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TGF-β1 and Serpine 1 expression changes in traumatic deep vein thrombosis

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ABSTRACT. The objective of this study was to investigate the expression changes of transforming growth factor β1 (*TGF-β1*) and Serpine 1 in rats with traumatic deep vein thrombosis (DVT). In total, 60 male Sprague Dawley rats were divided into model (N = 50) and control groups (Group A, N = 10). From the model group, 10 rats were randomly selected after modeling as the pre-thrombosis group (Group B, N = 10), and the remaining 40 rats in the model group were divided into the thrombosis (Group C) and no thrombosis groups (Group D) depending on whether DVT was apparent at 25 h after modeling. All rats were dissected and the total RNAs of the femoral veins were extracted. *TGF-β1* and Serpine 1 expression was detected by microarray and polymerase chain reaction (PCR) analyses, and the related signal pathways were analyzed using bioinformatic analysis. Of the 40 rats, DVT was evident in 23, yielding an incidence rate of 57.50%. *TGF-β1* and Serpine 1 expression increased significantly at 2.5 h after modeling, while DVT began to form at 25 h after modeling. Both PCR and microarray analysis showed that *TGF-β1* and Serpine 1 expression levels were significantly higher in the thrombosis group than in the other groups ($P < 0.05$). Bioinformatic analysis indicated

that *TGF-β1* was an upstream regulatory gene of Serpine 1 and could induce Serpine 1 overexpression. Together, these results suggested that *TGF-β1* and Serpine 1 overexpression might play an important role in DVT formation and have predictive values.

Key words: Deep vein thrombosis; Serpine 1; *TGF-β1*; Fibrinolysis

INTRODUCTION

Deep vein thrombosis (DVT) is a common clinical complication. Although the incidence of DVT has been reduced in recent years, it remains common in high-risk patients with long postoperative recovery times or who remain bed-ridden (Si et al., 2014). If not treated in a timely manner, DVT will develop into phlegmasia cerulea dolens, which leads to amputation or even pulmonary embolism, resulting in endangerment of patient lives. Populations at risk for DVT, therefore, must be subjected to early diagnosis. However, the mechanisms of thrombosis promotion and the establishment of imbalances of fribrinolysis/antiplasmin and coagulation/anticoagulant systems during early thrombosis are still not fully clear. To date, there is a lack of available reliable markers for DVT early diagnostics (Kang et al., 2014). In the present study, we focused on the differential expression of genes in the femoral vein during the early stages of DVT formation, with the goal of providing a basis for the development of markers for early clinical diagnosis.

MATERIAL AND METHODS

The 60 male Sprague Dawley rats used in this study were provided by the China Medical University Animal Center (Shenyang, China). The mean rat weight was 245 ± 25 g (range, 220-280 g) and their ages were between 8 and 12 weeks. The experiments were started after the rats had received adaptable feed for 1 week.

Methods

Modeling and grouping

The 60 rats were divided into model (N = 50) and control groups (Group A, N = 10) according to the random number table method. Rats in the model group were immobilized and dosed with 5 joule energy at the proximal lateral position of the double lower limbs. The limbs were fixed by plaster after fracture was confirmed. Rats in the control group were given a sham operation by suturing the skin immediately after incision. From the model group, 10 rats were randomly selected after modeling as the pre-thrombosis group (Group B, N = 10), and the remaining 40 rats in the model group were divided into thrombosis (Group C) and no thrombosis groups (Group D) according to whether DVT manifested at 25 h after modeling.

RNA extraction and detection

The femoral veins and surrounding tissues were collected and washed with normal saline. The femoral vein endothelial tissue was separated and cracked under a microscope

after removal of impurities and the thrombus. Total RNA was extracted and stored at -80°C.

Microarray

A GeneChip Rat Genome 230 2.0 gene chip (Affymetrix, Santa Clara, CA, USA) was used for microarray analysis. The Affymetrix V5.0 software was used for data analysis. An ABI 7900HT Fas system (Life Technologies, Carlsbad, CA, USA) was used for real-time polymerase chain reaction (PCR) amplification. The Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) was applied for primer design (Table 1). Gene expression levels were quantified relative to the expression of *GAPDH* using an optimized comparative Ct (ΔCt) value method (Schmittgen and Livak, 2008). Bioinformatic analysis on *TGF-β1* and Serpine 1 signaling pathways was performed through the KEGG pathway database (Shiraev et al., 2014).

Table 1. *TGF-β1* and Serpine 1 primers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)	Temperature (°C)
GAPDH	AAC CTG CCA AGT ATG ATG ACA TCA	TGT TTG AAG TCA CAG GAG ACA ACC T	111	55
TGF-β1	GAG GAT GAA AGA AAC AGC CAG CT	CAG ATC CCA TTG ATT TCC AC	155	52
Serpine 1	GAG GAT GAA AGA AAC AGC CAG CT	CCC GCT ATG AAA TTA GAT TCA CGT	145	56

Observation indexes

The DVT incidence and severity, as well as the microarray and PCR results were observed. The DVT classification standard utilized referred to Poley et al. (2014) as follows: level 0, no DVT; level 1, partial DVT formation; and level 2, complete DVT formation.

Statistical analysis

The SPSS 20.0 statistical software (SPSS, Chicago, IL, USA) was used for data analyses. Data counts were analyzed by the χ^2 test; measurement data are reported as means ± standard deviation5 of the mean and compared by ANOVA among different groups. Bonferroni's correction was applied for pairwise comparison, with statistically significant differences defined as $P < 0.05$.

RESULTS

DVT incidence rates and severity distribution

DVT manifested in 23 of the 40 rats, with an incidence rate of 57.50%. There were 15 rats at level 1 (65.22%) and 8 at level 2 (34.78%). The remaining 17 rats were grouped in the non-thrombosis group (Figure 1).

Comparison of real-time PCR results

The expression levels of *TGF-β1* and Serpine 1 were significantly higher in Group B

than in Group A (P < 0.05). Among the 4 groups, their expression was highest in Group C (P < 0.05) (Figure 2 and Tables 2 and 3).

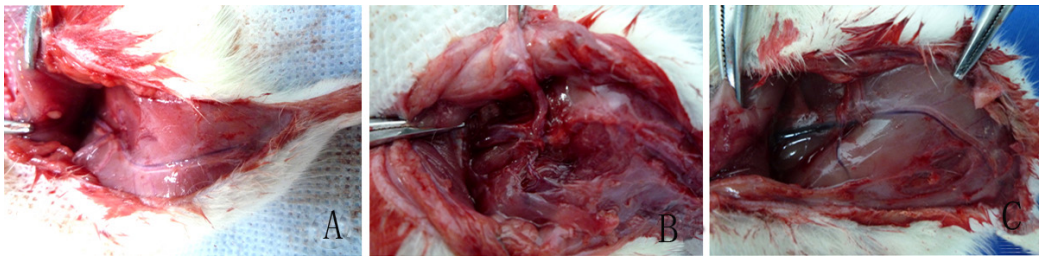

Figure 1. Variety of deep vein thrombosis (DVT) evident in the rat population. **A.** No DVT formation. **B.** Level 1 (partial DVT formation). **C.** Level 2 (complete DVT formation).

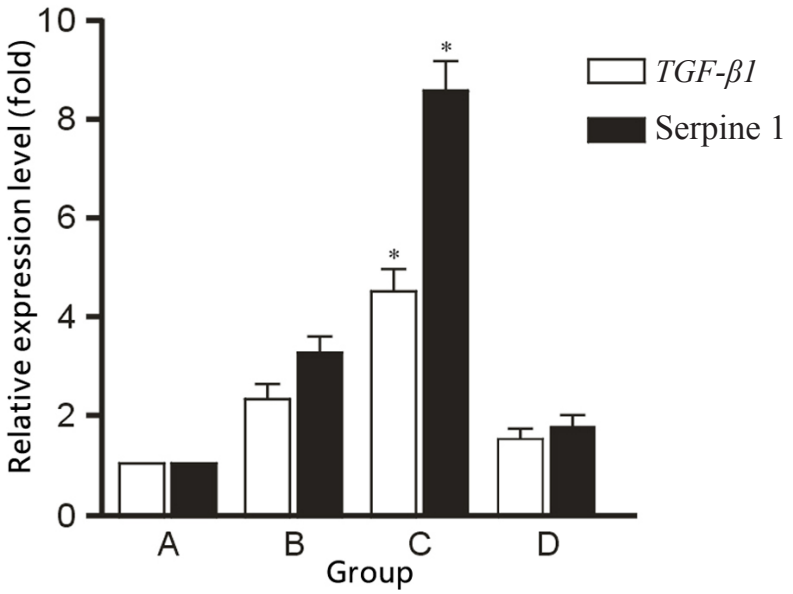

Figure 2. Comparison of expression levels of *TGF-β1* and Serpine 1 between rats from each group. *P < 0.05 compared with groups A, B, and D. Data are reported as means ± SD.

Table 2. *TGF-β1* and Serpine 1 expression in each group (means ± SD).

Group	Numbers	*TGF-β1*	Serpine 1
Group A	10	45.81 ± 12.79	19.73 ± 6.41
Group B	10	60.15 ± 16.78	48.64 ± 8.22
Group C	23	114.59 ± 17.41	133.26 ± 10.31
Group D	17	46.23 ± 13.48	20.17 ± 5.89

Table 3. Real-time PCR results (means ± SD).

Group	Numbers	*TGF-β1*		Serpine1	
		ΔCT	Relative expression	ΔCT	Relative expression
Group A	10	15.47 ± 0.49	1	11.06 ± 0.62	1
Group B	10	14.28 ± 0.29	2.33 ± 0.34	9.35 ± 0.77	3.28 ± 0.36
Group C	23	13.31 ± 0.43	4.48 ± 0.47	7.96 ± 0.55	8.56 ± 0.62
Group D	17	15.29 ± 0.38	1.16 ± 0.22	10.93 ± 0.52	1.08 ± 0.27

PCR = polymerase chain reaction.

Comparison of microarray results

The microarray results showed that the expression levels of *TGF-β1* and Serpine 1 were higher in Group B than in groups A and D ($P < 0.05$), while Group C exhibited the highest expression level among the 4 groups ($P < 0.05$; Figure 2).

Signaling pathway analysis

Bioinformatic analysis showed that *TGF-β1* was an upstream regulatory gene of Serpine 1, and that *TGF-β1* could induce Serpine 1 overexpression and inhibit fibrinolytic activity (Figure 3).

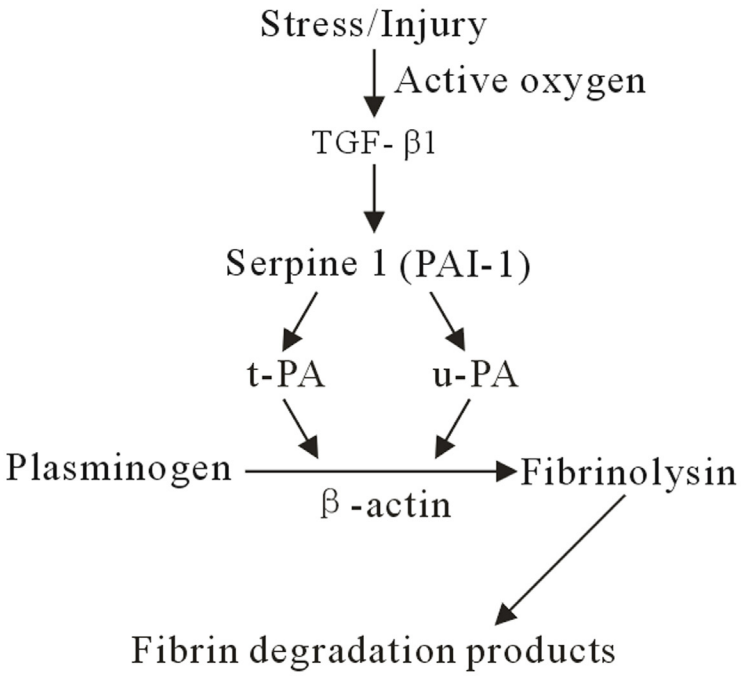

Figure 3. Bioinformatic analysis of *TGF-β1* and Serpine 1. t-PA = tissue plasminogen activator; u-PA = urokinase plasminogen activator.

DISCUSSION

Clinical research has shown that the incidence of DVT was 42-73% in elderly patients after hip replacement surgery, while it was 37-59% after knee replacement (Rodriguez et al., 2012). Thrombosis is prone to detachment, causing pulmonary embolism when it is in a state of flux, which endangers the life of the patient. In the clinic, laboratory examinations, imaging, physical examination, and clinical symptoms are the most frequently used methods to establish DVT diagnosis. However, clear diagnosis can only be made when a thrombosis is formed. At present, the mechanisms underlying the regulation and interaction among inflammatory cells, platelets, and venous endothelial cells are still being debated, as are those underlying the imbalance of the fribrinolysis/antiplasmin and coagulation/anticoagulant systems during early thrombosis that engender the body microenvironment conducive to thrombosis. To date, there are still no reliable early diagnosis and prediction markers for DVT (Martinod et al., 2013).

Previous animal studies have shown that many factors such as local deep vein blood flow clogging, accumulation of acidic metabolites, microenvironment dysfunction, and hypoxia can significantly upregulate endothelial cell activation factors such as tumor necrosis factor, interleukin-6, NF-κB, and reactive oxygen species, while reducing the expression of inhibitory factors such as nitric oxide and glutathione, leading to increased synthesis and secretion of ceramide and subsequent structural damage to DNA, mitochondria, lysosomes, the endoplasmic reticulum, and cell membranes (Guenther et al., 2013). Thus, local microenvironment changes might impact the generation and consumption balance of fribrinolysis/antiplasmin and coagulation/anticoagulant materials. The fibrinolytic system is composed of a fibrinolytic enzyme inhibitor, a fibrinolytic enzyme activator, and a fibrinolysis enzyme, of which the fibrinolytic inhibitor is comprised of thrombin-activated fibrinolysis inhibitor, α2-antiplasmin, and plasminogen activation inhibitors. Serpine 1, also known as plasminogen activation inhibitory factor 1, is a member of the serine protease inhibitor gene family. The Serpine 1 gene has been shown to be located at chromosome 7q21.2-q22 and to encode a protein with a relative molecular mass of 48,000-50,000 kDa (Baldwin et al., 2012). Serpine 1 is primarily synthetized by liver cells, fibroblasts, myocardial cells, macrophages, fat cells, and vascular endothelial cells. In-depth research has found that platelets can also store and synthetize Serpine 1 (Deatrick et al., 2013). Platelet activation would therefore be predicted to release a large quantity of Serpine 1 molecules, which could facilitate thrombosis by inhibiting thrombus degradation. The main physiological function of Serpine 1 is thought to be maintenance of the balance of the fribrinolysis/antiplasmin system through inhibition of the activity of tissue plasminogen activator (t-PA), urokinase plasminogen activator (u-PA), and serine protease. The fibrinolytic process includes the activation of fibrinogen, fibrin degradation, and plasminogen activation, while fibrinolysis is initiated through u-PA- and t-PA-mediated plasminogen activation. Plasminogen can be activated into active plasmin by t-PA through an extravascular pathway or by u-PA through an intravascular pathway. The compound formed by fibrin, t-PA, and plasminogen promotes fibrin degradation and the production of large amounts of plasmin. Serpine 1 can inhibit thrombolysis and plasmin formation by blocking the activity of uPA/tPA.

Serpine 1 is an important physiological regulatory factor that participates in the plasmin extracellular proteolytic cascade reaction. It can regulate vascular smooth muscle cell migration and also play very important physiological roles in the processes of tumor

malignancy, insulin resistance, obesity, inflammatory reactions, venous endothelial cell apoptosis, blood vessel aging damage, and atherosclerosis (Stolz et al., 2011). Some studies have found that Serpine 1 is overexpressed significantly in atherosclerosis and following vascular tissue damage, while interleukin-6 expression was decreased in Serpine 1 knockout rats (Jain et al., 2013). Recently, it has been found that elevated Serpine 1 levels can reduce overall fibrinolytic ability, which might comprise an important risk factor for DVT formation (Patterson et al., 2013). Additionally, DVT formation has a close relationship with Serpine 1 gene 4G/5G polymorphisms. Animal results have shown that Serpine 1 expression was increased in the DVT rat model compared with control animals (Houshmand et al., 2014). In the present study, it was found that Serpine 1 began to rise in the femoral vein of rats 2.5 h after trauma. Serpine 1 levels were increased significantly 25 h after trauma, while there was no significant difference in its expression between non-thrombosis group and control animals. Our results revealed that Serpine 1 might play an important role in the process of traumatic DVT development.

It had been found that Serpine 1 is regulated by numerous upstream factors such as angiotensin II, estrogen, reactive oxygen species, glucose, proinsulin, insulin, interleukin-1, tumor necrosis factor-α, and free fatty acids (Mishra et al., 2014). Of these, interleukin 1 and tumor necrosis factor-α increase Serpine 1 expression through stimulating fat cells; free fatty acids increase Serpine 1 synthesis and secretion by stimulating endothelial cells; proinsulin and insulin can increase Serpine 1 expression in liver cells; and glucose can induce Serpine 1 synthesis and its release in smooth muscle cells and endothelial cells (Cho et al., 2014). In recent years, the effect and mechanism of TGF-β regulation of downstream factors has received wide attention. TGF-β was shown to participate in the occurrence and developmental process of myocardial hypertrophy, diabetes, hypertension, and atherosclerosis through gene regulation mechanisms (Rovite et al., 2014). A previous study has shown that *TGF-β1* can stimulate Serpine 1 synthesis and secretion in smooth muscle cells (Le Moigne et al., 2014). In the present study, *TGF-β1* and Serpine 1 expression levels were found to be increased in rat femoral veins 2.5 h after trauma, and had increased significantly at 25 h after trauma. Further bioinformatic analysis showed that TGF-β1 can induce Serpine 1 overexpression and suppress fibrinolysis. Our results suggested that the significant increases in *TGF-β1* levels might in turn increase Serpine 1 secretion and synthesis to promote the formation of a thrombus microenvironment during the early phase of DVT formation. This model is in accord with previous clinical results (Alsina et al., 2014; Rolston et al., 2014).

In conclusion, the *TGF-β1* and Serpine 1 expression changes in the DVT rat model observed in this study were consistent with DVT formation processes. *TGF-β1* and Serpine 1 overexpression might play important roles in DVT formation. Their high expressions in the early phase of DVT development have predictive value for DVT, although the exact mechanism underlying this process and its reliability as a biomarker still require further investigation.

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