;94:1931–6.
7. Melter M, Reinders ME, Sho M, et al. Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis in vivo. Blood 2000;96:3801–8.
8. Reinders ME, Sho M, Robertson SW, Geehan CS, Briscoe DM. Proangiogenic function of CD40 ligand-CD40 interactions. J Immunol 2003;171:1534–41.
9. Deregibus MC, Buttiglieri S, Russo S, Bussolati B, Camussi G. CD40-dependent activation of phosphatidylinositol 3-kinase/Akt pathway mediates endothelial cell survival and in vitro angiogenesis. J Biol Chem 2003;278:18008–14.
10. Lutgens E, Gorelik L, Daemen MJ, et al. Requirement for CD154 in the progression of atherosclerosis. Nat Med 1999;5:1313–6.
11. Schonbeck U, Sukhova GK, Shimizu K, Mach F, Libby P. Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. Proc Natl Acad Sci U S A 2000;97:7458–63.
12. Kockx MM. Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects. Arterioscler Thromb Vasc Biol 1998;18:1519–22.
13. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. Circulation 1999;99:348–53.
14. Leroyer AS, Isobe H, Leseche G, et al. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. J Am Coll Cardiol 2007;49:772–7.
15. Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. Physiology (Bethesda) 2005;20:22–7.
16. Boulanger CM, Amabile N, Tedgui A. Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. Hypertension 2006;48:180–6.
17. Amabile N, Guerin AP, Leroyer A, et al. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. J Am Soc Nephrol 2005;16:3381–8.
18. Tamarat R, Silvestre JS, Durie M, Levy BI. Angiotensin II angiogenic effect in vivo involves vascular endothelial growth factor- and inflammation-related pathways. Lab Invest 2002;82:747–56.
19. Schonbeck U, Sukhova GK, Graber P, Coulter S, Libby P. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am J Pathol 1999;155:1281–91.
20. Simonini A, Moscucci M, Muller DW, et al. IL-8 is an angiogenic factor in human coronary atherectomy tissue. Circulation 2000;101:1519–26.
21. Bonderman D, Teml A, Jakowitsch J, et al. Coronary no-reflow is caused by shedding of active tissue factor from dissected atherosclerotic plaque. Blood 2002;99:2794–800.
22. Barry OP, Pratico D, Lawson JA, FitzGerald GA. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. J Clin Invest 1997;99:2118–27.
23. Mause SF, von Hundelshausen P, Zernecke A, Koenen RR, Weber C. Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium. Arterioscler Thromb Vasc Biol 2005;25:1512–8.
24. Boulanger CM, Scoazec A, Ebrahimian T, et al. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. Circulation 2001;104:2649–52.
25. Brill A, Dashevsky O, Rivo J, Gozal Y, Varon D. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. Cardiovasc Res 2005;67:30–8.
26. Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. Am J Pathol 2002;160:673–80.
27. Kim CW, Lee HM, Lee TH, Kang C, Kleinman HK, Gho YS. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res 2002;62:6312–7.
28. Mezentsev A, Merks RM, O'Riordan E, et al. Endothelial microparticles affect angiogenesis in vitro: role of oxidative stress. Am J Physiol Heart Circ Physiol 2005;289:H1106–14.
29. Huber J, Boechzelt H, Karten B, et al. Oxidized cholesteryl linoleates stimulate endothelial cells to bind monocytes via the extracellular signal-regulated kinase 1/2 pathway. Arterioscler Thromb Vasc Biol 2002;22:581–6.
30. Miguet L, Pacaud K, Felden C, et al. Proteomic analysis of malignant lymphocyte membrane microparticles using double ionization coverage optimization. Proteomics 2006;6:153–71.
31. Kolowos W, Gaipl US, Sheriff A, et al. Microparticles shed from different antigen-presenting cells display an individual pattern of surface molecules and a distinct potential of allogeneic T-cell activation. Scand J Immunol 2005;61:226–33.

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