Original Article
Expression and significance of NF-κB and MMP-2 in deep vein thrombosis

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Abstract: Deep venous thrombosis (DVT) is a severe vascular disease. The current study aimed to investigate expression and significance levels of NF-κB and MMP-2 in DVT. SD rats were adopted in constructing the traumatic DVT model. They were randomly divided into 3 groups, including the initial thrombosis group, peak thrombosis group, and peak non-thrombosis group. A total of 70 fracture patients, admitted to Qilu Hospital of Shandong University, between January 2017 and December 2017, were enrolled and divided into the non-thrombosis group and thrombosis group, according to postoperative thrombosis. NF-κB and MMP-2 mRNA expression levels in peripheral blood were tested via real time PCR and ELISA. NF-κB and MMP-2 expression levels significantly increased at initial and peak stages in the thrombosis group, compared with the normal control group (P < 0.05). NF-κB and MMP-2 expression levels in the peak stage were significantly different from the initial stage (P < 0.05). There were no statistical differences in expression levels of NF-κB and MMP-2 in the peak stage of the non-thrombosis group (P > 0.05). Compared with the normal control group, expression levels of NF-κB and MMP-2 in DVT patients were significantly increased with time dependence, reaching a peak at 72 hours (P < 0.05). No statistical differences were observed in NF-κB and MMP-2 expression levels in the non-thrombosis group (P > 0.05). NF-κB and MMP-2 expression levels increased during the formation of DVT. They were closely related to the formation and progression of DVT. Thus, they may be key factors affecting biological progression.

Keywords: Deep venous thrombosis, NF-κB, MMP-2, vascular disease

Introduction

Deep venous thrombosis (DVT), also known as thrombo-deep phlebitis, is a clinical form of venous thromboembolism (VTE) [1]. A common disease in peripheral vessels, DVT is a severe vascular disease. It is commonly seen in bone trauma, surgery, and acute disease [2, 3]. With the rapid development of industry, transportation, and construction, as well as aging of the population, DVT caused by trauma has become an important issue. DVT is the third highest cardiovascular disease, after coronary heart disease and strokes [4, 5]. DVT can be induced by other diseases as a complication. It can also occur alone with age dependence [6]. In addition to the above factors, tumors, severe infections, autoimmune diseases, and coagulation factor abnormalities can induce DVT [7]. DVT can occur in multiple parts of the body, espe-cially in the lower extremities. It occurs mainly in the deep veins of the lower extremities, including the femoral vein [8, 9]. It has no obvious symptoms in the early stages. Thus, it is easily ignored. Untimely diagnosis and treatment may lead to fatal pulmonary thromboembolisms, even sudden death [10].

The pathogenesis of DVT is very complicated, involving multiple factors. Inflammation, coagulation, and anticoagulant disorders, as well as vascular endothelial cells and fibrinolytic and anti-dissolved system imbalances, may be involved in DVT development and progression [11, 12]. However, definite molecular mechanisms of DVT have not been elucidated. It has been reported that matrix metalloproteinases (MMPs) may play an important role in the formation and evolution of DVT. However, the role of MMP-2 as an important member of the

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
NF-κB (Homo sapiens)	ATACTCCGATATGGATGG	TCACAGCTCGCTCGAGGTA
NF-κB (rats)	GTTAGTCACCGCTCATT	CGATATCGCACACTCTGGTA
MMP-2 (Homo sapiens)	GATATGGGACTCGACCTC	TATCGCGAGACTCGCTCC
MMP-2 (rats)	CCATGGATACTCGTCA	CTCGGCGAACTCCTCTGCTA

MMPs family in the process of DVT has not been found [13, 14]. A key transcription factor, NF-κB activation can be involved in the regulation of the inflammation network. Thus, it may be an important factor in DVT [15]. However, whether NF-κB plays a role in the pathogenesis of DVT remains poorly understood. Therefore, this study aimed to explore expression and significance levels of NF-κB and MMP-2 in the process of DVT.

Materials and methods

Experimental animals

A total of 60 Sprague-Dawley rats of SPF grade, aged 8-12 weeks and weighing 250 ± 30 g, were purchased from the Experimental Animal Center of Soochow University (Jiangsu, China). The rats were fed by the SPF Animal Experimental Center. Feeding conditions included maintaining temperature levels at 21 ± 1°C and relative humidity levels at 50-70%, with a 12-hour day/night cycle. The rats were free to eat and drink. The experiment was performed after 2 weeks of adaptive breeding.

Rats were used for all experiments. All procedures were approved by the Animal Ethics Committee of Qilu Hospital of Shandong University (Shandong, China).

General information

A total of 70 fracture patients, admitted to Qilu Hospital of Shandong University (Shandong, China), between January 2017 and December 2017, were included. The patients were diagnosed with computed tomography (CT) scans, combined with clinical symptoms. Patients included 42 males and 28 females, with a mean age of 45.1 ± 3.8 (20-65) years and mean body weight of 54.7 ± 6.8 (37-83) kg. Routine B-ultrasounds, electrocardiograms (ECG), and blood routine examinations were performed be-

fore surgery. Fracture types included femoral shaft fractures, tibial fractures, ilium fractures, and knee replacement and revisions. Exclusion criteria: Other serious complications; Significant impairment of vital organs, such as the liver and kidneys; Underage or excessive age; History of abnormal coagulation function; History of venous thrombosis; Pregnancy; Diabetes mellitus; Active bleeding in the last 2 weeks; Mental disorders or unable to cooperate with investigators [16]. In addition, 30 healthy volunteers were selected as the control group, including 18 males and 12 females, with a mean age of 44.1 ± 5.7 (20-62) years and mean body weight of 51.7 ± 3.6 (40-83) kg. This study was approved by the Ethics Committee of Qilu Hospital of Shandong University (Shandong, China). The overall study met the requirements of medical ethics. All subjects or family members provided informed consent.

Main reagents and instruments

Experimental surgical instruments were purchased from Shanghai Surgical Instrument Factory. RNA extraction and reverse transcription kits were purchased from RD Corporation (USA). Real-time PCR reagents were purchased from Thermo Fisher. Human and rat NF-κB and MMP2 ELISA kits were purchased from R&D Corporation (USA). Other commonly used reagents were purchased from Sangon (Shanghai, China). The Model 5424r Eppendrof centrifuge was purchased from Eppendorf (Germany). LabSystem Version 1.3.1 microplate reader was purchased from Bio-rad (USA). ABI9700 PCR instrument was purchased from Applied Biosystems.

Methods

Grouping: According to postoperative thrombosis, the patients were divided into the non-thrombosis group and thrombosis group. Another 30 cases were selected as the normal control group. There were no statistical differences in general clinical conditions, including age, gender, and blood pressure, among the three groups.
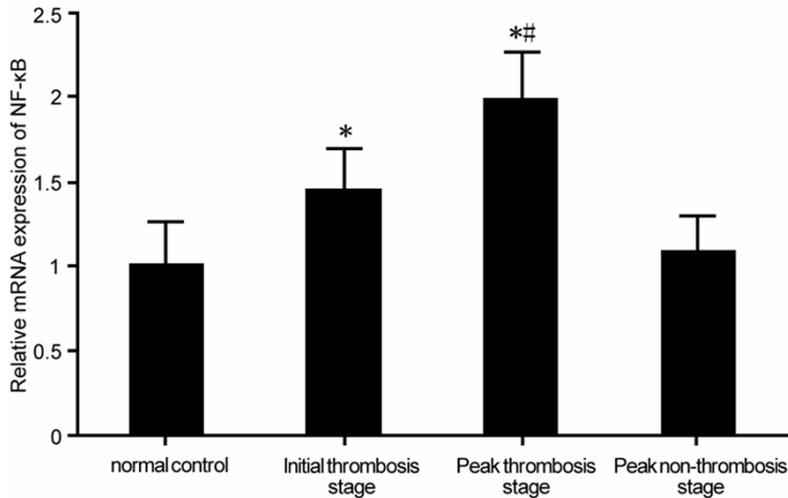
Figure 1. NF-κB mRNA expression changes at different periods in DVT rats. Total RNA was isolated from DVT rats at different stages, followed by analysis of NF-κB mRNA expression by real-time PCR. *P < 0.05, compared with normal control; #P < 0.05, compared with initial period.
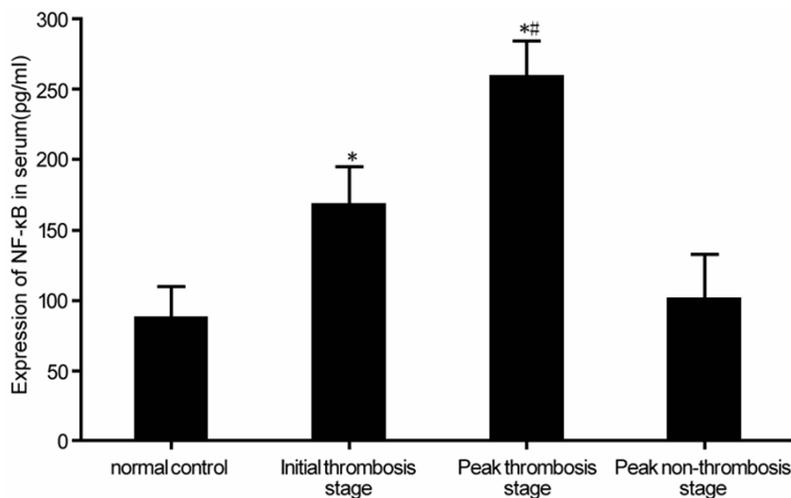
Figure 2. NF-κB expression in the serum of DVT rats at different periods. Serum was isolated from DVT rats at different stages, measuring NF-κB expression by ELISA. *P < 0.05, compared with normal control; #P < 0.05, compared with initial period.

re clamped with all-toothed mosquito vascular clamps in 3 places for 3 seconds each time. The skin incision was then sutured, layer by layer, using a surgical wire with no drainage tube. The same method was used to treat the opposite side. The rats were fixed after the operation with normal drinking and eating habits. No antibiotics were used.

Sample collection: A total of 6 mL of tail veins were extracted from each rat. A total of 3 mL were used for peripheral blood mononuclear cell (PBMCs) isolation. The remaining 3 mL of blood samples was centrifuged at 3,000 rpm for 15 minutes to separate nucleated cells and serum. They were placed in a refrigerator at -80°C for storage. Blood samples were collected at 24, 48, 72, and 96 hours postoperatively from DVT patients and 72 hours postoperatively from non-thrombosis patients. A total of 5 mL of blood was collected from the portal veins. After centrifugation at 3,000 rpm for 15 minutes, nucleated cells and serum were separated and placed in a refrigerator at -80°C.

Real-time PCR: Total RNA was extracted from the sa-mples using TRIzol Reagent, according to manufacturer instructions. It was reversely transcribed into DNA. PCR primer sequences were designed on Primer Premier 6.0 software and synthetized by Invitrogen (Shanghai, China) (**Table 1**). The PCR reaction procedure contained 55°C for 1 minute, followed by 35 cycles of 92°C for 30 seconds, 58-60°C for 45 seconds, and 72°C for 35 seconds. GAPDH was selected as the internal control. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Rat grouping and DVT modeling: According to references [14], the rats were randomly divided into the normal control group, DVT model containing initial thrombosis period group (3 hours after model establishment), peak thrombosis group (72 hours after model establishment), and peak non-thrombosis group, with 15 rats in each group. The skin was incised layer by layer. The medial incision was made in the inguinal region. Moreover, 3 cm of the femoral arterial vein was free and exposed. Femoral veins we-

Figure 3. MMP-2 mRNA expression changes at different periods in DVT rats. Total RNA was isolated from DVT rats at different stages, followed by analysis of MMP-2 mRNA expression by real-time PCR. *P < 0.05, compared with normal control; #P < 0.05, compared with initial period.
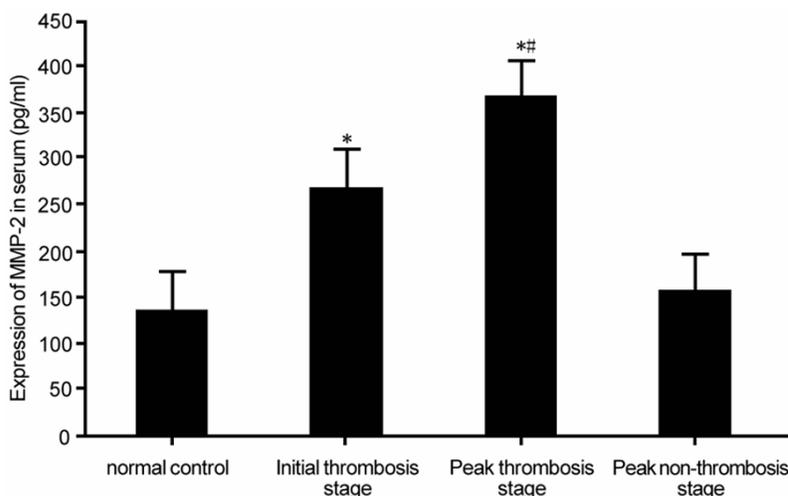
Figure 4. MMP-2 expression in the serum of DVT rats at different periods. Serum was isolated from DVT rats at different stages, measuring MMP-2 expression by ELISA. *P < 0.05, compared with normal control; #P < 0.05, compared with initial period.

ELISA: Serum levels of NF-κB and MMP-2 in each group were detected by ELISA. Collected peripheral blood was centrifuged and the supernatant was collected. The experimental procedure was performed according to ELISA kit instructions. The 50 µl diluted standard substance and samples were added to 96-well plates at 37°C for 30 minutes. After washing 5 times, the plates were added with 50 µl reagent A and 50 µl reagent B at 37°C for 10 minutes. Finally, the plates were added with 50 µl stop solution and tested at 450 nm. The standard curve was prepared based on OD values, calculating sample concentrations.

Statistical analysis

Data analyses were performed with SPSS 22.0 software. Measurement data are expressed as mean ± standard deviation (SD) and were compared by Student's t-tests and one-way ANOVA. The test level was taken as α = 0.05. P < 0.05 indicates statistical significance.

Results

Increased NF-κB mRNA expression in the serum of DVT rats

Real-time PCR and ELISA were used to detect expression levels of NF-κB mRNA in the serum of DVT rats at different stages. Expression of NF-κB in the thrombosis group was increased in the initial and peak period (P < 0.05). Compared with the initial stage of thrombosis, peak expression of NF-κB in the thrombosis group was obviously elevated (P < 0.05). There were no statistical differences in expression of NF-κB in the peak non-thrombosis group (P > 0.05) (**Figures 1**, **2**).

Elevated MMP-2 mRNA expression in the serum of DVT rats

Real-time PCR and ELISA were used to detect expression levels of MMP-2 mRNA in the serum of DVT rats at different stages. Expression of MMP-2 in the thrombosis group was increased in the initial and peak period (P < 0.05). Compared with the initial stage of thrombosis, peak expression of MMP-2 in the thrombosis group was obviously elevated (P < 0.05). There were
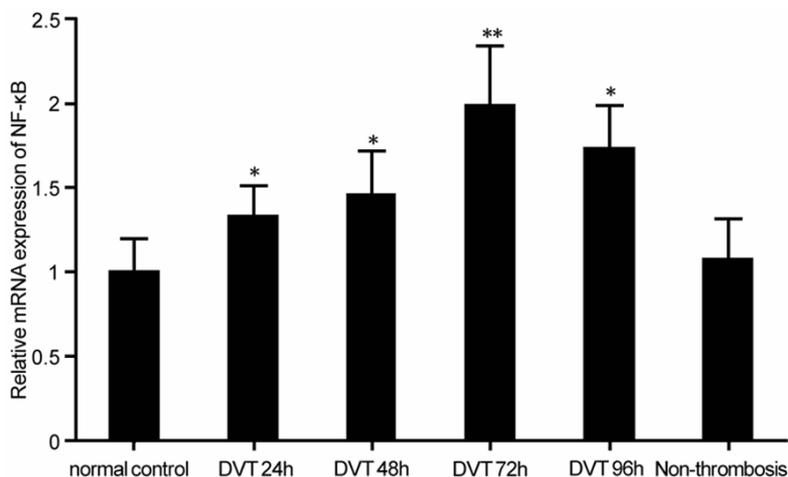
Figure 5. NF-κB mRNA expression in DVT patients at different stages. Total RNA was isolated from DVT patients at different stages, followed by analysis of NF-κB mRNA expression by real-time PCR. *P < 0.05; **P < 0.01, compared with normal control.
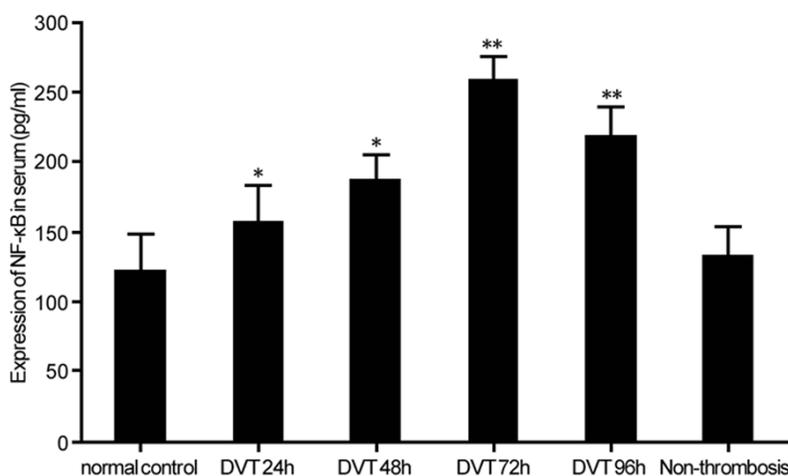
Figure 6. NF-κB expression in the serum of DVT patients at different stages. Serum was isolated from DVT patients at different stages, measuring NF-κB expression by ELISA. *P < 0.05; **P < 0.01, compared with normal control.

no statistical differences in expression of MMP-2 in the peak non-thrombosis group (P > 0.05) (**Figures 3**, **4**).

Increased NF-κB mRNA expression in the serum of DVT patients at different stages

Compared with the normal control group, expression of NF-κB in DVT patients was significantly increased with time dependence, reaching a peak at 72 hours (P < 0.05). No statistical differences were observed in NF-κB expression levels in the non-thrombosis group (P > 0.05) (**Figures 5**, **6**).

Elevated MMP-2 expression in the serum of DVT patients at different stages

Compared with the normal control group, expression levels of MMP-2 in DVT patients markedly increased with time dependence, reaching a peak at 72 hours (P < 0.05). No statistical differences were observed in MMP-2 expression levels in the non-thrombosis group (P > 0.05) (**Figures 7**, **8**).

Discussion

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that have a variety of biological functions. They may be involved in the regulation of tumors and cardiovascular diseases [17]. MMP-2 plays an important role in vascular repair. MMP-2 can regulate VEGF factor, as well as promote angiogenesis and collagen activity, during collagen conversion [18, 19]. Fractures and surgery in orthopedics are common causes of DVT [2, 3]. Therefore, in this study, patients undergoing fracture surgery were selected. A rat traumatic DVT model was prepared to analyze MMP-2 mRNA and protein level changes in peripheral blood. Results confirmed changes in MMP-2 mRNA and expression levels in serum of DVT rats at different stages. Expression of MMP-2 mRNA and protein in the thrombosis group was increased during the initial and peak thrombosis stages. It was higher in the peak stage. Expression of MMP-2 mRNA and protein was significantly elevated in DVT patients with time dependence, reaching a peak at 72 hours. However, there were no statistical differences in MMP-2 in patients without thrombosis, suggesting that MMP-2 participates in the formation of DVT. MMPs play a key role in endothelial injuries and inflammation.
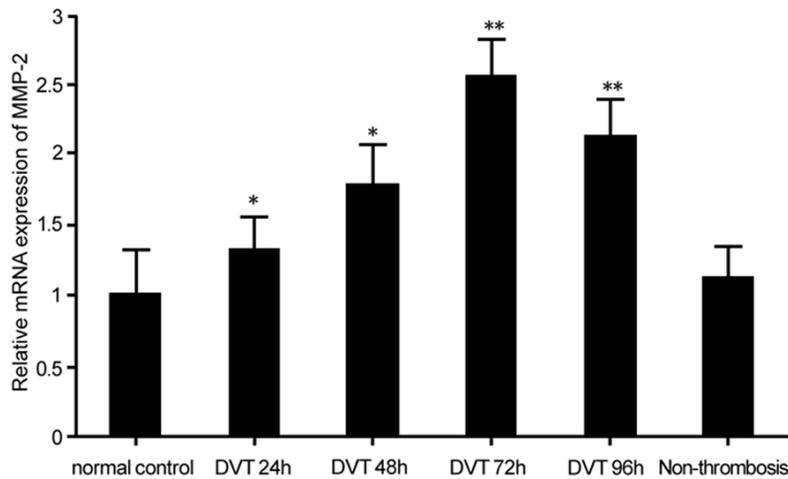
Figure 7. MMP-2 mRNA expression in DVT patients at different stages. Total RNA was isolated from DVT patients at different stages, followed by analysis of MMP-2 mRNA expression by real-time PCR. *P < 0.05; **P < 0.01, compared with normal control.
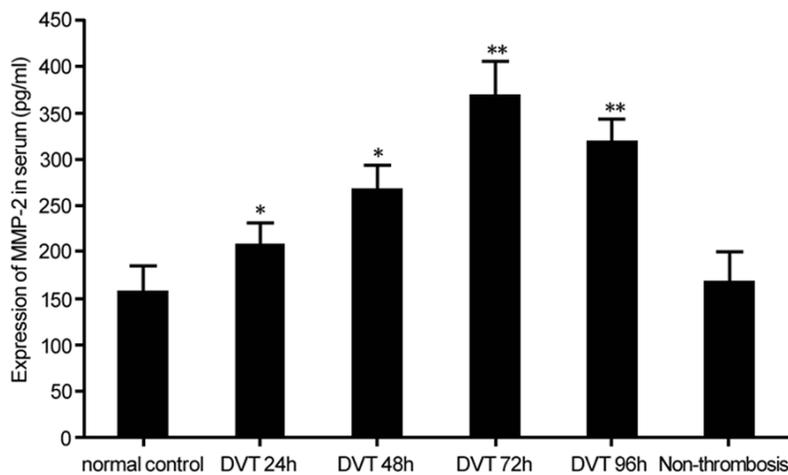
Figure 8. MMP-2 expression in the serum of DVT patients at different stages. Serum was isolated from DVT patients at different stages, measuring MMP-2 expression by ELISA. *P < 0.05; **P < 0.01, compared with normal control.

rates the structure of vascular endothelium. These factors are closely related to DVT [23]. It has been shown that vascular injuries, dysfunction of vascular endothelial coagulation, inflammation, and NF-κB activation induce exogenous coagulation pathways, causing intravascular thrombosis [24, 25]. The current study further analyzed expression levels of NF-κB mRNA and protein in the peripheral blood of fracture patients undergoing surgery, as well as in DVT rats. Results showed changes in NF-κB mRNA expression levels in the serum of DVT rats at different stages. Expression of NF-κB in the thrombosis group was increased in the initial and peak period. Compared with the initial stage of thrombosis, peak expression of NF-κB in the thrombosis group was significantly elevated. Compared with the normal control group, expression of NF-κB in DVT patients was significantly increased with time dependence, reaching a peak at 72 hours. No statistical differences were observed in NF-κB expression levels in the non-thrombosis group, suggesting that NF-κB par-

DVT is an interactive process of endothelial dysfunction and inflammatory response [20, 21]. Vascular injuries induce inflammation, resulting in enhanced paracrine and autocrine secretion of MMPs, increased endothelial permeability, and promotion of deep venous thrombosis [22].

In the process of inflammation, NF-κB activation can aggravate the accumulation and adhesion of neutrophils and macrophages. This releases many oxygen free radicals, destroys the integrity of blood vessel structure, and deterio-

ticipated in DVT process. The current study explored expression changes in NF-κB and MMP-2 in the formation of DVT, confirming their involvement in the progression of DVT. However, due to the limited number of patients enrolled in the present study, future large cohort clinical studies are required to confirm present findings.

Conclusion

NF-κB and MMP-2 expression levels increased during the formation of DVT. They were closely related to the formation and progression of

DVT, indicating that they may be key factors affecting the biological progression of DVT.

Disclosure of conflict of interest

None.

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