



Detection of targets and their mechanisms for early diagnosis of traumatic deep vein thrombosis

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ABSTRACT. The purpose of this investigation was to identify targets for the early diagnosis and predictors of deep venous thrombosis (DVT) and the role of these targets in the formation of venous thrombosis. A model of DVT was constructed in rats. Thromboses and venous walls were sampled for reverse transcription polymerase chain reaction study, and blood was sampled for enzyme-linked immunosorbent assay studies. Vein endothelial cells were cultured to observe the effects of interleukin (IL)-17 on the expression of tissue plasminogen activator (t-PA)/plasminogen activator inhibitor type 1 (PAI-1). IL-17 monoclonal antibody was used to study its effect on preventing the formation of DVT. One-hundred and twenty hours after the animal model was constructed, significant DVT started to form. Polymerase chain reaction tests showed that immediately after the model was created, the expression of IL-17 increased greatly, whereas the balance between t-PA and PAI-1 was disrupted just before DVT formed. The increase of serum IL-17 was positively related with the formation of DVT. Thus, the application of IL-17 monoclonal antibody could reduce the formation of DVT in

rats. IL-17 might be a target for the early diagnosis of DVT and should be further studied to assess its clinical value.

Key words: Deep Venous Thrombosis; Diagnostic indicator; Trauma

INTRODUCTION

Deep venous thrombosis (DVT) is formed in the deep vein cavity and blocks the main vein. Patients develop significant peripheral tissue congestion, which may lead to ecchymoses, amputation, and even pulmonary embolism if not treated in time (Brill et al., 2011). DVT is most often seen in the context of traumatic orthopedics, and amputation and mortality rates caused by DVT are increasing each year. Therefore, screening and early prevention of DVT has important clinical significance (Wang et al., 2013). Recent research on the mechanism of DVT formation has mainly focused on inflammation, and the fibrinolytic system and has shown that interleukin (IL)-17 plays an important role in connecting the inflammation and fiber formation/dissolution systems. Thus, we investigated IL-17 expression by constructing an animal model and measuring postoperative DVT formation time to study the relationship of IL-17 to the inflammation and fibrinolytic system response, its mechanism of action, and its value for the early diagnosis of DVT.

MATERIAL AND METHODS

Animals

Female Sprague-Dawley rats at 8-12 weeks old and weighing 280 ± 15 g were purchased from Weitong Lihua Animal Company, Shanghai, China. All animals were specified-pathogen-free grade and cultured at Gannan Medical College animal center. The experiments started after 1-week adaptation for the rats. All animals were raised in strict accordance with the Animal Ethical Standard, and this study was approved by the Experimental Animal Center of Gannan Medical College, Jiangxi Province Ethics Committee.

Grouping

After the model was created, the rats were grouped according to the following time points:
Group A: 10 rats; normal control with no treatment.
Group B: 10 rats; acute injury group, observed at 1 h after modeling.
Group C: 10 rats, early thrombosis group, observed at 72 h after modeling.
Group D: 30 rats; thrombosis group, observed at 120 h after modeling.
Group E: Rats without thrombosis as mentioned above; classified as no thrombosis group.

Modeling method

Rats were operated without anesthesia to reduce disturbance from exogenous factors. The quantitative targeting method was used. Briefly, 5 J of energy was applied to the proximal lateral aspect of the lower limbs of the rats (about 1.0 cm inferior to the greater trochanter of the femur). Modeling was finished if a fracture was observed through the rat movement (Diaz et al., 2012).

Sampling and detection

After each rat was anesthetized with 4% chloral hydrate at 0.3 mL/kg, a 3-cm incision was made in the skin of the lower limb along the direction of the femoral vein. The femoral vein branches were exposed, and the vessel with thrombosis or nearest to the fracture was excised according to the protocol from a previous study (Manly et al., 2011). The thrombosis was rinsed with a saline solution and quickly fixed with 4% paraformaldehyde. The blood vessel wall was saved in liquid nitrogen. For the 30 rats in group D, blood was collected from the angular vein for enzyme-linked immunosorbent assay (ELISA) testing at 1, 24, 48, 72, and 120 h after the operation.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from blood vessels by TRIzol (Takara), and cDNA was synthesized using reverse transcriptase (Takara) after quality was verified with a nucleic acid spectrometer. Real-time RT-PCRs were performed using the Takara PCR Kit and were detected on an ABI system according to a previously established protocol (Rodriguez et al., 2012). The primers used are listed in Table 1.

Table 1. Primers for RT-PCR detection.

Gene	Primer
IL-17 F	5'-GGTCAACCTCAAAGTCTTAACTC-3'
IL-17 R	5'-TTAAAAATGCAAGTAAGTTTGCTG-3'
t-PA F	5'-AGGATTGTGGGAATGG-3'
t-PA R	5'-TCAGATGAGATGACAGGAAAT-3'
PAI-1 F	5'-GTTTCGCTTCACCCCTCCAGA-3'
PAI-1 R	5'-GAAATAGAGGGCGTTCACCAGC-3'

IL-17 = interleukin-17; t-PA = tissue plasminogen activator; PAI-1 = plasminogen activator inhibitor type 1.

ELISA detection

Blood from the angular vein was collected in a tube containing an anticoagulant. After the specimen was centrifuged for 5 min at 1000 r/s at 4°C, the serum was extracted and transferred into a sterile Eppendorf tube. The supernatant was saved at -80°C after the serum was centrifuged for 10 min at 10,000 r/s at 4°C. IL-17, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor type 1 (PAI-1) were measured using an ELISA Kit (Oxford) (Martinod et al., 2013).

Cell stimulation test

Rats' femoral venous endothelial cells were extracted from the primary specimen. Normal rat femoral vein was extracted and rinsed with phosphate-buffered saline (PBS). Cells were digested by type I collagen enzyme for 20 min and centrifuged for sediment. The residual cells were cultured with special endothelial cell culture and 20% fetal calf serum. Cells fused over 90% were passaged after 2 weeks' culture, and the second generations were used for the experiment. The cells were classified into 3 groups: blank, control, and stimulation. A total of

50 ng/mL IL-17 (PeproTech) was added to the stimulation group, while an equal amount of PBS was added to the control group; no stimulating factors were added to the blank group. In the supernatant, t-PA and PAI-1 were detected using ELISA after 24 h stimulation. Each experiment was repeated 3 times (Guenther et al., 2013).

Animal intervention

Sixty rats were divided equally into 2 groups. In the control group, 30 rats were modeled as mentioned above; in the treatment group, each rat was injected with 50 μ g IL-17 monoclonal antibody (PeproTech) in the caudal vein before modeling.

Statistical analysis

Alterations between different subgroups were analyzed using chi-square and *t*-tests when appropriate. The Pearson test was used for correlation analysis. All statistical analyses were performed using the SPSS 17.0 software (Chicago, IL).

RESULTS

DVT model construction

No thrombosis was found in group B, and the formation rate was 0% as compared with group A. Only 1 rat in group C developed mild thrombosis, and the formation rate was 10%. In group D, 17 of 30 rats formed thromboses, and the formation rate was 56.67%. The remaining 13 rats without thrombosis were assigned to group E. Pathological sections are shown in Figure 1. Figure 1A represents a thrombosed rat vein, and Figure 1B depicts a normal vein.

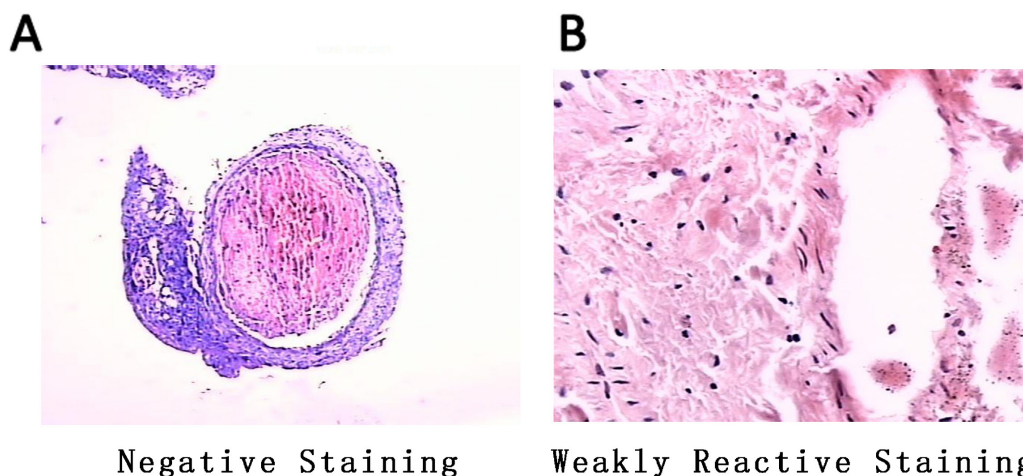


Figure 1. Deep vein thrombosis model construction. **A.** Thrombosed vein: Numerous mixed thrombi are seen in the vascular cavity, and the lumen is blocked. **B.** Normal vein: the vascular cavity is unobstructed, supported by the surrounding tissue naturally, and presents normal shape.

PCR detection in blood vessels

The PCR results for blood vessels from each group are presented in Table 2. IL-17 mRNA expression increased in the first 72 h after the trauma while t-PA decreased, and PAI-1 increased significantly after 72 h. IL-17 expression was higher in group D than in group E. PAI-1 also increased after 120 h ($P < 0.05$).

Table 2. PCR detection results.

Group	IL-17	t-PA	PAI-1
A	0.13	5.18	0.69
B	3.25	2.34	1.62
C	3.02	1.52	298
D	2.38	0.19	5.42
E	1.26	1.06	3.87

IL-17 = interleukin-17; t-PA = tissue plasminogen activator; PAI-1 = plasminogen activator inhibitor type 1.

ELISA detection in blood

Blood was sampled at different times in groups D and E. IL-17 expression significantly increased within 72 h after modeling in group D, whereas it was unchanged in group E. Accordingly, t-PA expression in group E was markedly higher than in group D at 120 h after surgery, whereas its inhibitor, PAI-1, was lower. This indicates that the antithrombotic ability of vessel endothelium at 120 h in group E was better than in group D (Figure 2A, B and C).

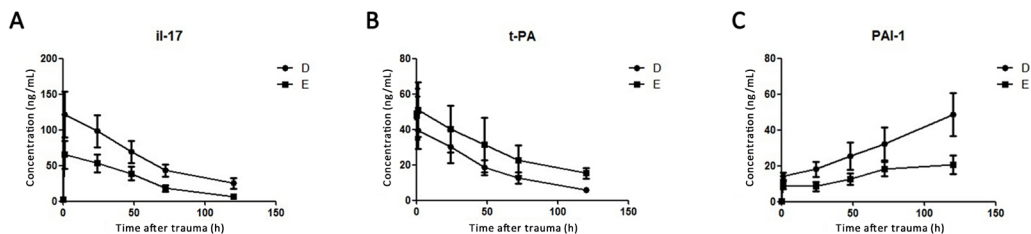


Figure 2. Enzyme-linked immunosorbent assay (ELISA) detection in blood. **A.** IL-17 measured with ELISA: IL-17 is higher in group D than in group E after modeling. **B.** t-PA measured with ELISA: t-PA secretion level is higher in group E. **C.** PAI-1 measured with ELISA: PAI-1 secretion level is higher in group D. IL-17 = interleukin-17; t-PA = tissue plasminogen activator; PAI-1 = plasminogen activator inhibitor type 1.

Cell stimulation test

After treatment with IL-17, the t-PA expression level significantly decreased in the supernatant of endothelial cells in the stimulation group compared with the blank and control groups, whereas the PAI-1 level notably increased (Figure 3A, B, $P < 0.05$). This indicates that the antithrombotic ability of the endothelial cells was attenuated under IL-17 stimulation.

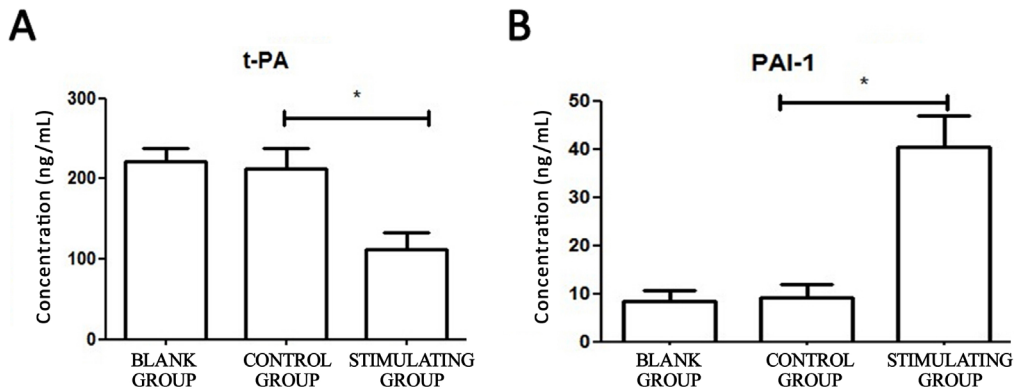


Figure 3. Cell stimulation test. **A.** t-PA from the supernatant measured with ELISA: Cells in the stimulation group secrete less t-PA after treatment with IL-17. **B.** PAI-1 from the supernatant measured with ELISA: Cells in the stimulation group secrete more PAI-1 after treatment with IL-17. t-PA = tissue plasminogen activator; ELISA = enzyme-linked immunosorbent assay; PAI-1 = plasminogen activator inhibitor type 1; IL-17 = interleukin-17.

Animal intervention effect

We dissected and observed thrombosis formation in rats after 120 h modeling. Thirty femoral veins (2 lower limb femoral veins modeled for each rat) from 16 of 30 rats in the control group formed thromboses, with a formation rate of 50.0%. In the treatment group, only 5 veins from 4 rats formed thromboses, with a rate of 8.33%. The formation rate in the treatment group was significantly lower than the rate in the control group ($P < 0.05$).

DISCUSSION

DVT incidence rate after joint replacement and fracture fixation can reach 40-60%. Amputation and death from pulmonary embolism caused by DVT are the most severe complications in traumatic orthopedics (Baldwin et al., 2012). However, the research on early diagnosis and prevention of DVT is still deficient. Although the classical theory of thrombogenesis explains the basic process of DVT formation, the theoretical explanation for DVT prediction and the need for intervention are still lacking. While DVT in the early stage progresses slowly, its definite diagnosis is mainly based on invasive and costly tests such as angiography (Galanaud et al., 2012). Furthermore, by the time DVTs are definitively diagnosed, the chance for preventive treatment is lost, and severe patient outcomes may still occur (Brill et al., 2011).

Recently, it has been proposed that inflammation in the early stage of injury and fiber repair balances in the late stage together constitute the DVT physiological process (Jain et al., 2013). Based on gene microarray, previous studies have found that many inflammatory cytokines are involved in the early inflammatory reaction of traumatic DVT. Further cell signal pathway analysis has proved that the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway participate in DVT formation (Patterson et al., 2013). The STAT signal pathway in the vessel endothelium is activated under trauma and acts on downstream target genes such as Akt and Cyclin D. It also regulates the antithrombotic ability and apoptosis of the vessel endothelial cell directly (Pabinger-Fasching et al., 2014). In our study,

we simulated the common traumatic DVT process clinically using the energy-fixed method (Martinod and Wagner, 2014). The success rate of modeling was 50%. Pathological evaluation showed mixed thrombus formation in the venous lumen. Many hemocytes were found within the large plasma fibrin skeleton, while inflammatory cells infiltrated the endothelial cells. The pathologic section also confirmed that inflammation and fiber formation coexist in the DVT microenvironment (Geddings et al., 2014).

To quantify the morphological evaluation index, we focused on the regulatory factors t-PA and PAI-1, which are commonly accepted for quantifying the local thrombosis state. PAI-1 is a type of serine proteinase inhibitor, and it can be secreted by vascular endothelial cells, fibroblasts, and macrophages (Deatrick et al., 2013). PAI-1 can inhibit t-PA activation and reduce fiber dissolving characteristics locally, thus regulating the fibrinolytic system balance. In the microenvironment, a higher t-PA leads to lower thrombophilia, while a higher PAI-1 causes opposite results (Heit et al., 2011). However, despite their application in thrombosis probability evaluation, changes in t-PA and PAI-1 are not time-sensitive - a localized area with a significant change in the t-PA/PAI-1 ratio would inevitably form a thrombus. Furthermore, the ratio change only exists at the point of injury, which makes it difficult to detect through routine clinical examination. Thus, this pair of regulatory factors can be used in the laboratory for thrombosis judgment but is not helpful for early clinical diagnosis of DVT (Agmon-Levin et al., 2013).

Inflammation plays an important role in thrombus formation and mostly occurs immediately after trauma. Acute inflammation often reaches or surmounts its peak before the thrombus appears. Numerous infiltrating inflammatory cells can activate local destruction and phagocytosis by releasing peroxidase, inflammation factors, complements, and other chemical mediators. Some scholars have pointed out that the levels of classic T helper cell type 1 (Th1) factors such as IL-1 β , tumor necrosis factor alpha, and IL-6 peak in the serum before DVT formation (Pabinger-Fasching et al., 2014). Although such factors can reflect local inflammation, the relationship between these traditional factors and the fiber formation/dissolution system is still unclear; thus, they cannot serve as warning molecules for early thrombosis despite their early appearance characteristics (Geddings et al., 2014).

A new type of inflammatory factor, IL-17, has attracted interest in recent years (Yus Teruel et al., 2012). It can be secreted by Th1 cells or by innate immune system cells during the acute inflammation period. It not only acts as a chemotaxis inflammatory cell to facilitate inflammation, but also has a crucial impact on the fiber formation/dissolution system. IL-17 has been reported to play an important role in promoting fibrinolytic activity in different types of compensatory local fibrosis seen in diseases such as psoriasis, pulmonary fibrosis, and abdominal adhesions (Golden et al., 2013). We investigated its role in the process of thrombosis and found that it achieved a peak in both the vascular endothelium and serum before thrombosis occurred. Most interestingly, the IL-17 peak value in group E was significantly lower than that in group D. This preliminary result suggests that higher expression of IL-17 could be an important predictive factor before thrombosis appears. When we further stimulated the vascular endothelial cell using IL-17, the result was downregulation of t-PA and upregulation of PAI-1. This indicates that IL-17 may facilitate thrombus appearance through regulating the t-PA and PAI-1 secreted by endothelial cells. Animal experiments have confirmed that the incidence of thrombosis can be effectively reduced if IL-17 peak appearance is blocked. We validated the status of IL-17 as a clinical predictor of early thrombosis and verified its physiological mechanism for promoting thrombosis, both *in vivo* and *in vitro*.

Inflammation appears early after trauma and can promote thrombosis. Study of the

inflammatory response is likely to be valuable for determining early predictors of thrombosis for the detection of thrombosis. Among numerous inflammatory factors, IL-17 is important for its close relationship with the fiber formation/dissolution system. We confirmed the value of the IL-17 peak level in rats as a predictor for thrombosis risk after surgery. Therefore, its clinical role and protein signal pathway warrant further investigation.

Conflicts of interest

The authors declare no conflict of interests.

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