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Microparticle Surface Proteins are Associated with Experimental Venous Thrombosis: A Preliminary Study

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SUMMARY

Microparticles (MPs) are small membrane vesicles released from activated cells and are associated with thrombosis and inflammation. Microparticles contain a unique subset of surface proteins derived from the parent cell and may be responsible for their diverse biological functions. To identify these proteins juvenile baboons (*Papio anubis*, n=4) underwent iliac vein thrombosis with six-hour balloon occlusion. Plasma samples were taken at baseline and at 2 days post thrombosis for MP analysis. Microparticles were extracted from platelet-poor plasma, digested separately with trypsin and tagged using iTRAQ reagents. The digests were subjected to 2-D LC separation followed by MALDI tandem mass spectrometry. Peak lists were generated and searched against all primate sequences. For protein identity, a minimum of two peptides at 95% confidence was required. Later, iTRAQ ratios were generated comparing relative protein level of day-2 to baseline. The proteomic analysis was performed twice for each blood sample, totaling 8 experiments. Proteins were considered elevated or depressed if the iTRAQ ratio deviated by 20% change from normal and a p-value less than 0.05. Significantly, 7 proteins were differentially expressed on day-2 compared to baseline, and appeared in at least two animals and regulated in at least 4 experiments, and appeared in at least three animals and regulated in at least four experiments. Among these 7 proteins, up-regulated proteins include various forms of fibrinogen and alpha-1-antichymotrypsin, and down-regulated proteins include immunoglobulins. These proteins influence thrombosis and inflammation through hemostatic plug formation (fibrinogen), inhibiting neutrophil adhesion (alpha-1-antichymotrypsin), and immunoregulation (immunoglobulins). Further studies are needed to confirm the mechanistic role of these proteins in the pathogenesis of venous thrombosis.

Keywords

microparticles; microparticle surface proteins; proteomics

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INTRODUCTION

Venous thrombosis is a significant health problem in this country. The pathophysiology of venous thrombosis has traditionally included stasis of the blood, hypercoagulability and changes in the vein wall (Virchow's triad). Recently, the role of inflammation in thrombosis has become better defined. One of the elements at the interface of inflammation and thrombosis is prothrombotic microparticles (MPs) which act to concentrate and contribute to thrombogenesis. Microparticles are fragments of phospholipid membrane released from activated prothrombotic cells and thought to contain no genetic materials. However, recent findings suggest that some MPs contained RNA acquired by horizontal gene transfer.¹ Microparticles may concentrate certain surface proteins, and have been characterized by evaluating platelet-derived and plasma-derived MPs obtained from healthy individuals.^{2, 3} However, there has been no description of MP protein up-regulation or down-regulation with actual venous thrombosis.

In the present study we hypothesize that MPs contain surface proteins that are regulated during the course of venous thrombosis and these proteins are responsible for directing MP activity in thrombogenesis. Therefore, the role of MPs can be elucidated by identifying and understanding the nature of these surface proteins. With that in mind, the purpose of this investigation was to determine the nature of the proteins which are both up-regulated and down-regulated on the MP surface in animals with experimentally induced venous thrombosis.

MATERIALS AND METHODS

Blood Collection and Isolation of Microparticles

Juvenile baboons (*Papio anubis*, n=4) underwent iliac vein thrombosis with temporary 6-hour balloon occlusion as previously described.⁴ The venous physiology of baboons is analogous to human and this fact was our justification for using baboons as our subject. Of all animals, one animal had a non-occlusive thrombus while the rest had occlusive thrombus. Blood samples taken at baseline and two days post thrombosis were evaluated for MP proteins. Due to the nature of this study aiming to elucidate the role of microparticles in early thrombogenesis blood samples were collected 2 days after induction of venous thrombosis. Primates had an 8.5-mL tube of whole blood drawn into 10% acid citrate dextrose (ACD) by butterfly antecubital stick. Platelet poor plasma (PPP) was obtained by centrifuging blood at 1,500×g and room temperature for 25 min, transferring the plasma to another tube and centrifuging it once more for 2 min at 15,000×g, to remove contaminating cells from the plasma. PPP (4-mL) obtained from each subject was stored in 1-mL aliquots at -70°C for 12 and 24 months. For proteomic evaluation, PPP was thawed and 400µL was spun down in 1-mL of HEPES buffer [10 mM Hepes/5 mM KCL/1 mM Mg CL₂/136 mM NaCl (pH 7.4)] for 2 h at 4°C, 35,000 RPM. Supernatant was removed and MP pellet was resuspended in 400-µl of 0.25M KBr and incubated on ice for 20 min. Samples were then spun down for 2 h at 4°C, 35,000 RPM. Supernatant was removed and pellet air dried before being resuspended in 250-µl of 1X PBS. The previous steps involving suspension in KBr followed by centrifugation were performed in the last two animals to remove soluble serum proteins. Protein concentration was determined using a standard colorimetric BCA total protein assay (Pierce, Rockford, IL).

Protein Isobaric Labeling with iTRAQ Reagents

Microparticles from 200 µL PPP were suspended in 20-µL 0.5M triethylammonium bicarbonate (TEAB) and 0.1% sodium dodecyl sulfate (SDS). For four-plex isobaric labeling, separate aliquots of proteins were treated in parallel, essentially as described by

Ross et al.⁵ Stock reagents and buffer (TEAB, SDS, Tris (2-carboxyethyl)phosphine (TCEP), *S*-methyl methanethiosulfonate (MMTS), and the four isobaric tagging reagents) were obtained in kit form (Applied Biosystems, Foster City, CA). Protein (10-100 µg) was reduced with 2.5 mM TCEP (60°C for 1 h) and cysteine residues blocked with 10 mM MMTS (room temperature for 15 min). Protein was digested with trypsin (porcine modified, Promega; 1:20, w/w) for 20 h. Isobaric tagging iTRAQ reagent (1 unit in 70-µL ethanol) was added directly to the protein digest (70% ethanol final) and the mixture incubated at room temperature for 1 h. The reaction was quenched by addition of 9 volumes 0.1% trifluoroacetic acid (TFA) in water. The reaction mixtures were combined and stored at 4°C.

SCX Peptide Fractionation

For the first dimension of the two-dimension chromatographic separation, an aliquot of the four-plex peptide mixture (200 µg) was applied to a sulfoethyl aspartamide SCX spin column (SEM HIL-SCX, PolyLC, The Nest Group, Inc. Southboro, MA) equilibrated with 10 mM KH₂ phosphate, pH 4.5, 20% CH₃CN. Excess reagent was washed from the column with 800-µL equilibration buffer. Peptides were eluted using 50 µL volumes of KCl in a stepwise gradient from 25-500 mM KCl in equilibration buffer (25, 50, 75, 100, 150, 225, 350, and 500 mM KCl). Fractions were dried in a vacuum centrifuge.

Reversed-Phase Liquid Chromatography

For the second dimension separation, peptides in SCX fractions were separated by C₁₈ nano LC using an 1100 Series nano HPLC equipped with µWPS autosampler, 2/10 microvalve, MWD UV detector (214 nm) and Micro-FC fraction collector/spotter (Agilent). Each SCX salt step was reconstituted with 43-µL 0.1% TFA, v/v in water and 40-µL injected. Sample was injected onto a C₁₈ cartridge (Zorbax300SB, 5 µm, 5 × 0.3 mm; Agilent), desalted with solvent C (CH₃CN:H₂O:TFA, 5:95:0.1) at 20-µL/min for 9 min with the effluent discarded. The enrichment cartridge was placed ahead of a C₁₈ column (Zorbax300SB, 3.5 µm, 150 × 0.1 mm; Agilent) equilibrated with solvent A (H₂O:TFA, 99.9:0.1). Peptides were eluted with a gradient of solvent B (CH₃CN:H₂O:TFA, 90:10:0.1) from 6.5% B to 50% B over 90 min at a flow rate of 0.4 µL/min. Column effluent was mixed (micro Tee, Agilent) with matrix (2 mg/ml α-cyano 4-hydroxy cinnamic acid (CHCA in CH₃OH:isopropanol:CH₃CN:H₂O:acetic acid (12:33.3:52:36:0.7) containing 10 mM ammonium phosphate) delivered with a PHD2000 infusion pump (Harvard Apparatus) at 0.9 µL/min. Fractions were spotted at 24 s intervals onto stainless steel MALDI targets (192 wells/plate, Applied Biosystems).

Mass Spectrometry

Mass spectra were acquired on an Applied Biosystems 4800 MALDI TOF/TOF Analyzer (TOF/TOF). MS spectra from 800-3500 Da were acquired for each fraction from 750 laser shots of a 200 Hz YAG laser operated in the 3rd harmonic (355 nm). The TOF/TOF was operated in positive ion reflectron mode. Seven point Gaussian smoothing was applied to spectra and S/N of 30 filter applied for peak picking. Calibration was done using default mode. Plate calibrants were glufib (m/z 1714.787), ACTH (m/z 2753.419), angiotensin (m/z 1440.790), bradykinin (m/z 1048.578), and ACTH 1-17 (m/z 2107.197). The twelve most intense peaks in each MS spectrum were selected for MS/MS analysis. MSMS spectra were acquired from 1500-400 laser shots using “quality dependent” mode (6 peaks at S/N 60); fragment peak picking used S/N 40. MSMS calibration was done with fragments of glufib (m/z 430.242, 684.346, 1056.475 and 1441.634). Fragmentation of the labeled peptides was induced by the use of atmosphere as a collision gas with a pressure of approximately 6 × 10⁻⁷ torr and collision energy of 2kV.

Peptide identifications were performed using GPS Explorer (v3.6, Applied Biosystems) which acts as a front end for the Mascot search engine (v2.1 MatrixScience, London UK). Each MS/MS spectrum was searched against NCBI mammal (Feb. 2, 2007). Trypsin specificity with one missed cleavage was selected. S-mercaptopomethylcysteine and the N-terminal and lysine iTRAQ labels were selected as fixed modifications. Oxidized methionine was considered as a variable modification. The precursor tolerance and MS/MS fragment tolerances were ± 0.7 and ± 0.3 Da, respectively. Individual peptide identifications were grouped into protein identifications using GPS Explorer and assigned a total ion C.I.%. Only the peptides with C.I.% at 95% or above for any MS/MS spectrum were retained for further analysis.

Statistical Analysis and Animal Use

The data were processed as follows. The raw peak areas for each experiment were normalized following the methodology in Keshamouni *et al.*,⁶ using a quantile method. Subsequently, the desired ratios of 2 day versus baseline for each experiment were obtained using the random effects ANOVA model introduced in Keshamouni *et al.*⁶ and Jagtap *et al.*⁷ This model considers the hierarchical structure in the data, namely that every protein is comprised of several peptides and the expression level of each peptide may be measured by multiple spectra. The outcome of this model is a ratio R of the treatment effect (2-day expression level) over the control effect (baseline expression level), along with its standard error, which allows us to obtain the P-value for testing the hypothesis R is in the range (0.8-1.2) –no change, versus the alternative one that R is outside that range. The choice of this range is supported by experience with previous experiments, where such ratios usually do not indicate biological activity. Proteins with P-values smaller than 0.05 that rejected the null hypothesis of no change were retained for further processing.

The P-values were adjusted since multiple experiments were performed and some proteins appeared significant in several of them. A false discovery rate⁸ approach was used to appropriately adjust the P-values, in order to make the appropriate differential expression calls. All baboons were housed and cared for the University of Michigan Unit for Laboratory Animal Medicine and participated on approved research protocol.

RESULTS

Protein identifications were based on two peptides with 95% confidence. Of the 116 proteins identified, 31 proteins displayed a greater than 20% increase or decrease in expression on MPs. Special significance was given to proteins appearing in at least three animals with their level of expression being regulated in at least five of the eight experiments. Seven proteins fit this criterion and are listed in Table 1. Among these 7 proteins, the first class of proteins represented most frequently were various polypeptides of fibrinogen. Fibrinogen alpha chain and fibrinogen alpha-E subunits were two variants of fibrinogen alpha polypeptides represented in this group. Other polypeptides of fibrinogen include fibrinogen beta chain isoform 4 and fibrinogen gamma chain isoform 2. Microparticles were enriched with fibrinogens on day two compared to baseline as indicated by iTRAQ ratios greater than 1.2. Fibrinogen alpha chain and fibrinogen gamma chain isoform 2 appeared in all the animals and were regulated in seven experiments. Fibrinogen alpha-E subunit and fibrinogen beta chain isoform 4 appeared in three animals, and were regulated in five and six experiments, respectively.

Immunoglobulin M heavy chain and immunoglobulin lambda-like polypeptide 1 precursor were additional proteins identified. All the immunoglobulins were observed in all animals; however, immunoglobulin lambda-like polypeptide 1 precursor was regulated in five experiments while immunoglobulin M heavy chain was regulated in six experiments. All the

immunoglobulin proteins were decreased by day two compared to baseline as indicated by the iTRAQ ratios less than 0.8. Other protein noted includes alpha-1-antichymotrypsin (ACT) which appeared in all animals and was regulated in seven experiments. As indicated by the iTRAQ ratios greater than 1.2, ACT was up-regulated.

DISCUSSION

Microparticles are fragments of phospholipids from cell membranes that can be prothrombotic, and have been found to modulate a number of inflammatory cell vessel wall interactions. Recent investigations suggest that MPs, which are prothrombotic in part by virtue of tissue factor on their surface,^{9, 10} are important in early venous thrombogenesis.¹¹ Microparticles are recruited to the area of thrombosis,¹² where they amplify coagulation via tissue factor and factor VIIa.^{10,13-17} Co-localization of fibrin, platelets, and leukocytes in the developing thrombus^{10, 18} and the leukocyte-platelet interactions generating tissue factor¹⁹ support the central role of inflammation in thrombogenesis. Clinically, platelet-derived MPs are involved in venous thrombosis in the syndrome of heparin-induced thrombocytopenia.²⁰ Microparticles are found in healthy individuals, and have also been hypothesized to have an anticoagulant function by promoting the generation of low amounts of thrombin which activates protein C, supporting protein C's anticoagulant function.²¹ Microparticles have also been suggested to play a role in inflammation during severe sepsis, and their reduction was found to correlate with organ dysfunction and mortality.²² The current study attempted to shed light on the influence of MPs in early point of thrombogenesis. Inherent in this approach was the assumption that proteins crucial for influencing thrombogenesis are regulated during the course of venous thrombosis, and therefore, identifying and understanding the nature of these proteins can serve to provide valuable insight into the mechanistic role of MPs in venous thrombogenesis.

Up-regulated Proteins: *Fibrinogen*

Fibrinogen is a six-chain protein precursor to the clot structural protein, fibrin and dimer of α , β , and γ polypeptides.^{23,24} Proteomic analysis detected all three of these polypeptides on MP surface. The presence of fibrinogen suggests a number of potential mechanisms regarding the role of MPs in the thrombotic process. Among all the polypeptides, the γ chain is of special interest because it contains multiple epitopes that interact with growth factors and integrins (Figure 1), and plays a critical role in platelet aggregation, inflammation, and wound healing.^{24, 25}

The fibrinogen γ chain sequence γ 400-411 binds to $\alpha_{IIb}\beta_3$ on platelets.^{24, 26} Thus, it is highly likely that MPs participate in platelet aggregation by binding to platelet integrins using this sequence. Additionally, the γ chain contains a sequence from γ 346-358 that binds to endothelial integrin $\alpha_v\beta_3$ and plays a key role in anchoring MPs to the endothelium.²⁷ The presence of sequences γ 400-411 and γ 346-358 render MPs the ability to associate with endothelium and form a hemostatic plug. Recent studies have identified two sequences, γ 370-383 and γ 316-324, that interact with $\alpha_{IIb}\beta_3$ on platelets and leads to platelet-mediated clot retraction; thus, suggesting potential involvement of MPs in clot retraction.²⁷

The γ chain contains yet other regions that may facilitate MPs to take a part in inflammation. The sequence γ 190-202 and γ 377-395 are important in recognizing leukocyte integrin $\alpha_M\beta_2$.²⁸ This binding interaction may mediate the recruitment of phagocytes during inflammation. Moreover, the γ region contains sites for several different growth factors and cytokines, including vascular endothelial growth factor, fibroblast growth factor-2 and interleukin-1 β .²⁹⁻³¹ Recent studies indicate that these binding sites may serve as storage depots to concentrate growth factors and cytokines in the fibrin clot to function in inflammation and wound healing.

Besides the γ chain, the α and β chains of fibrinogen may lend MPs further ability to influence clot formation. These chains have specific domains that can bind to basement membrane proteins.³² This type of binding to fibrinogen confer further important physiological role for MPs such as anchoring fibrin clots at the site of injury where the basement membrane is exposed.¹⁰ Moreover, these interactions could modulate other biological activities. For instance, it has been demonstrated that the binding of fibrinogen to basement membrane proteins stimulate chemotaxis and enhances neutrophil phagocytosis.³² Therefore, by virtue of having fibrinogen on their surface, MPs are likely pivotal in both clot formation, its adherence and accelerating the removal of a fibrin clot.

There may be concern that the fibrinogens detected on MP surfaces are contamination from the thrombus itself rather than up-regulated proteins on the MP surface. We do not believe such is the case because the MP samples were washed with KBr, a chaotropic salt, to reduce non-specific binding of soluble proteins that were not anchored to MPs. More stringent wash conditions (e.g., carbonate at pH 10) completely disrupt and solubilize MPs (data not shown).

Up-regulated Proteins: *Alpha-1-antichymotrypsin*

Alpha-1-antichymotrypsin (ACT), a serine protease inhibitor, was up-regulated in this experiment. Microparticles containing this protein may control adhesion of neutrophils to fibronectin (FN) through the action of ACT. Neutrophil accumulation within inflammatory sites is an adhesion-dependent process where the integrins on neutrophils associate with high affinity domains on FN.³³ However, the high affinity domains are exposed after proteolytic processing by serine proteinase cathepsin G on the surface of neutrophils. ACTs are known to block proteinases including cathepsin G, preventing the proteolytic cleaving of FN by neutrophil.³⁴⁻³⁶ This subsequently prevents the exposure of high affinity domains on FN and hinders the binding of neutrophils to FN. Cathepsin G on neutrophil granules are often released at the site of inflammation and are involved in killing and degrading pathogens, remodeling tissues and activating pro-inflammatory cytokines.^{34, 37, 38} Thus, the inhibition of cathepsin G by ACT may prevent induction of an inflammatory response.

Down-regulated Proteins: *Immunoglobulins*

A number of immunoglobulin proteins were observed on MPs and they were down-regulated on day two compared to baseline. These identifications are less likely due to experimental contaminations as our findings are consistent with another earlier study on MPs which had identified Immunoglobulin M (IgM) associated with the MPs.² It is well established that immunoglobulin proteins are key players for controlling inflammation in various pathological conditions; however, their roles in the context of thrombosis have not been studied extensively. A recent study has demonstrated the ability of immunoglobulin lambda light chain to bind to fibrinogen and inhibit thrombin-catalyzed fibrin polymerization. It is proposed that the lambda light chain non-covalently associates with the alpha region leading to significant polymerization defect.³⁹ Immunoglobulin M has been implicated in venous and arterial thrombosis. Studies with animal models have demonstrated that passive infusion of IgM can increase clotting after a mechanical injury to the vessel wall.⁴⁰ High levels of IgM have also been observed in patients with deep venous thrombosis (DVT).⁴¹ The facts on these proteins together with their down-regulated status suggest that MPs may play a role as anticoagulant agent.

Additional Observations and Explanations

Unexpectedly, a number of proteins such as tissue factor (TF), P-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1) were not observed on MPs. This is interesting since we have found that the amount of tissue factor activity on MPs correlate with the presence or

absence of experimentally induced venous thrombosis. Potential reasons for this absence include a low density of surface proteins, the saturation of these proteins by their receptors, or glycosylation. Highly glycosylated proteins might not be detected by proteomic analysis,² and PSGL-1 as a receptor for P-selectin, a member of lectin family, is glycosylated. A more likely explanation for their absence is a low abundance of these proteins in the protein pool. Earlier studies have shown that these types of proteins are generally found on platelet-derived MPs and not plasma-derived MPs.² Since the number of plasma-derived MPs are approximately 6-fold greater than platelet-derived MPs when prepared in our laboratory,⁴² the abundance of these proteins may be too low compared to the ones enriched on the plasma-derived MPs to be considered as significant.

Additionally, blood samples from the last two animals were washed in KBr to remove soluble serum proteins that are not physically attached to MPs. There may be concern that the origin of some of the proteins identified is non-MP derived. However, we believe such is not the case because all the proteins identified in our study are seen in both KBr and non-KBr washed samples.

CONCLUSIONS

In this preliminary study, we have identified a diverse group of proteins associated with circulating MPs in our primate model of venous thrombosis. These proteins may influence thrombosis through hemostatic plug formation (fibrinogen), inhibiting neutrophil adhesion (ACT), and immunoregulation (various immunoglobulins). Proteomic data from this investigation will be used to design future studies aimed at identifying novel biomarkers to target future therapies for venous thrombosis. This study is the first to demonstrate the proteome of MPs in a large animal model of venous thrombosis. Further human studies characterizing MP proteins are underway.

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Figure 1. Interaction sites in the fibrinogen γ chain. This figure shows critical amino acid residues and domains in the γ chain 5 that are shown according to their location in the primary sequence. The circle represents the microparticle body and the rod represents the fibrinogen γ chain.

List of 7 proteins that are present in at least two experiments. Proteins with iTRAQ ratio greater or equal to 1.2 are up-regulated while less than or equal to 0.8 are down-regulated. N[†] indicates the number of animals in which the particular protein is observed. N is the number of experiments in which the protein was either up-regulated or down-regulated compared to control.

Table 1

Accession Number	Protein Name	N [†]	N	Average iTRAQ	P
85701319	Fibrinogen alpha chain	4	7	1.43	≤0.05
971177	Fibrinogen alpha-E subunit	3	5	1.47	≤0.05
109075981	Fibrinogen beta chain isoform 4	3	6	1.56	≤0.05
109075977	Fibrinogen gamma chain isoform 2	4	7	1.55	≤0.05
109093570	Immunoglobulin lambda-like polypeptide 1 precursor	4	5	0.69	≤0.05
4105439	Immunoglobulin M heavy chain	4	6	0.63	≤0.05
109084779	Alpha-1-antichymotrypsin	4	7	2.60	≤0.05