Exosomal Myeloperoxidase as a Biomarker of Deep Venous Thrombosis

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Research

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Abstract

**Background:** Deep vein thrombosis (DVT) often occurs following major orthopedic surgery. In this study, we investigated specific exosomal proteins as potential diagnostic biomarkers of DVT.

**Methods:** Proteomic analysis of exosomes from four DVT patients and healthy controls (n = 4) was performed by mass spectrometry. The model animals were evaluated at 1 (IVCL-1D), 3 (IVCL-3D) and 7 (IVCL-7D) days after inferior vena cava ligation (IVCL). Endothelial cells in the thrombus segment were examined using TUNEL assays and hematoxylin and eosin staining. Myeloperoxidase (MPO) expression in the damaged vessel was detected by immunofluorescence staining. Exosomes were co-cultured with human umbilical vein endothelial cells (HUVECs) and cell proliferation was estimated using CCK-8 assays.

**Results:** Totally, 78 differentially expressed proteins (38 downregulated and 40 upregulated) were identified in the DVT group. In the rat DVT model, endothelial cells were damaged continuously after thrombosis, with the most serious injury in the IVCL-3D group, after which signs of endothelial repair were apparent. The IVCL-1D group showed the highest levels of vascular endothelial cell apoptosis and MPO increased sharply in the IVCL-1D and IVCL-3D groups, but had almost disappeared in the IVCL-7D group. In co-culture, plasma exosomes isolated from DVT model rats were efficiently absorbed by HUVECs, with markedly lower HUVEC growth and higher levels of apoptosis in the IVCL-1D and IVCL-3D groups compared with the control group.

**Conclusions:** Our findings suggest that Exosomes may be involved in endothelial cell injury during DVT. The exosomal protein MPO is a potential biomarker of early stage DVT.

**Background**

Deep venous thrombosis (DVT) is a common complication following major orthopedic surgery such as hip, knee replacement, and spinal surgery and can cause death due to secondary pulmonary embolism. At present, the diagnosis of DVT depends mainly on vascular ultrasound and digital subtraction angiography technology. Although both methods have good diagnostic value, the former cannot diagnose intra-peritoneal venous embolism, and the latter is highly invasive and costly.

Currently, there are no definitive laboratory indicators and detection methods for early diagnosis and prediction of DVT in clinic. Most doctors routinely use anticoagulants for patients undergoing major orthopedic surgery of the lower extremities; this is not only an economic expense, but can also induce severe complications such as intracranial bleeding [1]. Therefore, definitive laboratory indicators for early diagnosis of DVT are urgently required.

Recent studies have indicated the potential of exosomes as diagnostic markers for some diseases, probably providing new ideas for DVT biomarkers. Exosomes contain unique microRNAs (miRNAs) and proteins, which not only function in substance transport and signal transmission, but also protect the
substances contained within from degradation [2–4]. Some unique miRNAs and proteins are highly expressed in exosomes than in plasma. Studies have shown that exosomes can initiate endogenous and exogenous coagulation pathways by transmitting different signals, and play an important role in blood coagulation. Furthermore, there is accumulating evidence that exosomes play an important role in coagulation in trauma, tumors, and vascular sclerosis [5, 6].

Exosomes can affect vascular endothelial cell function as well as platelet activation and aggregation, increasing blood coagulability [7]. Vascular endothelial cells play an important role in the stabilization of blood flow, and procoagulant factors secreted by endothelial cells directly and quickly participate in coagulation and thrombosis [8]. Cytokines produced by endothelial cells after stimulation can induce monocytes to adhere to endothelial cells and stimulate platelet activating factors to participate in thrombosis [9]. In this study, we explored potential exosomal biomarkers in the blood of DVT patients and investigated the effect of exosomes on vascular endothelial cells in cell culture and animal models.

Materials And Methods

Collection of blood in DVT patients

EDTA-anticoagulant venous blood was collected from four DVT patients (two males and two females; mean age, 56 ± 15 years) after total hip arthroplasty and four healthy volunteers (two males and two females; mean age, 56.5 ± 12.5 years). Blood samples were centrifuged at 3000 × *g* for 10 min at 4°C to remove cells. The plasma was collected and immediately frozen in liquid nitrogen for 15 min; 250 µL aliquots were stored at −80°C prior to analysis. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Shantou University Medical College (China), and all participants provided written informed consent.

Isolation and evaluation of exosomes

The plasma sample was centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatant was then centrifuged at 100,000 × *g* for 70 min at 4°C. The resulting supernatant was discarded and the pellets were washed in a large volume of phosphate-buffered saline (PBS) and centrifuged at 100,000 × *g* for 70 min at 4°C. The resulting pellet was collected and resuspended in PBS. After separation, the ultrastructure, concentration, and size distribution of exosomes were analyzed by electron microscopy and nanoparticle tracking analysis (NTA).

Transmission electron microscopy (TEM)

The purified plasma exosomes were resuspended in PBS, dropped into the electron microscopy grid, which was switched to the absorb mode for 10 min, and negatively stained with 2% phosphotungstic acid (pH 6.8) for 5 min. After air-drying under an incandescent lamp, the plasma exosomes were examined under an electron microscope (Hitachi-H7650, Tokyo, Japan) at 120 kV.

Nanoparticle tracking analysis
The size distribution and number of isolated exosomes were examined with the Nanosight N3000 system and the data were analyzed using NTA 3.2 Dev Build 3.2.16.

**Proteomic analysis of exosomes**

Total proteins were extracted from plasma exosomes using radioimmunoprecipitation lysis buffer (BioSharp, HeFei, China), and the concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of each protein sample were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane before incubation overnight with primary antibodies against CD63 and CD9 (1:1500; Abcam, Cambridge, MA, USA), SHIP1, CDKN1B, and SOCS1 (1:800; Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin and phosphorylated NF-κB p65 (1:1000; Cell Signaling Technology, Beverly, MA, USA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000; Cell Signaling Technology). The protein bands were visualized using IMAGISOLANE LAS4000 Mini (GE Healthcare, Piscataway, NJ, USA). Image-Pro Plus 6.0 software (Media-controlnetics, Silver-Spring, MD, USA) was used to measure the scale values of protein bands, and the relative expression of the indicated proteins was normalized to the internal control β-actin.

**Studies of an animal model of venous thromboembolism (VTE)**

Adult male Sprague–Dawley rats (8–10 weeks, 300–400 g) were purchased from the Experimental Animal Central of Southern Medical University (Guangzhou, China, 44002100019745). All protocols were approved by the Animal Experimentation Ethics Committee of Shantou University Medical College and carried out in accordance with the Guide for the Care and Use of Laboratory Animals. The experimental animals were anesthetized with 1% sodium pentobarbital (300–400 μL/100 g body weight).

**Establishment of the rat model of DVT**

An inferior vena cava (IVC) ligation model was established as described previously [10]. The IVC and all visible side branches (usually two or three) were ligated with nonreactive 4−0 silk sutures. After 2 days, the IVC and associated thrombus in each group were removed, weighed, and measured for thrombus length. The IVC and its branches in the control group rats were not ligated.

**Plasma exosome isolation and characterization**

Rat blood was collected into 5-mL EDTA anticoagulant tubes and plasma was obtained by centrifugation at 3000 × g for 10 min at 4°C to remove cells and debris. The supernatant was then centrifuged at 20,000 × g for 30 min at 4°C and the resulting supernatant was centrifuged at 100,000 × g for 70 min at 4°C. The supernatant was discarded and the pellets were washed in a large volume of PBS and centrifuged at 100,000 × g for 70 min at 4°C. Finally, the pellet was collected and resuspended in PBS. After separation, the ultrastructure, concentration, and size distribution of the plasma exosomes were analyzed by electron microscopy and NTA.

**Hematoxylin and eosin (HE) staining**
Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned (thickness, 4 mm) for dewaxing and dehydration. HE staining was performed as previously described [11].

**TUNEL assays**

After the cells or tissues were fixed with 4% paraformaldehyde and sectioned, *in situ* nick end labeling of nuclear DNA fragments was performed using a TUNEL apoptosis assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The samples were then analyzed under a laser scanning confocal microscope (Olympus, Japan).

**Western blot analysis**

Proteins were extracted from exosomes or tissues using radioimmunoprecipitation lysis buffer (Biosharp, China) and concentrations were determined using the BCA protein assay kit (Absin, China). Equal amounts of protein samples were separated by 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and incubated overnight with the following primary antibodies (1:1000): mouse monoclonal antibodies against GAPDH (Proteintech, China) and CD63 (Abcam) and rabbit monoclonal antibodies against Alix (Abcam), MPO (Proteintech), and TSG101 (Abcam). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:4000; Invitrogen, Chicago, IL, USA) at room temperature for 1 h. Protein bands were visualized using an OPTIMAX X-Ray Film Processor (PROTEC, Germany).

**Immunofluorescence**

For immunofluorescence staining, tissues were fixed with 4% paraformaldehyde, and antigen repair was achieved by incubation with 0.25% citrate (ShiFeng Biology, Shanghai, China) for 16 h at 60°C. Sectioned tissues were blocked with goat serum at room temperature for 0.5 h before incubation overnight at 4°C with the following primary antibodies (1:500): mouse monoclonal antibody against CD31 (Proteintech) and rabbit monoclonal antibody against MPO (Proteintech). Sections were incubated for 1 h at room temperature with fluorescently labeled anti-rabbit or anti-mouse secondary antibody (1:1000, Invitrogen). Nuclei were stained with DAPI for 10 min. The images were captured under a laser scanning confocal microscope (Olympus).

**Uptake of exosomes by human umbilical vein endothelial cells (HUVECs)**

Exosomes were fluorescently labeled using the PKH67 kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. After washing with PBS (Gibco, Grand Island, NY, USA), exosomes were centrifuged at 100,000 × g for 70 min. Exosomes (1 µg) were then co-cultured with 100,000 HUVECs for 4 h at 37°C and 5% CO₂. The uptake of PKH67-labeled exosomes by HUVECs was analyzed under a laser scanning confocal microscope (Olympus).

**HUVEC culture**
HUVECs were purchased from American Type Culture Collection and cultured in endothelial cell complete medium containing endothelial cell growth supplement (AllCells, Alameda, CA, USA) at 37°C in a humidified atmosphere containing 5% CO$_2$.

**CCK-8 assay**

HUVECs were seeded in 96-well plates with 5000 cells in each well. Exosomes (0.05 µg) were added into each well and co-cultured with HUVECs for 72 h. Subsequently, 10 µL CCK-8 solution was added to each well and the cells were incubated for 3 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (BioTek, Carlsbad, CA, USA) and data were analyzed using Gen 5 software.

**Statistical analysis**

Experimental data are described as the mean ± standard deviation, and a two-tailed Student's $t$-test (GraphPad Prism software Version 5.0) was used to assess statistical significance. $P<0.05$ was considered to indicate statistical significance.

**Results**

**Extraction and verification of exosomes from DVT patients**

Exosomes were isolated by density gradient centrifugation from the blood of four DVT patients after total hip arthroplasty and four healthy controls. Evaluation by TEM and NTA showed that the diameter of the isolated exosomes (100–150 nm) was consistent with the size of exosomes (Fig. 1). Detection of the exosome marker ALG 2-interacting protein X (Alix) revealed that the content of exosomes in the DVT group was higher than that in the control group (Fig. 1).

**Label-free quantitative proteomic analysis of exosomes in the plasma of orthopedic DVT patients**

A large number of differentially expressed exosomal proteins were distributed in the plasma of the DVT group compared with the control group (Fig. 2). In total, 78 differentially expressed proteins were identified in the DVT group; 38 were downregulated and 40 were upregulated. Bioinformatics analysis revealed five significant differentially expressed proteins (S100, MPO, aminopeptidase N, haptoglobin, and histone H4) related to thrombosis (Table 1).
Table 1
Differential expression of ve exosome proteins in thrombosis group and control group (P < 0.01)

| Protein name     | DVT group     | Healthy group | FC (DVT group/ Healthy group) | P-value   |
|------------------|---------------|---------------|------------------------------|-----------|
| Protein S100-a-A9| 213744522     | 8835305.5     | 24.19209183                  | 0.000602  |
| Myeloperoxidase  | 330053495.5   | 2103409       | 156.9136081                  | 0.000075  |
| Aminopeptidase N | 290852279     | 24308406      | 11.96509055                  | 0.004872  |
| Haptoglobin      | 78457229874   | 12762601692   | 6.1474323                    | 0.000071  |
| Histone H4       | 667621969     | 6234318.5     | 107.0882036                  | 0.000762  |

HE staining showed IVC vascular injury in an animal model of DVT

Venous thrombosis was confirmed in VTE model rats. In the IVCL-1D group, there was almost no gap between the venous thrombus and the vessel wall (Fig. 3). By contrast, there was a visible gap between the blood vessel wall and the thrombus in the IVCL-3D and IVCL-7D groups. The blood vessel walls in the experimental groups were thinner than those in the control group. Among the experimental groups, the blood vessel walls were thinnest in the IVCL-3D group and thickest in the IVCL-7D group. The vessel walls were smooth and intact in the control group. Nevertheless, the intima of the vessel walls in each experimental group was damaged and the continuity was interrupted in the IVCL-3D group, while intima repair and thickening was observed in the IVCL-7D group. These results showed that endothelial cells are damaged continuously after thrombosis, with the most serious injury occurring on the third day after ligation, after which signs of endothelial repair were apparent.

Detection of vascular endothelial cell apoptosis in animal models with thrombosis by TUNEL staining

TUNEL staining in VTE model rats showed low levels of apoptotic cells in the IVC of the control and IVCL-7D groups, with no marked difference between the two groups. Higher levels of TUNEL staining were detected in the IVCL-1D group. The highest level of apoptosis was detected in the IVCL-3D group (Fig. 3).

Western blot analysis of myeloperoxidase (MPO) content

Western blot analysis showed that, compared with the control group, MPO expression in vascular tissues increased sharply in the IVCL-1D and IVCL-3D groups, and decreased in the IVCL-7D group (Fig. 4). Expression of the exosome markers CD63 and Alix was increased in the IVCL groups.

Immunofluorescence staining of MPO distribution in the IVC of VTE model rats
The distribution of MPO in blood vessels was detected using immunofluorescence following IVC thrombosis (Fig. 4). DAPI staining was performed to visualize the cell nuclei and CD31 was detected as a marker of endothelial cells. The content of MPO increased after venous thrombosis and was distributed mainly on the blood vessel wall in the IVCL-1D group, and mainly in the thrombus in the IVCL-3D group, while MPO expression had almost disappeared in the IVCL-7D group (Fig. 4).

**MPO content in plasma exosomes isolated from VTE model rats increased following IVC thrombosis**

Exosomes were extracted and separated from the plasma of each group of animals. Exosomes were identified by TEM and NTA, and the MPO content was measured by western blot analysis of Tsg101 and the exosome marker Alix. Compared with the control group, the exosomal MPO content in the thrombus groups increased, with the highest MPO levels detected in the IVCL-3D group (Fig. 5).

**HUVECs take up plasma exosomes**

The exosomes extracted from plasma were labeled with PKH67 and co-cultured with HUVECs for 4 h. As shown in Fig. 5, a large amount of exosomes was absorbed by HUVECs.

**MPO-containing plasma exosomes promote HUVEC injury and apoptosis**

Plasma exosomes isolated from VTE model rats were co-cultured with HUVECs for 72 h and examined by light microscopy. As shown in Fig. 6A, the proliferation of HUVECs co-cultured with plasma exosomes from the IVCL-1D and IVCL-3D groups was significantly poorer than those co-cultured with exosomes from the control and IVCL-7D groups. HUVECs co-cultured with exosomes from the IVCL-3D group exhibited the lowest proliferation ability, with predominantly rounded cell morphology typical of naive cells (Fig. 6B). CCK-8 assays were conducted to detect the number of HUVECs in each group. The highest and lowest numbers of HUVECs were detected in the control and IVCL-3D groups, respectively (Fig. 6). TUNEL staining revealed visibly more apoptotic cells in the IVCL-1D and IVCL-3D groups than in the control and IVCL-7D groups (Fig. 6C).

**Discussion**

A recent study found that soluble coagulation factors are as important as endothelial cell damage, platelet adhesion, aggregation, and release in venous thrombosis [12]. DVT is a complex inflammatory response process involving a large number of inflammatory cells and inflammatory factors [13]. Abnormal expression of inflammatory factors can cause excessive apoptosis of endothelial cells and lead to vascular wall damage [14]. In the present study, we confirmed that impaired endothelial cell growth combined with increased apoptosis occur following venous thrombosis. In clinical practice, some patients show no symptoms in early stage DVT, which is called occult thrombus. Early diagnosis of clinical occult venous thrombosis not only reduces late complications of venous thrombosis but also reduces bleeding events in patients caused by the use of anticoagulant drugs. By studying the role of
exosomes during venous thrombosis in this study, we identified exosomal MPO as a potential biomarker for early diagnosis of DVT.

MPO is mainly derived from neutrophils and is a lysosomal protein that occurs in azophilic granules [15]. Its properties in promoting inflammatory responses and oxidative stress are well known [16]. A host of inflammatory cells and inflammatory factors such as tumor necrosis factor alpha and interleukin 1 are involved in the inflammatory response [17]. In arterial thrombosis, a large amount of MPO is found in atherosclerotic plaques [18]. Fecal MPO has been proposed as a diagnostic biomarker of inflammatory bowel disease [19]. MPO also mediates inflammatory responses and oxidative stress that induce cardiovascular disease [20]. Thus, MPO may play an important role in DVT. In this study, we investigated the role of MPO in the pathogenesis of orthopedic surgery patients with DVT. We found that MPO was abundantly distributed in the vessel walls and emboli within 1 to 3 days of DVT. MPO in exosomes isolated from the plasma of DVT model rats remained at a high concentration within 1 to 3 days of DVT, but decreased significantly after 7 days. Interestingly, exosomes isolated from the plasma of model rats at 3 days after DVT induced damage to vascular endothelial cells and promoted apoptosis. However, exosomes isolated from the plasma of model rats at 7 days after DVT did not induce endothelial cell injury or apoptosis. Based on these observations of the synchronous relationship between the MPO content of plasma exosomes and the damage to endothelial cells induced by these exosomes, we speculated that the increased levels of exosomal MPO might indicate damage to endothelial cells in early stage DVT. Thus, exosomal MPO may be a potential biomarker for early diagnosis of DVT.

The functions of exosomes are influenced by external stimuli. Under different environmental stimuli, exosomes can play important roles throughout the body by transporting different substances. The microparticles in patients with β-thalassemia can activate vascular endothelial cells and increase the adhesion of monocytes, thereby increasing the risk of thrombosis [21]. In patients with sepsis, nitric oxide (NO) and bacteria stimulate the production of platelet-derived exosomes. These exosomes can induce endothelial cell apoptosis by producing superoxide, NO, and peroxynitrite anions [22]. MPO is known to play an important role in promoting inflammation and oxidative stress and the results of the present study indicate that exosomal MPO plays a role in vascular endothelial cell damage.

Conclusion

During DVT formation, blood vessel walls are damaged by the release of plasma exosomes. We identified differences in exosomal MPO levels in orthopedic DVT and non-DVT patients. Thus, our results implicate exosomal MPO as a potential biomarker for early diagnosis of DVT. However, the molecular mechanism underlying the role of exosomal MPO in DVT remains to be elucidated.

Abbreviations

DVT, deep venous thrombosis
Declarations

Acknowledgements
Not applicable.

Authors’ contributions
XW and XB accomplished the conception and design of the research; YH, XW performed the experiments; YH, XW and XB analyzed and interpreted the data; YH drafted the manuscript; XW and XB edited and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Second Affiliated Hospital of Shantou University Medical College (China).
Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**
Figure 1

A, Transmission electron micrograph of exosomes. B, Nanoparticle tracking analysis. Exosome diameters ranged from 100 to 150 nm. C, Higher expression of Alix was observed in the thrombus group compared with the control group.
Figure 2

Proteomics analysis of plasma exosomes from patients with DVT and healthy individuals. Heat maps of 24 significantly differentially expressed exosomal proteins obtained from the plasma of patients in the thrombus and control groups.
Figure 3

Images of inferior vena cava injury at different time points (1, 3, and 7 days) after ligation. A, HE staining showing that the continuity and integrity of blood vessels were disrupted following inferior vena cava ligation, and the vessel wall was most severely damaged 3 days after ligation. B, TUNEL staining showing that venous blood vessel apoptosis was most obvious at 3 days after ligation.
Figure 4

The distribution of MPO content in the inferior vena cava over time after inferior vena cava thrombosis. A, Western blot analysis of MPO expression at different time points after venous thrombosis (1, 3, and 7 days). MPO was higher in the IVCL-1D and IVCL-3D groups, but significantly lower in the IVCL-7D group. Expression of the exosomal markers CD63 and Alix increased after venous thrombosis. B, Immunofluorescence analysis of the distribution of MPO in the inferior vena cava at different time points.
after thrombosis. MPO was mainly distributed in the blood vessel wall in the IVCL-1D group and in the emboli in the IVCL-3D group, while it had almost disappeared in the IVCL-7D group.

Figure 5

HUVEC uptake of plasma exosomes containing MPO. A, Levels of CD63 and Alix in the IVCL-1D, IVCL-3D, and IVCL-7D groups was increased relative to levels of the control group. The content of MPO in the IVCL-1D and IVCL-3D groups increased significantly. B, Exosomes labeled with green fluorescence were absorbed in large quantities by HUVECs.
Figure 6

Exosomes containing MPO in the plasma of rats from different groups of thrombus models were cocultured with HUVECs. A, Relative growth status of HUVECs (IVCL-1D < IVCL-3D < IVCL-7D < control group) observed under a light microscope. B, CCK-8 assay showing the highest and lowest numbers of HUVECs in the control and IVCL-3D groups, respectively; data are presented as mean ± SD, *P < 0.05. C, TUNEL staining of apoptosis of HUVECs: IVCL-3D > IVCL-1D > IVCL-7D.