Leukocyte Microparticles Stimulate Endothelial Cell Cytokine Release and Tissue Factor Induction in a JNK1 Signaling Pathway*

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A role of membrane microparticles (MP) released by vascular cells in endothelial cell (EC) activation was investigated. Flow cytometric analysis of blood samples from normal volunteers revealed the presence of an heterogeneous MP population, which increased by 2-fold after inflammatory stimulation with the chemotactic peptide, N-formyl-Met-Leu-Phe (2,799 ± 360 versus 5241 ± 640, p < 0.001). Blood-derived MP stimulated release of EC cytokines interleukin (IL)-6 (377 ± 68 pg/ml) and MCP-1 (1, 282 ± 79) and up-regulated de novo expression of tissue factor on the EC surface. This was associated with generation of a factor Xa-dependent procoagulant response (2.28 ± 0.56 nM factor Xa/min/10⁶ cells), in a reaction inhibited by a monoclonal antibody to tissue factor. Fluorescent labeling with antibodies to platelet GPIIb or leukocyte lactoferrin demonstrated that circulating MP originated from both platelets and leukocytes. However, depletion of platelet MP with an antibody to GPIIb did not reduce EC IL-6 release, and, similarly, MP from thrombin-stimulated platelets did not induce IL-6 release from endothelium. EC stimulation with leukocyte MP did not result in activation of the transcription factor NF-κB and was not associated with tyrosine phosphorylation of extracellular signal-regulated protein kinase, ERK1. In contrast, leukocyte MP stimulated a sustained, time-dependent increased tyrosine phosphorylation of ~46-kDa c-Jun NH₂-terminal kinase (JNK1) in EC. These findings demonstrate that circulating leukocyte MP are up-regulated by inflammatory stimulation in vivo and activate a stress signaling pathway in EC, leading to increased procoagulant and proinflammatory activity. This may provide an alternative mechanism of EC activation, potentially contributing to dysregulation of endothelial functions during vascular injury.

Vascular endothelial cells (EC)¹ respond to environmental and cellular stimuli with profound changes of adhesive, procoagulant, and inflammatory phenotypes (1–3). This process of EC activation results in release of inflammatory and chemotactic cytokines IL-6, IL-8, and MCP-1 (2, 4–6), expression of procoagulant tissue factor (TF) (3, 7), and enhanced leukocyte recruitment via expression of adhesion molecules E-selectin, ICAM-1, and VCAM-1 (1). These responses may originate from signal transduction by released cytokines, i.e. TNFα (8, 9), shear stress during vascular remodeling (10), or cross-talk with different vascular cells, including leukocytes and platelets (11). Although of critical importance to preserve immune inflammatory responses and leukocyte trafficking (1, 12), dysregulated EC activation may contribute to vascular injury and exacerbate the onset and progression of atherosclerosis in vivo (13).

Considerable interest has recently focused on alternative mechanisms of EC activation by vascular cells. Recent work has suggested that released membrane microparticles (MP) from platelets (14, 15) or leukocytes (16) may provide such an alternative pathway of EC activation. In these studies, platelet or leukocyte MP stimulated increased expression of various adhesion molecules on EC, up-regulation of inflammatory and chemotactic cytokines, and increased monocyte adhesiveness (14–16). For platelet MP, this pathway was recapitulated by arachidonic acid, consistent with the presence of bioactive lipids in platelet MP, and their ability to influence gene expression in target cells (14, 15). Although the existence of platelet MP in vivo and their potential contribution to procoagulant and/or anticoagulant responses has long been established (17–20), little is known about leukocyte MP or their potential ability to stimulate EC in vivo.

In this study, we sought to investigate a potential role of leukocyte MP in EC responses and to identify signaling requirements involved in gene expression. We found that although both platelet and leukocyte MP are present in the normal circulation in vivo, only the leukocyte fraction initiates signal transduction and stimulates inflammatory and procoagulant responses in the endothelium.

MATERIALS AND METHODS

Cells and Cell Cultures—Polymorphonuclear leukocytes (PMN) were isolated from heparin sodium-anticoagulated blood drawn after informed consent from normal healthy volunteers by differential centrifugation on Ficoll/Hypaque gradient and dextran sedimentation as described (16). Human umbilical vein EC were prepared by collagenase treatment, maintained in medium 199 (BioWhittaker, Walkersville, MD) supplemented with 20% heat-inactivated fetal bovine serum (Bio-Whittaker), l-glutamine (2 mM), and 1% endothelial cell growth supplement, pH 7.4, and used between passages 2 and 4.

Flow Cytometric Detection of MP in the Normal Circulation—Heparin sodium-anticoagulated blood was drawn from normal healthy volunteers after informed consent. Aliquot (1.5 ml) of undiluted blood or samples diluted 1:5 in PBS, pH 7.4, were incubated with the fluorescent labeling dye, quinacrine mustard (mepacrine, 0.1 µM; Sigma), in the presence or in the absence of FMLP (1 µM) for 2 h at 37 °C. Blood samples were centrifuged at 1500 × g for 20 min at 22 °C, and the cell-free supernatant was collected and analyzed by flow cytometry.

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* The abbreviations used are: EC, endothelial cell(s); MP, microparticle(s); FRP, platelet-rich plasma; TF, tissue factor; IL, interleukin; TNF, tumor necrosis factor; PMN, polymorphonuclear leukocyte(s); PBS, phosphate-buffered saline; FMLP, formyl-methionyl-leucyl-phenylalanine; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

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some experiments, cell-free supernatants prepared as described above were passed through 100-kDa cut-off nanospongs (Millipore, Bedford, MA) according to the manufacturer’s specifications. Aliquots of 0.5 ml were analyzed on a Becton-Dickinson (Mountain View, CA) flow cytometer as described (16). To identify the MP population, samples were gated for light scatter, fluorescence and red fluorescence. The light scatter and fluorescence channels set at logarithmic gain, samples were analyzed for 6-s intervals for forward light scatter, right angle light scatter, and mepacrine fluorescence. The light scatter profile of mepacrine-positive MP typically demonstrated one single low light scatter population, in agreement with previous observations (16). In distinguishable results were obtained using a Beckman Coulter (Life Technologies, Inc.) using oligo(dT) or gene-specific primer (5′-CAGCTGGCCTTTCAC-3′). The reverse transcription reaction containing 5 μg of total RNA or 50 ng of control RNA, 0.5 μg of oligo(dT) or 2 μg GSP, 1 × 10^6 unit/ml reverse transcriptase in the presence of 25 mM MgCl₂, 10 mM dNTP mix, and 0.1 mM dithiothreitol was incubated for 50 min at 42 °C. At the end of the incubation, samples were heated for 15 min at 70 °C, chilled on ice, mixed with 1 μl of RNase H for 20 min at 37 °C, and amplified by polymerase chain reaction with oligonucleotides 5′-GTGAAAGAGAGCAACACT-3′ (forward) and 5′-CAGCTGGCCTTTCAC-3′ (reverse) derived from the sequence of human TF. Thirty-five cycles of amplification were carried out in a Perkin-Elmer 480 thermal cycler with denaturation for 30 s at 94 °C, annealing for 40 s at 55 °C, and extension for 1 min at 37 °C. MgCl₂ was used at a final concentration of 1.5 mM. Polymerase chain reaction products were analyzed on 1% agarose gels by ethidium bromide staining.

In another series of experiments, EC in a 48-well plate were washed and incubated in the presence or absence of 10 ng/ml TNFα, cell-free supernatant from FMLP-stimulated PMN (4 × 10^5/ml), or equivalent purified MP prepared as described above for 6 h at 37 °C. After washes, EC were incubated with 100 μl of phenol red-free RPMI 1640 medium containing 150 ng/ml human factor X (Alexis), 5 ng/ml activated factor VII (Alexis), and 2 mM CaCl₂ for 20 min at 37 °C. In some experiments, EC monolayers were preincubated with anti-TF mAb 5G9 or control mAb 14E11 (20 μg/ml) for 30 min at 22 °C before addition of factors X and VIIa. Generation of activated X (factor Xa) was stopped by addition of 10 mM EDTA to each incubation reaction. Samples were transferred to a 96-well plate, and factor Xa activity was determined by hydrolysis of the chromogenic substrate S-2222 (Chromogenix, Mölndal, Sweden) at a final concentration of 0.2 mM. The optical densities were read at 405 nm using a plate-reader spectrophotometer (ThermoMax, Molecular Devices). The amount of factor Xa generated under the various conditions was determined by extrapolation of standard curves. A standard curve was generated using the factor Xa standard of the manufacturer.

Signal Transduction Mediated by PMN MP in EC—Serum-starved quiescent subconfluent EC in 6-well plates were stimulated with PMN (4 × 10^5/ml) supernatants for increasing time intervals at 37 °C. EC were washed and lysed with lysis buffer containing 10 mM Tris, 140 mM NaCl, 1% Triton, 0.5% deoxycholate, 0.05% SDS, 100 mM NaF, 200 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 mM pepstatin, and 1 μg/ml leupeptin. EC extracts were centrifuged at 14,000 × g for 30 min, precleared with protein A-Sepharose and immunoprecipitated with antibodies (1 μg/ml) to extracellular signal-regulated kinase 1 (ERK1, Santa Cruz Biotechnologies, Santa Cruz, CA), c-Jun NH₂-terminal kinase 1 (JNK1, Santa Cruz), or phosphotyrosine proteins 20 (ICN Pharmaceuticals Inc., Costa Mesa, CA) for 2 h at 4 °C. The immune complexes were electroprecipitated in a 10% SDS gel, electrobotted to nylon membranes, and immunoblotted with 1 μg/ml phosphotyrosine antibody Py20 or anti-JNK1 antibody. After washes, reactive bands under the various conditions were detected by addition of alkaline phosphatase-conjugated goat anti-mouse IgG (1:2000) and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). For electrophoretic mobility shift assays, confluent EC monolayers were either left untreated (control) or incubated with PMN (4 × 10^5/ml) supernatant or TNFα (10 ng/ml) for 90 min at 37 °C. Nuclear extracts were prepared as described previously (21). Nuclear extracts were normalized for protein concentration, and 10 μg were incubated with 4 μg of poly(dI-dC) (Amersham Pharmacia Biotech) for 20 min at 22 °C and then transferred to nitrocellulose filters (092269, Schleicher and Schuell, Keene, NH) using a 32P-labeled probe in a final volume of 15 μl. The oligonucleotide used in these studies for the b binding was 5′-AGTGTAGGGAGACTTTCAGGG-3′. Double-stranded oligonucleotide probe was end-labeled with γ-32P/ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). DNA-protein complexes were resolved on a 6% nondenaturing polyacrylamide gel containing 7.5% glycerol in 0.25% Tris borate.
EDTA buffer, pH 8.3 (1× TBE: 89 mM Tris borate, 89 mM boric acid, 20 mM EDTA). Dried gels were exposed to x-ray film (Kodak Co., Rochester, NY), and relevant bands were visualized by autoradiography.

**RESULTS**

**Inflammatory Stimulation of PMN Induces Membrane MP Release**—Treatment of freshly isolated PKH26-GL-labeled PMN with inflammatory/chemotactic stimuli fMLP (10 nM) or IL-8 (100 ng/ml) induced release of an heterogeneous membrane MP population, as determined by flow cytometry (Fig. 1), and in agreement with previous observations (16). Pretreatment with 100 ng/ml pertussis toxin (22) did not reduce MP release from fMLP- or IL-8-stimulated PMN suspensions (Fig. 1).

**Detection of MP in Vivo**—Flow cytofluorimetric analysis of whole blood supernatants revealed the constitutive presence of a discrete population with low forward scatter consistent with MP (Fig. 2A, top left quadrant). Stimulation of blood samples with fMLP resulted in a 2-fold increase in the MP population (Fig. 2A, top right quadrant), in agreement with previous observations (16). In parallel experiments, fMLP stimulation increased the number of fluorescent MP following mepacrine labeling of whole blood samples (Fig. 2A, lower left and right quadrants). Consistent with these in vivo data, cytofluorimetric analysis of cell-free supernatants collected from 23 normal volunteers revealed the constitutive presence of an MP population in nonmanipulated blood samples (events, 2799±360). Stimulation of whole blood samples with fMLP resulted in a 2-fold increase in the number of MP detected by flow cytometry (events, 5241±640; p = 0.001; Fig. 2B).

**Modulation of EC Activation by Blood-derived MP**—Incubation of quiescent EC with cell-free supernatants from unstimulated whole blood samples did not result in release of cytokines, IL-6, and MCP-1 (Fig. 3A). In contrast, cell-free supernatants from fMLP-stimulated whole blood supernatants caused a 2–4-fold increased release of cytokines MCP-1 (1282 ± 79 pg/ml) and IL-6 (377 ± 68 pg/ml), respectively (Fig. 3A). A 2-fold increase in IL-8 release was also observed under the same experimental conditions (not shown). Filtration of cell-free supernatants through 100-kDa filters prior to incubation with EC completely abolished the MP-stimulated release of IL-6 and MCP-1 by EC (Fig. 3A), in agreement with previous observations (16). Consistent with these findings, filtration of cell-free supernatants resulted in complete depletion of the blood MP population, identified by forward and side scatter parameters and quantitated by flow cytometry (Fig. 3B). In control experiments, preincubation of PMN-derived MP with a neutralizing mAb to TNFα did not significantly reduce EC IL-6 release (unstimulated, 28 pg/ml; MP-stimulated plus control mAb 14E11, 422 pg/ml; MP-stimulated plus anti-TNFα mAb, 378 pg/ml). In contrast, the anti-TNFα antibody inhibited by ~50% EC IL-6 release induced by TNFα stimulation (TNFα alone plus control mAb 14E11, 615 pg/ml; TNFα plus anti-TNFα mAb, 305 pg/ml).

**Induction of EC Procoagulant Activity by Circulating MP**—A...
6-h exposure of EC to supernatants from fMLP-stimulated PMN resulted in moderate but consistent increased surface expression of TF, as determined by flow cytofluorimetric staining with mAb 5G9 (Fig. 4A). Similar results were obtained using whole blood-containing MP (not shown). EC stimulation with blood-derived MP also resulted in de novo expression of TF mRNA, as determined by appearance of a 352-base pair TF RNA transcript detected by reverse transcriptase-polymerase chain reaction amplification of EC RNA (Fig. 4A, inset). In control experiments, EC stimulation with TNFα resulted in maximal increase in TF surface expression and TF RNA (Fig. 4A), in agreement with previous observations (23). In parallel experiments, EC stimulation with MP-containing PMN supernatant, or purified MP resulted in the generation of 2.28 ± 0.56 and 2.75 ± 0.07 nM of factor Xa/min/10⁴ EC, respectively (Fig. 4B). This response was abolished in the absence of factor VIIa or by addition of anti-TF mAb 5G9 (0.51 ± 0.07 nM of factor Xa/min/10⁴ EC) (Fig. 4B). In contrast, EC preincubation with control mAb 14E11 did not reduce EC procoagulant activity (Fig. 4B). In control experiments, EC stimulation with 10 ng/ml TNFα resulted in the formation of 6 ± 1.5 nM factor Xa/min/10⁴ EC (not shown), in agreement with previous observations (23).

Characterization of Circulating MP by Flow Cytometry—To identify the potential cell(s) of origin of released MP in vivo, flow cytofluorimetric experiments were carried out with antibodies to platelet GPIbα or leukocyte lactoferrin. Trypsin digestion and ionization mass spectrometry had previously identified the main ~85-kDa component in purified MP (16) as lactoferrin.2 In these experiments, unstimulated blood-derived MP reacted with antibodies to GPIbα and lactoferrin, as compared with control nonbinding antibody (Fig. 5). Furthermore, fMLP stimulation increased by ~10-fold the reactivity of the MP population with the lactoferrin antibody, whereas no significant differences in the binding of anti-GPIbα mAb were observed with unstimulated or fMLP-stimulated samples (Fig. 5).

Functional Dissociation between Platelet and Leukocyte MP in EC Stimulation—Preincubation of blood samples with 20 µg/ml anti-GPIbα mAb resulted in a significant reduction in the amount of MP released after fMLP stimulation (Fig. 6A). In contrast, no differences in fMLP-induced MP release were observed in the presence of control mAb 14E11 or the antibody to lactoferrin (Fig. 6A). In parallel experiments, preincubation of cell-free supernatants with antibodies to lactoferrin or GPIbα did not reduce EC IL-6 release, as compared with untreated samples or treated with control mAb 14E11 (Fig. 6B). A potential differential ability of platelet or leukocyte MP to induce EC IL-6 release was further investigated. Thrombin stimulation of PRP resulted in MP release, as determined by flow cytometry (Fig. 6C), in agreement with previous observations. However, thrombin-stimulated platelet MP or unstimulated PRP supernatant did not stimulate EC IL-6 release (Fig. 6D) and data not shown). In contrast, TNFα stimulation of EC resulted in the generation of 599 ± 142 pg/ml IL-6 after a 12-h culture at 37 °C (Fig. 6D).

Signal Transduction Initiated by PMN-derived MP in EC—EC stimulation with TNFα resulted in strong activation of NF-κB, as compared with unstimulated EC extracts, as determined by electrophoretic mobility shift assay (Fig. 7A). In contrast, leukocyte MP did not induc e NF-κB activation (Fig.

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2 M. Mesri and D. C. Altieri, our unpublished observations.
A). Similarly, no significant differences in tyrosine phosphorylation of ERK1 were observed in EC treated with leukocyte MP, as compared with unstimulated cultures (Fig. 7B). The identity of the ~46-kDa band was further investigated. Immuno precipitation of EC lysates with an antibody to JNK1 followed by Western blot with Py20 antibody revealed a ~2-fold increase in tyrosine phosphorylation of JNK1 in MP-stimulated cultures but not in control untreated EC (Fig. 7C). In contrast, EC serum treatment did not result in tyrosine phosphorylation of immunoprecipitated JNK1 (Fig. 7C). In control experiments, Western blot of JNK1 immunoprecipitates demonstrated a comparable amount of JNK1/lane, under the various conditions tested (Fig. 7C).

DISCUSSION

In this study, we have shown that leukocyte-derived MP circulate in the bloodstream under normal conditions and are rapidly up-regulated by inflammatory stimulation. In contrast, EC serum treatment did not result in tyrosine phosphorylation of immunoprecipitated JNK1 (Fig. 7C). In control experiments, Western blot of JNK1 immunoprecipitates demonstrated a comparable amount of JNK1/lane, under the various conditions tested (Fig. 7C).

The notion that vascular cells release MP in response to disparate environmental stimuli is well established and has been experimentally validated for platelets (24, 25), monocytes (26), and endothelium (27). Through their ability to assemble a functional prothrombinase complex, platelet (26, 28) and monocyte (29) MP may amplity cellular procoagulant responses, thus potentially contributing to aberrant fibrin deposition in vivo (30, 31). However, it has also been recently proposed that platelet (14, 15), and leukocyte (16), MP may function as gen-
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Fig. 6. Differential EC activation by leukocyte or platelet MP. A, diluted blood samples were incubated with 20 μg/ml aliquots of control mAb 14E11 or antibodies to GPIbα or lactoferrin for 30 min at 22 °C before stimulation with 1 μM fMLP for 1 h at 22 °C. Samples were centrifuged at 1500 × g for 20 min, and the cell-free supernatants were analyzed for MP content by flow cytometry. B, the experimental conditions are the same as those in A except that antibody-treated supernatants from blood samples were added to EC monolayers for 12 h before determination of IL-6 release by ELISA. C, diluted PRP samples were incubated in the absence or presence of 1 unit/ml thrombin and 2.5 mM CaCl2 for 10 min at 37 °C with gentle shaking. After platelet aggregation, PRP samples were centrifuged at 1500 × g for 20 min, and platelet-free supernatants were analyzed for MP content by flow cytometry. D, the experimental conditions are the same as in C except that supernatants from thrombin-stimulated PRP were added to EC monolayers for 12 h before determination of IL-6 release by ELISA.

Leukocyte microparticles (MP) induce complex EC responses. In this context, platelet MP induce COX-2 and prostacyclin production in EC (14) and stimulated increased monocyte adherence through up-regulation of adhesion molecules ICAM-1 (15). A similar paradigm has been also proposed for leukocyte MP for their ability to stimulate EC expression of adhesion molecules ICAM-1, E-selectin, and VCAM-1 and promote release of cytokines IL-6 and IL-8 (16). In expanding these observations, leukocyte MP released by inflammatory/chemotactic mediators fMLP or IL-8 in a pertussis toxin-insensitive pathway mediate a procoagulant response in EC by increasing the expression of TF mRNA and up-regulating functional TF at the cell surface. This is consistent with the ability of leukocyte MP to directly affect EC gene expression, as reflected by the ∼18-fold up-regulation of IL-6 mRNA, under these experimental conditions (16). Combined with the presence of leukocyte MP in vivo, and their rapid quantitative up-regulation by inflammatory stimuli, these data suggest that leukocyte MP may cooperate with locally released cytokines (2) and leukocyte-EC intercellular signaling (7, 32) to stimulate a broad proadhesive, procoagulant, and proinflammatory phenotype in EC. This pathway may be potentially relevant to EC dysfunction during vascular diseases, invariably associated with increased leukocyte recruitment, platelet activation, and fibrin deposition at the site of vascular injury (13).

As shown here, this mechanism of EC stimulation appears selective for leukocyte MP, because depletion of platelet MP did not decrease cytokine induction in EC, and thrombin-stimulated platelet MP did not stimulate EC IL-6 release. Although induction of EC ICAM-1 by platelet MP required treatment with phospholipase A2 (14, 15), a potential role of platelet MP on EC cytokine release has not been previously investigated (15). In contrast, EC stimulation by leukocyte MP did not require phospholipase A2 treatment, and similarly, arachidonic acid failed to stimulate IL-6 release, whereas it recapitulated EC activation by platelet MP (15). Altogether, these data suggest that EC activation by platelet (14, 15) or leukocyte (16) MP may involve separate or only partially overlapping signaling pathways.

On the other hand, fMLP stimulation was also associated with platelet MP release in vivo, in a reaction inhibited by an antibody to GPIbα. This suggests that inflammatory challenges, directly or through intercellular collaboration (33),
may result in both platelet and leukocyte MP release, thus further amplifying EC procoagulant and proinflammatory gene expression (14–16).

In investigating potential downstream signals of this EC activation pathway, we found that leukocyte MP did not stimulate NF-κB activation or promote ERK1 tyrosine phosphorylation. In contrast, MP stimulated a prominent and sustained tyrosine phosphorylation of c-Jun NH2-terminal kinase, JNK1, in EC. A member of the mitogen-activated kinase gene family, JNK1, phosphorylation through upstream activators SEK and MEKK has been observed in response to growth factors, cytokines, and stress signals, including ultraviolet lights or alkylating agents (34–37). JNK1 phosphorylation has also been implicated in apoptosis of neuronal PC12 cells after nerve growth factor deprivation, TNFα treatment, or ischemia (34, 38) and in stimulatory phosphorylation of c-Jun, a component of the transcription factor, AP-1 (39). This suggests that MP may target JNK1 activation in the context of a broad EC stress signaling response, culminating with proinflammatory, proadhesive, and procoagulant gene expression through AP-1 activation. In this context, it is of interest that AP-1 has been implicated in regulated transcription of the TF gene in endothelium (40), and in platelet-derived growth factor-dependent IL-6 gene transcription in osteoblasts (41). Whether this pathway may also influence EC apoptosis (42), thus further exacerbating vessel wall dysfunction and procoagulant activity (43), is cur-
rently unknown and may depend on the differential expression of AP-1 components or activation of other modulatory transcription factors (34, 39).

In summary, we have identified a pathway of EC signaling and gene expression centered on the release of leukocyte MP. Future studies will dissect the molecular requirement(s) of this stress response mechanism and its potential contribution to EC dysfunction in vascular diseases (13).

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