Mast Cell–Derived Exosomes Activate Endothelial Cells to Secrete Plasminogen Activator Inhibitor Type 1
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Objective—Previous studies supported the contribution of exosomes to an acellular mode of communication, leading to intercellular transfer of molecules. In this study we provide evidence that mast cell-derived exosomes induce plasminogen activator inhibitor type 1 (PAI-1) expression in endothelial cells, detectable at the level of PAI-1 mRNA and protein synthesis. The stimulating effect was also measured at the level of PAI-1 promoter activity.

Methods and Results—To identify components responsible for this activity, exosome proteins were separated by 2-dimensional PAGE, and protein spots were identified by microsequencing using electrospray (ISI-Q-TOF-Micromass) spectrometer. Components of 3 independent systems that can be involved in activation of endothelial cells, namely the prothrombinase complex, tumor necrosis factor-α, and angiotensinogen precursors were identified. Procoagulant activity of exosomes was confirmed by a thrombin generation assay using a specific chromogenic substrate. Because the potential of mast cell-derived exosomes to induce PAI-1 expression was completely abolished by hirudin, thrombin generated on exosomes seems to be responsible for this activity.

Conclusions—It can be concluded that mast cell-derived exosomes via significant upregulation of PAI-1 secretion from endothelial cells may provide feedback between the characteristically increased PAI-1 levels and procoagulant states, both observed in diverse syndromes manifesting as endothelial cell dysfunction. (Arterioscler Thromb Vasc Biol. 2005; 25:1744-1749.)

Key Words: exosomes ■ mast cells ■ plasminogen activator inhibitor type 1 ■ prothrombinase complex

Formation of exosomes is a common mechanism of membrane shedding by activated cells.1–3 Circulating formed elements, like platelets and leukocytes, have been shown to constitutively shed microparticles, the process that is enhanced during cardiopulmonary bypass,4 unstable angina and lacunar infarcts5,6 or diabetes mellitus,7 and contributes to procoagulant state in these and other conditions.

Mast cells exert profound pleiotropic effects on immune cell reactions at inflammatory sites, where they are most likely influenced not only by the extracellular matrix and inflammatory mediators but also by the proximity of activated T lymphocytes. These cells have been implicated in 2 contrasting types of immune responses, the immediate hypersensitivity reactions associated with allergic phenomena and their acute activation by bacterial products during infection.8 Mast cells are localized near blood vessels and are involved in the activation of the clotting system during inflammation to contain the injury and initiate tissue repair. This concept is supported by studies of the reverse passive Arthus reaction in mast cell-deficient mice cells, which showed that these cells contributed to the exudation of clotting factors resulting in fibrin deposition and enhancement of fibrin cross-linkage.9,10

In view of the role of mast cells in deposition of fibrin during inflammation near the site of injury, the aim of the present study was to examine the effect of mast cell-derived exosomes on expression and secretion of plasminogen activator inhibitor type 1 (PAI-1) from endothelial cells. Because exocytosis is the process by which stimulation of plasma membrane receptors on secretory cells results in the release of proteins and/or peptides from the intracellular stores into the extracellular space,11 we attempted to identify active components of exosomes by proteomic analysis. Our results show that mast cell-derived exosomes contain all proteins required to attach to endothelial cells and that their activation may be accomplished through the potential involvement of mast cell-derived exosomes in the pathogenesis of endothelial cell dysfunction.

Materials and Methods
Elisa-Imulyse PAI-1 immunoenzymatic kit was from Biopool. All standard tissue culture reagents including DMEM, fetal bovine serum (FBS), and LipofectAMINE Plus reagent were from GIBCO. The Luciferase Assay System was from Promega. Plasmid p800LUC with PAI-1 promoter was obtained as a gift from Dr David J. Loskutoff from the Department of Cell Biology, The Scripps Research Institute, La Jolla, California. Protein assay reagents and polyacrylamide gel chemicals were from BioRad. Reagents for 2-dimensional gel electrophoresis were from Amersham Biosciences.
Cell Cultures
Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords by collagenase treatment and cell cultures were maintained as described previously. For the experiments, confluent cultures used at the third passage. Human endothelial cell line EA.hy 926, derived by fusion of HUVECs with continuous human lung carcinoma cell line A549, was obtained as a gift from Professor Cora-Jean S. Edgell (Pathology Department, University of North Carolina at Chapel Hill). This endothelial hybrid cell line is presently the best-characterized macrovascular endothelial cell line (for review see Bouis et al).

The cells were cultured in DMEM with high glucose, supplemented with 10% FBS, HAT (100 μmol/L hypoxanthine, 0.4 μmol/L aminopterin, and 16 μmol/L thymidine), and antibiotics in a 90% to 95% humidified atmosphere of 5% CO2 at 37°C. Monocytic cells (U937) cells were from American Type Culture Collection (Rockville, Md) and cultured as described by the supplier.

Purification of Exosomes
Exosomes were isolated from media of HMC-1 cultures, as previously described. HMC-1 cells used in these studies were obtained from Dr Gunnar Nilsson (Uppsala University) and recently characterized. HMC-1, a cell line established from peripheral blood cells of a patient with mast cell leukemia, expresses a juxtamembrane activator.

mRNAs for PAI-1, von Willenbrand factor, tissue-type plasminogen activator, α1-acid glycoprotein, and β-actin, respectively. All samples were incubated at 50°C for 2 minutes and at 95°C for 10 minutes and cycled at 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute for 40 cycles.

SYBR Green I fluorescence emission data were captured and mRNA levels were quantified using the critical threshold (C) value. Analysis was performed with ABI Prism 7000 (SDS Software). Controls without reverse-transcription and with no template cDNA were performed with each assay. To compensate the variations in input RNA amounts and efficiency of reverse-transcription, β-actin mRNA was quantified and results were normalized to these values. Relative gene expression levels were obtained using the ΔΔCt method. Amplification-specific transcripts were further confirmed by obtaining melting curve profiles.

Measurements of PAI-1 Antigen
HUVECs, EA. hy926, or U937 cells were planted in 48-well microplates at the density of 2×104 cells/mL. The next day, they were starved overnight in medium containing 0.1% FBS and then stimulated for 24-hour with different concentrations of exosome proteins. Afterward, the supernatants were collected and assayed for PAI-1 antigen by enzyme-linked immunoassorbent assay using the Elisa-Imulysa PAI-1 kit.

Transfection of EA. hy 926 cells
Semiconfluent cells cultures (EA. hy926) in 6-well tissue culture plates were transfected with plasmid p800LUC with PAI-1 promoter using the lipofectAMINE method. Cells were transfected with 4 μg of p800LUC with PAI-1 promoter or its mutated form (p800LUCmut) and 5 μg of pSV vector containing the β-galactosidase gene to evaluate transfection efficiency. For exosome studies, increasing concentrations of exosome proteins were added to wells, and the cells were incubated for 24 hours, washed, and harvested in the lysis buffer. After centrifugation for 5 minutes at 4°C, the supernatants were transferred into fresh vials and used for enzymatic assays. Luciferase assay kits (GIBCO) were used according to the manufacturer's instructions using a 1420 Multilabel Counter–Victor (Wallac, Perkin Elmer). β-galactosidase activity from a constitutively expressed internal control was assayed with a β-galactosidase Enzyme Assay System (GIBCO) according to the manufacturer's instruction, and with an EL340 plate reader (BIO-TEK Instruments, Inc). In parallel experiments, EA. hy926 were transfected with luciferase reporter vector pGL3 (Promega) and used as control cells to test whether the effect of inhibitors was specific for the PAI-1 promoter.

Sample Preparation and 2-Dimensional Polyacrylamide Gel Electrophoresis
Exosomes obtained from HMC-1 were solubilized using Lysis Buffer (9 mol/L urea, 1% CHAPS, 1% DTT, 2% Phenolm, and Complete Inhibitors; Roche). A buffer volume approximately equal to the packed cell volume was used. To improve resolution and recovery of proteins in 2-dimensional gel electrophoresis, cell extracts were treated with a PlusOne 2D Clean-up Kit (Amersham Biosciences). Concentration of proteins in the supernatant was determined using PlusOne 2D Quant Kit and bovine serum albumin as a standard. Exosome lysates were stored at ~70°C until analyzed. Proteins were separated by 2-dimensional gel electrophoresis using ready-made gels with immobilized pH gradients (Amersham-Biosciences). For the first dimension, samples containing 100 μg of soluble proteins in the Lysis Buffer were mixed with the IPG Reswelling Solution (8 mol/L urea, 1% CHAPS, 0.1% DTT, 0.5% Phenolm) to obtain the final volume of 450 μL. Then, they were loaded onto 24-cm immobilized pH linear gradient strip gels (pH 3 to 10). IEF strips were allowed to rehydrate for 5 hours and isoelectric focusing was performed according to the manufacturer's protocol by a gradual increase of voltage (30 V for 5 hours, 500 V for 1 hour, 1000 V for 1 hour, followed by 70 kVh at 8000 V) using an IPGphor system (Amersham-Biosciences). SDS electrophoresis was performed on 12.5% polyacrylamide gels using the Ettan Dalt
vertical system. Protein spots were visualized by staining with silver according to the method compatible with the analysis of proteins by mass spectrometry. Major spots were excised from the gel and subjected to in-gel digestion with trypsin.

Mass Spectrometry, Data Base Search, and Data Processing
Proteins in each gel slice were subjected to reduction with 10 mmol/L dithiothreitol, alkylation with 50 mmol/L iodoacetamide, and trypsin digestion with the modified trypsin (10 μg/mL; Promega) at 37°C for 14 hours. After in-gel digestion, the product peptides were extracted stepwise with 3 portions of 60 mL 0.1% TFA-2% acetonitrile and loaded on an RP-18 pre-column (LC Packings). Peptides were eluted to a nano–high-performance liquid chromatography RP18 column (75 μm x 15 cm capillary; LC Packings) by acetonitrile gradient in the presence of formic acid and directly applied into an electrospray (ISI-Q-TOF-Micromass) spectrometer. The spectrometer was working in the regime of data-dependent MS to MS/MS switch, giving peptide sequencing data in addition to mass fingerprint data. NCBI nonredundant protein database was searched with the Mascot program (Matrix Science). The list of top 20 candidates for each sample was verified manually by inspection of the quality of sequencing data. We examined their automatic ordering manually in terms of their reliability scores and MS spectrum profiles to pick up only highly reliable peptide data (sorted data).

Procoagulant Activity of Mast Cell-Derived Exosomes
The ability of exosomes to generate thrombin was analyzed using a modification of the measurement of the procoagulant activity of endothelial microparticles. Exosome samples, 50 μL, were incubated in a final volume of 200 μL at 37°C in the presence of 0.3 mmol/L Spectrozyme fXa (methoxy carbonyl-D-hexahydroxy eryosyl-D-Ala-D-Arg-p-nitroanilide diacetate; American Diagnostica, Greenwich, Conn) or Chromozym-TH (D-Phe-L-Pro-L-Arg-p-nitroanilide; Roche Diagnostics) in HEPES-buffered saline, pH 7.4, 0.1% BSA, and either 5 mmol/L CaCl2 or 5 mmol/L EDTA. Factor Xa activity and thrombin generation was monitored by continuous measurement of absorbance at 405 nm. The chromogenic assay was standardized with pure human α-thrombin (20 nmol/L). Activities are expressed as the rate of absorbance change in the chromogenic assay, which is proportional to thrombin concentration.

Results
Induction of PAI-1 Expression by Mast Cell-Derived Exosomes
Mast cells are found in atherosclerotic lesions, which are known to have consistently increased PAI-1 expression. Therefore, in current experiments, we analyzed the effect of mast cell-derived exosomes on PAI-1 synthesis and secretion from endothelial cells, both primary HUVECs and immortalized endothelial cell line EA. hy926. In parallel experiments we used U937 as control cells. Exosomes were purified by

Figure 1. Mast cell-derived exosomes induce expression of PAI-1 mRNA in cells. A, B, and C, Changes in mRNA levels in HUVECs, EA. hy926 cells, and U937 cells, respectively. In these experiments, cells were stimulated for 24 hours with different concentrations of exosome proteins, and then total cellular RNA was extracted. mRNAs of PAI-1 and control proteins, such as α1-acid glycoprotein, tissue-type plasminogen activator, and von Willenbrand factor, were quantified by real-time PCR. This experiment repeated 3 times with similar results.

Figure 2. Mast cell-derived exosomes induce PAI-1 secretion from cells. A, Release of PAI-1 from HUVECs (●), EA. hy926 cells (○), and U937 cells (○). These cells planted in 48-well microplates were starved overnight in medium containing 0.1% fetal bovine serum, and then stimulated for 24 hours with different concentrations of exosome proteins. Afterward, the supernatants were collected and assayed for PAI-1 antigen by enzyme-linked immunosorbent assay using an Elisa-Lumlyse PAI-1 kit. The graph shows the average:SD of duplicate measurements performed during 3 experiments. B, Changes in PAI-1 promoter activity induced by exosomes. EA. hy 926 cells were transiently transfected with p800LUC. Eighteen hours after the transfection, cells were washed and transferred to DMEM media containing 0.5% BSA. After 24 hours, exosomes were added at the indicated protein concentration and the cells were harvested after an additional 7 hours. Luciferase and β-galactidase activities were assayed as described in Materials and Methods. Values are expressed as relative light units (RLU) of luciferase normalized to β-galactidase activity with data of 2 experiments shown as the mean of 3 separate samples plus the SE.
positions from containing the PAI-1 promoter fragment corresponding to transfected with high efficiency (transfection of the PAI-1 promoter. Figure 2A). To determine the molecular level at which PAI-1 expression can be regulated, we used a transient transfection approach to ask if exosome components can influence the activity. Figure 1 shows that treatment of the cells with exosomes specifically increased PAI-1 mRNA signal, as determined by real-time PCR measurements. There was no induction of mRNAs for tissue-type plasminogen activator, von Willebrand Factor, or β-actin. There was also a significant induction of α1-acid glycoprotein mRNA, which we recently described to form a complex with active PAI-1 and stabilize its activity.23 The stimulating effect of exosomes on PAI-1 synthesis was further evidenced by measuring the amount of PAI-1 released to culture media from these cells, as determined by enzyme-linked immunosorbent assay (Figures 2A and 2B). To determine the molecular level at which PAI-1 expression can be regulated, we used a transient transfection approach to ask if exosome components can influence the transcription of the PAI-1 promoter. EA. hy926 cells were transfected with high efficiency (>80%) with p800LUC containing the PAI-1 promoter fragment corresponding to positions from −800 to +71 and the firefly luciferase gene as a reporter. Incubation of such cells with the increasing doses of exosome proteins for 24 hours resulted in significant activation of the PAI-1 promoter, as reported by luciferase activity (Figure 2B). This demonstrates that mast cell-derived exosomes contain components that primarily regulate PAI-1 gene expression at the point of transcription.

Proteomic Analysis of Exosome Proteins

To identify these components, total proteins were extracted from mast cell-derived exosomes and separated by 2-dimensional gel electrophoresis. The first dimension was performed using pH ranging from 3.0 to 10.0, and 12.5% gels were used for the second dimension. After silver staining, protein spots were excised and subjected to microsequencing using an electrospray spectrometer. Figure 3 shows representative 2-dimensional gel obtained after the separation of exosome proteins with locations of those studied in this work (Table). Criteria for positive protein identification were set as follows: at least 3 matching peptide sequences, molecular weight of identified protein should match estimated values, and top score given by software not <70. Using these criteria, we found ~400 proteins to be components of mast cell-derived exosomes. Proteomic analysis showed that mast cell-derived exosomes concentrate several components that may be responsible for the activation of PAI-1 expression, particularly tumor necrosis factor-α precursor, angiotensinogen, and prothrombinase complex components, such as factor V and prothrombin. Although random sampling for proteomic analysis of the exosome proteins did not reveal factor Xa, its presence in exosomes is evidenced by procoagulant activity detected by chromogenic assay using Spectrozyme FXa, specific for factor Xa (Figure 4A). Incubation of exosomes with Spectrozyme FXa resulted in a cleavage of the chromogenic substrate. This amidolytic activity was inhibited by p38/50 MAPK inactivator.

Selected Protein Components of Mast Cell–Derived Exosomes

Table 1. Selected Protein Components of Mast Cell–Derived Exosomes

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Whole exosome protein extracts were separated by 2-dimensional electrophoresis and protein spots identified after silver staining were analyzed by sequencing as described in Materials and Methods. Annotation indicates annotations in the data bases for assigned proteins; gi, gi accession numbers of the identified proteins in the protein database; mass, predicted molecular masses of identified proteins; peptide matches, numbers of peptides identified from the amino acid sequences of assigned proteins; TNF, tumor necrosis factor; total score, total probability scores from the Mascot search for identified proteins.
by the synthetic direct factor Xa inhibitor DX-9065a. Furthermore, we determined in vitro activity of exosomes in thrombin generation assay using Chromozym-TH. As shown in Figure 4B, incubation of increasing amounts of mast cell-derived exosomes with the chromogenic substrate resulted in a significantly increased rate of thrombin generation, proving that they contain the entire prothrombinase complex and anionic phospholipids. This effect was in part dependent on the presence of Ca ions, indicating that in addition to the prothrombinase, exosomes may contain active thrombin as well. To evaluate whether under this conditions thrombin is involved in stimulation of PAI-1 synthesis, in subsequent experiments exosomes were pre-incubated with thrombin specific inhibitor, hirudin, and then used to treat endothelial cells. Figure 5 shows that neutralization of thrombin completely abolished the stimulating effect of exosomes on PAI-1 measured at the level of mRNA and protein synthesis.

**Discussion**

Mast cells are considered to be major players in IgE-mediated allergic responses but have also recently been recognized as active participants in both innate and specific immune responses. They are able to activate B and T lymphocytes through the release of nanometer-sized vesicles called exosomes. Although emphasis has been placed on their involvement in immune modulation, exosomes’ potential for more wide-ranging biological effects has also been reported. In this report, the effect of mast cell-derived exosomes on PAI-1 expression in endothelial cells was analyzed. The reason to perform such studies is supported by observations that increased numbers of mast cells are found in atherosclerotic lesions, particularly in fatty streaks and shoulder regions of atheromas. These structures are known to have consistently increased PAI-1 expression. Our present data show that mast cell-derived exosomes induce PAI-1 expression in endothelial cells at the level of mRNA, protein synthesis, and a promoter activity. Furthermore, proteomic analysis showed that these exosomes consist of several components that are known to activate PAI-1 expression. First, they contain tumor necrosis factor-α precursor, which can be converted into an active cytokine. Tumor necrosis factor-α is one of the most potent stimulators of PAI-1 and plays an important role in the regulation of PAI-1 synthesis in endothelial cells during inflammation. Second, they contain substantial amounts of angiotensinogen, which can be converted into angiotensin II, a vasoactive peptide that exerts a variety of effects on vascular cells and tissues, including activation of PAI-1 expression. Third, they contain components of the prothrombinase complex, factor V and prothrombin, which, on activation, lead to generation of thrombin. Thus, similar to endothelial microparticles, mast cell-derived exosomes have procoagulant properties. Because thrombin is known to stimulate PAI-1 synthesis, mast cell-derived exosomes may provide a link for an intimate relationship between thrombin and PAI-1 generation under inflammatory conditions. Interestingly, the rate of in vitro thrombin generation is accelerated significantly by microparticles obtained from the cultured HUVECs pretreated with PAI-1. However, mast cell-derived exosomes significantly induce PAI-1 expression in endothelial cells and its secretion, providing feedback between the characteristically elevated PAI-1 levels and procoagulant state, both observed in diverse syndromes.
manifesting as endothelial cell dysfunction. However, because these data were obtained using HMC-1, it remains to be confirmed whether they apply to other human mast cells.

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