

Expression changes and roles of matrix metalloproteinases in a rat model of traumatic deep vein thrombosis

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【Abstract】 Objective: To study the expression changes of matrix metalloproteinases (MMPs) in traumatic deep vein thrombosis (TDVT) in a rat model with the aid of gene chip technology and to explore the roles of MMPs in TDVT.

Methods: Totally 150 Sprague Dawley rats were randomly divided into control group ($n=10$) and model group ($n=140$). Rat models of TDVT were established by clamping the femoral vein and fixing the bilateral hind limbs. Then fixation of the hip spica with plaster bandage was conducted. According to the observation phases and/or biological situations of the femoral vein thrombosis, the model rats were further divided into 7 groups. Vascular tissues were obtained from each group through noninvasive incision into the femoral vein at corresponding time points. We adopted the Trizol one-step method for total RNA extraction, Affymetrix RAT 230 2.0 array for detection of RNA expressions and fold change (FC) analysis for changes of differential expressions of MMPs in each group. The main outcome parameters measured included expressions of MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-16, MMP-23 and MMP-

24. Gene array data of these MMPs were analyzed by the Affymetrix Microarray Analysis software (Version 5.0).

Results: FC analysis showed differential expressions of MMPs in each group during the course of TDVT. At the initial period of thrombosis, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, and MMP-24 had significantly high expression, while MMP-12, MMP-13, MMP-14, MMP-16 and MMP-23 had relatively low expression. MMPs were all highly expressed at the peak time of thrombosis. In the process of thrombus resolution, MMP-2, MMP-10, MMP-16 and MMP-24 have relatively low expression, while MMP-12, MMP-13, MMP-14, MMP-16 and MMP-23 have significantly high expression.

Conclusion: MMPs may affect the process of TDVT through transcription regulation of the fibrinolysis-anti-fibrinolytic system during the course of thrombosis and thrombus resolution.

Key words: Rats; Wounds and injuries; Venous thrombosis; Genes; Matrix metalloproteinases

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Traumatic deep vein thrombosis (TDVT) refers to deep vein thrombosis resulting from trauma or operation. It is common and can bring about multiple complications such as phlegmasia cerulea dolens that requires limb amputation and life-threatening pulmonary thromboembolism. Studies at cellular and molecular levels reveal that local cells change after

trauma and generate corresponding expression products that further change cell function and transmit messages around according to increased, decreased, activated or unactivated gene expressions. Previous studies have shown that TDVT is a complex disease with multiple factors, genes and systems involved in.^{1,2} Matrix metallo-proteinases (MMPs), which result in excessive degradation of the endothelial basement membrane, are one of the main factors that affect thrombosis and thrombus resolution and the mechanisms have become a hotspot in recent years.

METHODS

Animals

This study was approved by the local animal experimentation and ethics committee. A total of 150

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Sprague Dawley (SD) rats, specific pathogen free, 8-12 weeks old, weighing 250-300 g, were purchased from the Animal Center of Kunming Medical College [License: SCXK (Yunnan) 2005-0008].

Establishment of TDVT rat models³

The 150 SD rats were randomly divided into normal control group (Group A, $n=10$) and model group ($n=140$). The model rats were anesthetized with 3% pentobarbital sodium for 1 mg/kg. Their bilateral thighs were depilated. Along the longitudinal axis of the thigh, an about 2 cm-long incision was made on each side to expose the femoral vein. Each femoral vein was clamped in three different positions within 3 seconds by 12# mosquito forceps. Then, the incision was stitched and rats were fixed with hip spica cast. After completion of the modeling, these rats had access to water and grain. Antibiotics were not used.

Grouping

According to different observation phases and biological situations of the femoral vein thrombosis, model rats were further divided into 7 subgroups: Group B (0.5 hour after trauma, the initial period of thrombosis), Group C (2.5 hours after trauma, the peak time of thrombosis), Group D (25 hours after trauma, thrombus at peak time), Group E (25 hours after trauma, no thrombus at peak time), Group F (72 hours after trauma, thrombus resolution), Group G (72 hours after trauma, thrombus insolubilization) and Group H (168 hours after trauma, non-thrombosis, Figure 1). At each corresponding phase, every 10 rats with the particular pathological features were selected into model groups through gross observation.

Materials

At each corresponding time point, vascular tissues about 2 cm-long from the bilateral femoral vein of each group were obtained and flushed with 0.9% normal saline to remove blood and intraluminal thrombosis. Then the tissues were frozen in freezing tubes within 30 seconds in vitro and preserved in liquid nitrogen canisters for total RNA extraction.

RNA extraction, chip hybridization and scanning

The total RNA was extracted using the Trizol one-step method and preserved at -80°C after its quality and quantity had been confirmed by agarose gel electrophoresis (AGE) and spectrophotometer testing. Then the valid RNA samples were sent to Shanghai Biochip

Co. Ltd (Shanghai, China) within 48 hours where biotin labeling for hybridization was done.

Data analysis of gene array

Data of the gene array were analyzed by the Affymetrix Microarray Analysis software (Version 5.0, Shanghai Biochip Co. Ltd, Shanghai, China). Through fold change (FC) analysis, we established the selection standards of differentially expressed genes as follows: for up-regulation, \log_2 ratio=1 and change was marked as I; for down-regulation, \log_2 ratio=-1 and change was marked as D. After combination of FC analysis, we could screen out differentially expressed genes in each model group. The main parameters to evaluate outcomes in this study were the expressions of MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-16, MMP-23 and MMP-24 at different critical time points of TDVT.

RESULTS

Animal model and grouping

Totally two rats died due to excessive bleeding, one died from unknown causes and the others survived. In this model, the femoral vein thrombogenesis started at 2.5 hours after trauma. At 25 hours after trauma, the rate of thrombogenesis reached 50.5% and non-thrombogenesis was 49.5%. At 168 hours after trauma, the rate of thrombus resolution was 56.7% and 43.3% of thrombus constantly did not dissolve.

RNA extraction and electrophoretogram of RNA samples

Total RNA of vascular tissues of the femoral vein from each group was extracted by the one-step Trizol method. AGE analysis showed that the two strands of 28S RNA and 18S RNA were clear. This confirmed that the RNA samples were in good integrity, high quality and had no degradation (Figure 2).

Differential expression genes at different phases

As shown in Table 1, genes of the experimental groups had differential expressions at different time points. Compared with normal control group, the number of up-regulated genes was much larger than that of down-regulated ones in model group according to FC analysis. This suggested that the change of local micro-environment was an important factor that affected thrombosis and thrombus resolution in TDVT.

MMPs expressions

Gene chip detection showed that MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-16, MMP-23, and MMP-24 in TDVT at each time point had different levels of expression (Table 2 and Figure 3).

FC analysis

Figures 4-6 show that MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-16, MMP-23, and MMP-24 in TDVT had different levels of expressions at different time points.

Table 1. Frequency of differential expression genes at different phases

Genes	BvsA	CvsA	DvsA	HvsA	EvsA	FvsA	GvsA
Up-regulated genes	214	1386	945	1685	1222	1536	1235
Down-regulated genes	135	1007	798	1105	691	1029	614
Total	349	2393	1743	2790	1913	2565	1849

Table 2. MMPs expressions in TDVT at each time point (*n*=10 for each group).

Groups	Genes												
	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-10	MMP-11	MMP-12	MMP-13	MMP-14	MMP-16	MMP-23	MMP-24
A	181.6	100.7	7.5	114.0	61.7	14.1	34.4	113.5	53.0	1313.8	88.2	836.7	109.2
B	174.1	634.4	4.4	280.9	92.8	23.4	79.0	116.6	48.0	958.8	47.8	748.8	133.5
C	315.5	1167.9	12.3	547.1	220.8	19.1	95.6	1398.7	1363.7	7980.9	90.9	968.6	119.4
D	272.2	2140.3	16.2	452.6	233.0	34.9	52.8	606.0	1545.8	6935.8	67.9	766.7	149.0
E	123.8	54.1	4.6	50.6	8.4	10.9	127.3	117.2	12.6	1076.6	412.6	130.0	118.3
F	112.5	464.8	9.4	201.3	86.8	17.3	41.4	200.1	355.9	4486.1	75.8	794.1	98.3
G	229.1	1087.8	15.9	121.7	88.3	27.3	140.4	480.7	498.7	6326.8	131.0	889.1	102.3
H	260.7	291.0	14.2	93.7	70.4	22.0	29.4	162.2	24.7	2968.1	117.4	855.9	98.1

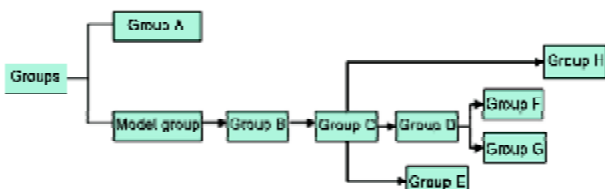


Figure 1. Block diagram of control group and model groups.

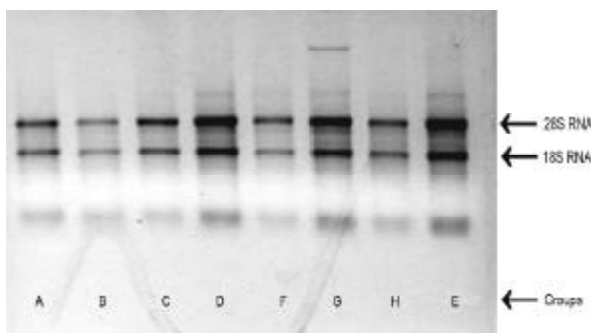


Figure 2. AGE analysis showed that the strands of 28S RNA, the thicker band, and 18S RNA, the thinner one, were clear, verifying that the RNA samples had good integrity, high quality and no degradation.

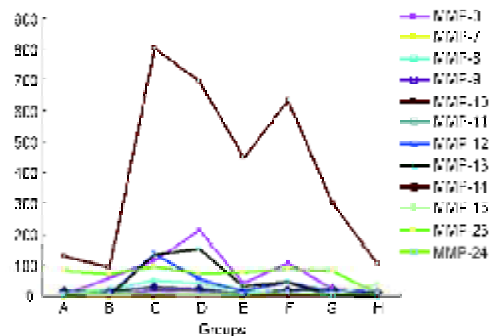


Figure 3. MMPs had different expressions in TDVT at each time point.

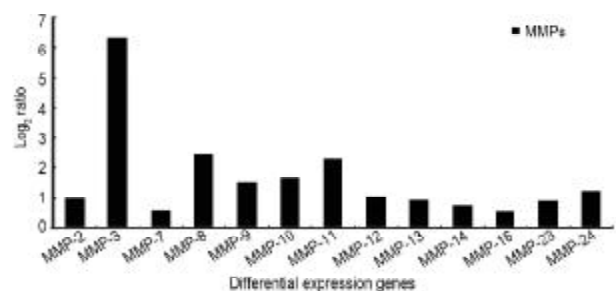


Figure 4. Changes of MMP expressions in BvsA during the formation of traumatic deep vein thrombus.

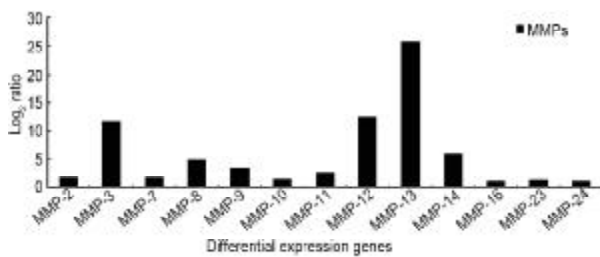


Figure 5. Changes of MMP expressions in CvsA during the formation of traumatic deep vein thrombus.

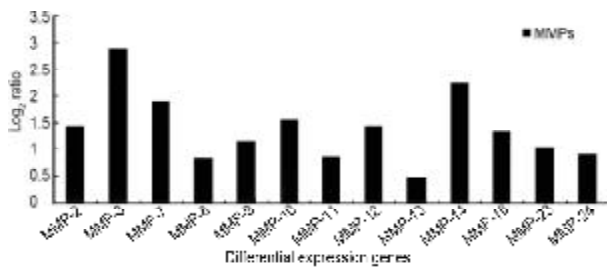


Figure 6. Changes of MMP expressions in EvsA during the resolution of traumatic deep vein thrombus.

DISCUSSION

Multiple genes are often activated by traumas of body tissues. These genes, as an important component of cell response and regulatory elements, further activate multiple signal transduction pathways and bring about corresponding changes. Some of them play the role of protection or repair, and some could lead to cell apoptosis or necrosis. MMPs, which stimulate a cascade reaction of gene activation at different time points, play an important role in the process of TDVT.

Structural and biochemical characteristics of MMPs

MMPs are a family of Zn²⁺-dependent endopeptidase, and also the major media involved in degradation and remodeling of the extracellular matrix (ECM). They participate in many physiological and pathological processes of human bodies,^{4,5} and are widely expressed *in vivo*.⁶ In 1962, Wu and Eyre⁷ found the first MMP (MMP-1). To date, nearly 30 species have been found, including 26 already identified and sequenced. MMPs are secreted in the form of zymogen, activated after protein hydrolysis and inhibited by some specific tissue inhibitors of metalloproteinase (TIMPs) and ethylene diamine tetraacetic acid.^{8,9} The activated MMPs further promote the plasminogen activation through positive feedback, thus arousing an activation cascade reaction.¹⁰ The extracellular activated MMPs could degrade all the extracellular matrix components except

polysaccharide, and participate in the physiological processes of growth and tissue repair.¹¹

MMPs in regulating thrombosis and thrombus resolution

Studies show that activities of matrix degradation markedly increase with the increased expressions of MMPs.¹² It has been reported that MMP-2 and MMP-9 are involved in the regulation of vascular wall modeling and migration of collagen and matrix during early wound repair.¹³ Visse et al¹⁴ confirmed that MMP-2 and MMP-9 are closely related to neovascularization and activity of proteins converted from collagen fibers in the process of thrombolysis. This might due to that the expressions of MMP-2 and MMP-9 have been activated by the urokinase-type plasminogen activator (u-PA).

In this experiment, gene chip test found that MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11 and MMP-24 had significantly high expression, while MMP-12, MMP-13, MMP-14, MMP-16 and MMP-23 had relatively low expression at the initial period of thrombosis. This shows that the majority of MMPs are activated immediately and thus have persistent high expression due to posttraumatic systemic inflammatory response. However, a small number of MMPs are at an inhibited state and thus show a low expression.

In this study, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-16, MMP-23 and MMP-24 were all highly expressed at the peak time of thrombosis. The mechanism may be that these genes promote the proliferation of vascular smooth muscle cells, which leads to narrowness of the blood vessels and further promotes the formation of traumatic deep vein thrombus.

In the process of thrombus resolution, MMP-2, MMP-10, MMP-16, and MMP-24 have relatively low expressions, while MMP-12, MMP-13, MMP-14, MMP-16 and MMP-23 have significantly high expressions, showing that different MMPs could play different roles in the resolution of TDVT. The mechanism is that the u-PA receptor located on the surface of endothelial cells shows biological activity after combined with u-PA and converts single chain urokinase-type plasminogen activator (scu-PA) into two chain urokinase-type plasminogen activator (tcu-PA), and converts plasminogens into activated fibrinolytic enzymes to positively regulate thrombolysis.¹⁵⁻¹⁸ Moreover,

u-PA further activates the majority of MMPs. These activated MMPs then facilitate the activation of their zymogens by positive feedback and thus start an activation cascade reaction. Furthermore, they promote the migration and proliferation of endothelial cells, smooth muscle cells, fibroblasts, neointimas and new blood vessels and promote thrombus resolution in the end. This is in accordance with reports in the literature.¹⁹⁻²¹

In conclusion, this experiment revealed that MMPs might play important roles in TDVT. MMPs are activated during the course of thrombosis, and then they promote the activation of plasminogens through positive feedback and further start an activation cascade reaction. In the course of thrombus resolution, most MMPs continue to show high expressions, indicating that they may also play a positive role in repair. Therefore, MMPs may be one of the most important factors that influence the biological states of TDVT, but its mechanisms still need further studies.

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