Developmental Endothelial Locus-1 (Del-1) Mediates Clearance of Platelet Microparticles by the Endothelium

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Background—Phosphatidylserine-expressing microparticles circulate in blood with a short half-life of <10 minutes. We tested the role of an endothelium-derived phosphatidylserine-binding opsonin, developmental endothelial locus-1 (Del-1), in the uptake of platelet microparticles.

Methods and Results—Cultured human umbilical vein and microvascular endothelial cells avidly engulf BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-maleimide–labeled platelet microparticles. Microparticle uptake was inhibited by a monoclonal antibody to Del-1 (P<0.027) and by annexin A5 (P=0.027), abciximab (P=0.027), a monoclonal antibody to integrin αVβ3 (P=0.027), and chlorpromazine (P=0.027). These results suggest that Del-1 mediates phosphatidylserine- and integrin-dependent endothelial uptake of microparticles by endocytosis. To assess the in vivo significance, we infused fluorescent platelet microparticles into the inferior vena cava of mice and harvested endothelial cells from the pulmonary and systemic circulation. Compared with their wild-type littermates, Del-1–deficient mice had decreased uptake in endothelial cells in lung (3.07±1.9 versus 1.09±1.3, P=0.02) and liver (2.85±1.1 versus 1.35±0.92, P=0.01). Furthermore, after endotoxin administration, Del-1–deficient mice displayed an increase in the level of microparticles compared with wild-type mice (P=0.02).

Conclusions—These studies show a physiological role for Del-1 in the clearance of phosphatidylserine-expressing microparticles by endothelium. (Circulation. 2012;125:1664-1672.)

Key Words: endothelium ■ platelet-derived factors ■ platelets

Microparticles are submicron-sized vesicles released from cells in response to activation or during apoptosis. Formation of microparticles is accompanied by externalization of phosphatidylserine, which is normally present only in the inner leaflet of the membrane bilayer. In platelets, microparticle formation and phosphatidylserine exposure have been studied extensively. Phosphatidylserine, on activated platelets and platelet microparticles, plays an essential procoagulant role in normal hemostasis by providing a surface for the assembly of enzyme-cofactor complexes. In addition to their hemostatic role, platelet microparticles have been shown to elicit cytokine responses from synovial fibroblasts via interleukin 1,5 stimulate hematopoietic cells,6 and transfer platelet-specific receptors to the surface of other cells.7 Recently, several pathophysiological roles have been identified for microparticles derived from other cells. Monocyte-derived microparticles activate endothelial cells in an interleukin-1β–dependent manner.8 Tumor-derived microparticles promote activation of macrophages9 and induce immune suppression by promoting regulatory T-cell expansion.10 Furthermore, the recent finding that circulating microparticles carry microRNA11 raises the possibility that their uptake may modify gene expression in host cells.

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Developmental endothelial locus-1 (Del-1), a 52-kDa glycoprotein (also termed Edil3) is secreted by endothelial cells and is expressed in a developmentally restricted pattern during embryogenesis. In adults, Del-1 is expressed in endothelium and in a subset of macrophages,13,14 It consists of 3 epidermal growth factor–like repeats at the amino terminus followed by 2 discoidin I–like domains at the C-terminus. The second epidermal growth factor–like repeat contains the canonical Arg-Gly-Asp (RGD) motif that enables Del-1 to bind integrins αVβ3 and αVβ5. The discoidin I–like domains mediate its binding to phosphatidylserine. Del-1 can act as a bridging molecule between integrin on endothelial cells and phosphatidylserine-containing microparticles. In addition, Del-1 acts as an inhibitor of leukocyte recruitment, and Del-1–deficient mice have increased inflammatory cell accumulation. Here, we investigated the role...
of Del-1 as a mediator of clearance of phosphatidylserine-expressing platelet microparticles by endothelium.

Methods

Reagents

BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-maleimide was purchased from Invitrogen Corp (Carlsbad, CA). Vitronectin was isolated from normal human plasma as described previously. Recombinant annexin A5 was isolated as described previously. Human thrombin was purchased from Hematologic Technologies (Essex Junction, VT). Collagen was purchased from Helena Laboratories (Beaumont, TX). Phycocyanin-labeled monoclonal anti-human CD144 and polyyclonal antibodies to CD63 were obtained from eBioscience (San Diego, CA). A rat monoclonal antibody to mouse CD144 (clone 11D4.1) and phycoerythrin-Cy5–labeled CD42B were purchased from BD Biosciences (San Jose, CA). Electron-coupled dye–labeled CD14 (clone REM052) was from Beckman Coulter (Fullerton, CA). A murine monoclonal antibody to human glycophorin (CD235a) and anti-integrin αVβ3 (clone LM609) were obtained from Millipore Corp (Billerica, MA). D345 is a monoclonal murine anti-Del-1 IgG derived from BALB/c mice expressing clone was expanded, and Del-1 was isolated by affinity screening for expression by Western blots with monoclonal anti-Myc cDNA by electroporation, and the culture supernatants were obtained from eBioscience (San Diego, CA). The amplified fragments were digested with HindIII and ligated into the plasmid vector pPICZ (Manassas, VA). Lipopolysaccharide (LPS) from Escherichia coli 0111:B4, chlorpromazine, and amiloride were from Sigma-Aldrich (St Louis, MO).

Mouse Strains

C57BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The generation of Del-1–deficient mice has been described previously. The Del-1–/– mice were rederived at Baylor College of Medicine (Houston, TX) in C57BL/6 mice and backcrossed 6 times to C57BL/6 background. The Institutional Animal Care and Use Committee of Baylor College of Medicine approved all animal protocols.

Isolation of Recombinant Del-1

The cDNA for Del-1 (OriGene Technologies, Inc, Rockville, MD) was amplified by polymerase chain reaction with the primers 5′-TTTGAATTC-GGCA-AAGG-TGAT-ATTT-GTGATCCC-3′ and 5′-GGC-GGCGG-CTTCC-TCTCTGTGC-AGGCC-CAGCAG-3′. The amplified fragments were digested with EcoRI and NotI and ligated into the plasmid vector pPICZa, which contains Myc and His tags at the C-terminal end. Pichia pastoris GS115 strain (Invitrogen) was transformed with the plasmid-containing Del-1 cDNA by electroperoration, and the culture supernatants were screened for expression by Western blots with monoclonal anti-Myc antibody (clone MYC1-9E10.2 from ATCC). Recombinant Del-1–expressing clone was expanded, and Del-1 was isolated by affinity chromatography with an Ni-NTA column.

ELISA for Del-1

A sandwich ELISA for Del-1 was developed with D345 as the capture antibody and a polyclonal rabbit antibody to Del-1. The standard curve was linear from 1 to 250 ng/mL Del-1.

Isolation, Characterization, and Labeling of Microparticles and Exosomes

Circulating microparticles from peripheral blood were isolated by differential centrifugation. Blood was drawn from healthy volunteers, after receipt of informed consent as approved by the Committee for Protection of Human Subjects. The blood was immediately transferred to polypropylene tubes, and platelet-rich plasma was obtained by centrifugation at 1000 g for 3 minutes at room temperature. The platelet-rich plasma was centrifuged at 5000 g for 15 minutes at 4°C to obtain platelet-poor plasma. The platelet-poor plasma was further centrifuged at 20 000g for 20 minutes to sediment the microparticles. The pellet containing microparticles was suspended in modified Tyrode buffer for analysis in a flow cytometer (Coulter FC500, Beckman Coulter) with CXP software. The flow cytometer was calibrated for size with nonfluorescent microspheres (Invitrogen) and with isolated unlabeled platelet microparticles. Particles <1 μm in diameter were gated as microparticles.

Uptake of Platelet Microparticles by Endothelium In Vivo

BODIPY–labeled platelet microparticles were infused into the inferior vena cava of mice under isoflurane anesthesia. After 5 minutes, the mice were euthanized, and lung endothelial cells were isolated as described by Fehrenbach et al with minor modifications. The abdomen and the thoracic cavities were exposed, and 10 mL of phosphate-buffered saline (0.1 mol/L NaCl, 0.05 mol/L phosphate,
pH 7.4) was infused via the right ventricle to perfuse the lung. One milliliter of collagenase (from Clostridium histolyticum; 10 mg/mL; Sigma-Aldrich) solution was instilled into the trachea, and the trachea was tied off. The lungs were subsequently dissected out and incubated with 5 mL of collagenase solution in a 50-mL tube for 30 minutes at 37°C in a water bath. After 30 minutes of incubation, 25 mL of phosphate-buffered saline was added, and the tube was vigorously shaken for 30 seconds; the resulting cell suspension was filtered through a cell strainer (80 mesh). The cell suspensions were centrifuged at 2000 g for 20 minutes, and the pellet was solubilized in 1% SDS, subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane, probed with the murine monoclonal antibody to Del-1, and developed by a peroxidase-labeled goat anti-mouse antibody.

Statistical Analysis
All data are expressed in a box plot with outliers identified as closed circles. Comparisons between individual groups were performed with nonparametric tests using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA). Paired experiments were compared with Wilcoxon signed rank tests; for comparisons among multiple individual groups, probability values were adjusted with a Bonferroni step-down procedure as described previously30 with SPSS software (IBM Corp). Unpaired 2-group experiments were performed with a Mann-Whitney U test. P≤0.05 was considered statistically significant.

Results
Circulating Microparticles Contain Del-1
We tested for the presence of Del-1 on the surface of circulating peripheral blood microparticles with a murine monoclonal antibody to human Del-1 (D345) by flow cytometry. Del-1 was readily demonstrated on the surface of microparticles (Figure 1A). Prior treatment with the detergent Triton X-100 decreased the reactivity to >75%, which indicates that most of the signals were derived membrane-enclosed structures.21 The microparticles were resolved according to the cell of origin (endothelial cell, monocyte, platelet, or red cell) based on expression of cell-specific markers. Del-1 was present in all populations; expressed as a percentage of baseline values and analyzed at 10, 20, and 30 minutes. The investigators performing and analyzing thrombosis experiments remained blinded as to the mouse genotype.
(32±6%), followed by platelets (27±3%), monocytes (7±2%), and endothelium (5±1.2%; Figure 1B). Furthermore, microparticle-bound Del-1 was detected in peripheral blood microparticles by immunoblotting with the monoclonal antibody D345 (Figure 1C).

**Endothelial Cells Internalize Platelet Microparticles**

Del-1 has been shown to be associated with the surface of endothelial cells.16 We examined whether endothelial cells in culture secrete Del-1. To this end, the serum-free culture supernatant from HUVECs was examined by immunoblotting. A single band at 52 kDa was detected in the culture medium (Figure 2). Using an ELISA, we detected 2.6±2.8 ng/mL Del-1 in endothelial cell conditioned medium. In addition, we also measured 78±23 ng/mL in the plasma of healthy individuals, presumably derived from endothelium. To determine whether endothelium can internalize platelet microparticles, we isolated platelet microparticles and endosomes from washed human platelets (Figure 3). Endothelial cells were incubated with BODIPY-labeled platelet microparticles, and morphological evidence of uptake was obtained by confocal fluorescence microscopy. To distinguish between cell-surface binding and actual internalization, extracellular and cell-surface fluorescence was quenched by addition of trypan blue (0.4 mg/mL), and the unquenched fluorescence, which represented the internalized microparticles, was examined for cytoplasmic fluorescence.31 Platelet microparticles were avidly engulfed by the endothelial cells (Figures 4A and 4B). Initially, the microparticles were distributed diffusely throughout the cytoplasm. Within 15 minutes, the microparticles were localized predominantly to the periphery, to 1 pole of the cells. Uptake was complete within the time interval of 30 minutes. Virtually no internalization was seen at 4°C.

We quantified the internalization of BODIPY-labeled platelet microparticles by measuring the cytoplasmic fluorescence associated with endothelial cells by flow cytometry after quenching the cell-surface fluorescence with trypan blue. Uptake was dependent on the amount of microparticles added (Figure 5). In HUVECs (Figure 6A), the monoclonal antibody to Del-1 inhibited uptake compared with the buffer control (2.5±0.6 versus 3.6±1.0; adjusted \(P=0.027, n=9\)); under similar conditions, an irrelevant control monoclonal antibody had no significant effect (3.3±0.8 versus 3.6±1.0; adjusted \(P=0.027, n=9\)). In human microvascular endothelial cells (Figure 6D), the Del-1 antibody also inhibited uptake (2.8±0.6 versus 5.4±0.5; adjusted \(P=0.02, n=9\)). Because Del-1 has the integrin-binding RGD motif, we examined the effect of...
LM609, a monoclonal antibody to integrin αVβ3. LM609 inhibited the uptake (adjusted \( P=0.03 \)). Abciximab, a human–mouse hybrid Fab fragment that reacts with platelet integrin αIIbβ3 and endothelial cell integrin αVβ3, also inhibited uptake compared with buffer control in HUVECs (2.06±0.9 versus 3.6±1.0; adjusted \( P=0.027, n=9 \)) and human microvascular endothelial cells (3.08±0.7 versus 5.3±0.5; adjusted \( P=0.02, n=9 \)) respectively (Figures 6A and 6D). Furthermore, annexin A5 also inhibited internalization in HUVECs (1.76±0.4 versus 3.6±1.0; adjusted \( P=0.027, n=9 \)) and human microvascular endothelial cells (2.8±0.7 versus 5.3±0.5; adjusted \( P=0.02, n=9 \)), respectively (Figures 6A and 6D). The effects of annexin A5 and abciximab were not additive. Accordingly, these results show the phosphatidylserine- and integrin-mediated uptake of microparticles by the endothelium. The Del-1–dependent uptake was not restricted to platelet microparticles. Phosphatidylserine-expressing microparticles from red blood cells are also endocytosed in a Del-1– and integrin-dependent manner (Figure 6C). In contrast, under similar conditions, the uptake of labeled exosomes was not inhibited by Del-1 antibody (Figure 6B).

To characterize the uptake pathway, we incubated HUVECs for 30 minutes with compounds that inhibit particle uptake, namely, chlorpromazine, which inhibits clathrin-dependent endocytosis, or amiloride to block macropinocytosis. Chlorpromazine (10 μmol/L) decreased the intracellular content of labeled vesicles compared with the buffer (1.17±0.4 versus 3.6±1.0; adjusted \( P=0.027, n=9 \)), whereas under similar conditions, amiloride caused a slight decrease (Figure 6A). Thus, the clathrin-dependent endocytotic process plays a predominant role in the uptake process.

**Del-1–Deficient Mice Have Defective Uptake of Platelet Microparticles by Endothelial Cells In Vivo**

To assess the in vivo significance, we infused labeled platelet microparticles into the inferior vena cava of anesthetized mice. After 5 minutes, the mice were euthanized, and endothelial cells from lung, liver, and spleen were isolated and examined by flow cytometry. We compared the cytoplasmic fluorescence of endothelial cells from Del-1–deficient mice to the fluorescence in endothelial cells from their wild-type littermate controls, which were subjected to the same experimental procedure simultaneously (Figure 7). Del-1–deficient mice had significantly less fluorescence than wild-type mice in pulmonary endothelial cells (1.09±1.3% versus 3.0±2.0%; \( P=0.02, n=9 \)) and in liver endothelial cells in 9 separate pairs of experiments (2.85±1.1 versus 1.35±0.9; \( P=0.01, n=9 \)). There was no significant difference in uptake by splenic endothelial cells. These experiments show that Del deficiency leads to impaired uptake of microparticles by lung and liver endothelial cells in vivo.

**Increased Circulating Microparticles After LPS Administration in Del-1–Deficient Mice**

Del-1–deficient mice have normal levels of microparticles compared with their wild-type littermate controls. Endotoxemia induces increased microparticles in the blood in human and in mice. \(^{32,33}\) Hence, we tested the effect of LPS administration on the level of circulating microparticles in Del-1–deficient mice and their littermate controls. As seen in Figure 8, LPS induced significantly higher levels of microparticles in Del-1–deficient mice \( (P=0.02, n=9) \) than in their wild-type littermate controls when measured 6 hours after administration.

In spite of the increased microparticles, when tested in a carotid artery injury/thrombosis model, no significant differ-
ence was observed between Del-1–deficient mice and their littermate controls. Cessation of blood flow occurred in 4 of 12 Del-1–deficient mice and 5 of 11 littermate controls. Carotid blood flow, expressed as a percentage of baseline values, did not differ statistically between Del-1–deficient mice and littermate controls at 10 minutes (62 ± 100% versus 45 ± 14%), 20 minutes (52 ± 11% versus 45 ± 13%), or 30 minutes (52 ± 12% versus 40 ± 12%).

**Discussion**

Circulating microparticles were first described in 1967 as platelet membrane fragments detected in human plasma. Although initially considered as cell debris, a central role in hemostasis has been identified for platelet microparticles. Microparticles from different cells have been identified in the circulating blood. Despite their physicochemical characterization, very little is known about the fate of microparticles in the circulation. Phosphatidylserine-expressing platelet microparticles have a short half-life of <10 minutes when infused, which implies that microparticles are continuously formed in the circulation. The mechanism of clearance of platelet microparticles from the circulation is not well understood; some clues may be obtained from studies on the clearance of phospholipid liposomes, which have been used extensively as vehicles for drug delivery. The clearance of liposomes is affected markedly by the lipid composition of the liposomes; phosphatidylserine markedly enhances the clearance rate of liposomes. Even in intact red blood cells, incorporation of as little as 3% mol of phosphatidylserine in the outer leaflet results in rapid clearance.

The present experiments show that Del-1 secreted by the endothelium plays a role in the clearance of phosphatidylserine-expressing microparticles. Del-1 has been demonstrated on the endothelial cell surface, and we have detected 78 ± 23 ng/mL Del-1 in the plasma of healthy control subjects, presumably derived from endothelium. The endothelium

![Figure 6. Inhibition of uptake of microparticles by endothelium.](http://circ.ahajournals.org/)

Discrete uptake of platelet microparticles by endothelium. Washed human umbilical vein endothelial cells (HUVEC; A, B, and C) or human microvascular endothelial cells (HMVEC; D) were incubated with BODIPY-labeled platelet microparticles (A and D), platelet exosomes (B), or red blood cell (RBC) microparticles (C) in the presence of various agents. The cells were washed and then detached with trypsin-EDTA, and cell-surface fluorescence was quenched with trypan blue. Cytoplasmic fluorescence was analyzed by flow cytometry. The effects of various agents were compared with buffer alone by Wilcoxon signed rank test, based on 9 separate experiments. P values shown in the Figure reflect adjustment for multiple comparisons by Bonferroni step-down procedure; the raw significant P values ranged from 0.004 to 0.009. NS indicates not significant.
Spleen has a unique open circulation designed to filter phagocytic functions. Furthermore, spleen is rich in lactadherin, and splenic endothelial cells are endowed with phagocytic activity. Platelets release exosomes on activation, and platelet-derived exosomes can modulate endothelial cell functions under certain experimental conditions. However, exosomes do not express phosphatidylserine on their surface, which is consistent with the fact that the Del-1 antibody does not inhibit their uptake by endothelium. Activated platelets and endothelial cells also generate a significant proportion of microparticles that do not express phosphatidylserine, and the clearance of these microparticles will be Del-1 independent.

Figure 8. Increased microparticles in developmental endothelial locus-1 (Del-1)–deficient mice. Plasma was collected from Del-1–deficient mice or their littermate wild-type controls, and microparticles were quantified by flow cytometry. P values are derived from 9 separate experiments, based on Mann-Whitney U test. NS indicates not significant.

Exosomes are 40- to 100-nm membrane vesicles secreted by most cell types that contain proteins involved in membrane transport or vesicle biogenesis, as well as different heat shock proteins, tetraspanins, and integrins. Platelets release exosomes on activation, and platelet-derived exosomes can modulate endothelial cell functions under certain experimental conditions. However, exosomes do not express phosphatidylserine on their surface, which is consistent with the fact that the Del-1 antibody does not inhibit their uptake by endothelium. Activated platelets and endothelial cells also generate a significant proportion of microparticles that do not express phosphatidylserine, and the clearance of these microparticles will be Del-1 independent.

The uptake of microparticles by the endothelium mainly involves the clathrin-dependent endocytic pathway, because chlorpromazine inhibited this process to 20%, whereas amiloride (an inhibitor of micropinocytosis) did not significantly decrease uptake. These data suggest that the clathrin-dependent endocytic pathway plays a major role in uptake. Because microparticles are heterogeneous in size and in the expression of surface markers, depending on the cells of origin, it is likely that multiple distinct pathways are engaged in their uptake.

Antibodies to Del-1 inhibit microparticle uptake only in part, and Del-1–deficient mice have normal levels of microparticles under baseline conditions. Furthermore, in a carotid artery injury model, we could not demonstrate a hypercoagulable state despite an increase in microparticles. The phosphatidylserine-dependent phagocytic process is also involved in clearance of apoptotic cells and plays a vital role in organogenesis and tissue remodeling. For important homeostatic mechanisms, redundant mechanisms or parallel systems must necessarily exist. Therefore, it will not be surprising to find other mediators, which can compensate in part for Del-1 deficiency. Several phosphatidylserine-binding molecules, such as β2-glycoprotein I and lactadherin, are associated with circulating microvesicles. In fact, multiple phosphatidylserine-dependent phagocytic receptors that mediate clearance of phosphatidylserine-expressing apoptotic cells have been identified on macrophages. Many of these receptors and opsonins are also present in endothelial cells and may play a role in the clearance of microparticles. Thus, Del-1 deficiency may contribute to the procoagulant state only at times of pathological increases in microparticle generation.

In summary, we have identified Del-1 as one of the mediators of microparticle clearance in vivo.

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Disclosures
None.
References


Microparticles are submicron-sized, membrane-enclosed fragments released from cells in response to activation or during apoptosis. Platelet-derived microparticles, released on platelet activation, constitute a major fraction of microparticles in the circulating blood. Platelet microparticles have a procoagulant function in normal hemostasis because of the expression of phosphatidylserine on their surface. In addition, platelet microparticles have been shown to stimulate hematopoietic cells, transfer platelet-specific receptors to the surface of other cells, and elicit cytokine responses from synovial fibroblasts. Microparticles are rapidly cleared from the circulation, and their fate is largely unknown. In flowing blood, microparticles are pushed toward the plasma-endothelial interface because of their size, and they are poised to interact with the endothelial cell surface. Here, we studied the clearance of microparticles by endothelium and the role of developmental endothelial locus–1 (Del-1), a 52-kDa glycoprotein (also termed Edil3) secreted by endothelial cells. Del-1 binds to platelet microparticles (via phosphatidylserine) and anchors them to integrins on cultured endothelial cells (via the RGD motif) for efficient endocytosis. After infusion of fluorescent microparticles in vivo, Del-1–deficient mice have an impaired uptake by the endothelium. In addition, Del-1–deficient mice also have increased microparticles after endotoxin administration. These results suggest that microparticles are taken by the endothelium and that Del-1 functions as a physiological mediator of microparticle clearance by the endothelium.
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