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Novel distribution pattern of fibrinolytic components in rabbit tissues extract: a preliminary study

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Abstract: Objective: The purpose of this work was to investigate the distribution pattern of fibrinolytic factors and their inhibitors in rabbit tissues. Methods: The components of the fibrinolytic system in extracts from a variety of rabbit tissues, including tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), plasminogen (Plg), plasmin (Pl) and α_2 plasmin inhibitor (α_2PI), were determined by colorimetric assay. Results: The tissue extracts in renal, small intestine, lung, brain and spleen demonstrated strong fibrinolytic function, in which high activity of tPA, Plg and Pl was manifested; whereas in skeletal muscle, tongue and stomach, higher activity of PAI-1 and α_2PI showed obviously. Also excellent linear correlations were found between levels of tPA and PAI-1, Pl and α_2PI, Plg and Pl. In related tissues, renal cortex and renal marrow showed distinctly higher activity of tPA and lower activity of PAI-1, with the levels of Plg and Pl in renal cortex being higher than those in renal marrow, where the α_2PI level was higher than that in renal cortex. Similarly, the levels of tPA, Plg and Pl in small intestine were higher than those in large intestine, but with respect to PAI-1 and α_2PI, the matter was reverse. In addition, the fibrinolytic activity in muscle tissue was lower, however, the levels of tPA, Plg, and Pl in cardiac muscle were obviously higher than those in skeletal muscles, and the levels of PAI-1 and α_2PI were significantly lower than those in skeletal muscle. Conclusion: Our data demonstrate that a remarkable difference of the fibrinolytic patterns exists in rabbit tissues, which has probable profound significance in understanding the relationship between the function of haemostasis or thrombosis and the physiologic function in tissues.

Key words: Tissue extract, Fibrinolytic factors, Fibrinolytic inhibitors
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INTRODUCTION

Clinical studies on coagulation and fibrinolysis have been only confined to blood samples over a long period of time. However, the factors in blood participating in coagulation and fibrinolysis are all from cell secretions so that the activities of coagulation and fibrinolysis in tissues which present the biological characteristics of tissues become an indispensable element in physiological coagulation and fibrinolysis, and especially, are closely associated with the behavior of tumor (Casslen *et al*., 1994; Schmitt *et al*., 2006; Zekanowska *et al*., 2004; Lu *et al*., 2003; Hatton *et al*., 2006). Because the abnormality of fibrinolysis detected by serum tests is correlated with the imbalance between fibrinolytic factors and their inhibitors excreted by local lesion or cells (Gesualdo *et al*., 1999; Kurose *et al*., 1992; Stenberg *et al*., 1983), hence, investigating fibrinolysis from other aspects such as its role in tissues is helpful for revealing the pathophysiologic mechanism of various diseases. Our present work aims to elucidate the distribution pattern of fibrinolytic factors and their inhibitors in rabbit tissues extract through determining their activities.

MATERIALS AND METHODS

Animal

Fifteen (8 males and 7 females) adult New Zea-

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land white rabbits weighing 2.1 to 3.0 kg were provided by the Center of Animal Experiment, School of Medicine, Zhejiang University, China.

Tissue preparation

The experimental rabbits were killed by the method of air embolism. The tissues, including liver, lung, spleen, brain, cardiac muscle, skeletal muscle, tongue, stomach, small intestine, large intestine, renal cortex and renal marrow, were taken out, then washed with 0.9% normal saline, dried by gauze and cut into pieces. Gastrointestinal tissues were removed and washed with 0.9% normal saline. Cell homogenizer or morta was used to triturate the tissue according to the ratio, which was 1 g of wet weight plus 2 ml 0.01 mol/L pH 7.2 phosphate buffered saline (PBS). Then, the homogenate was put into a plastic tube in 4 °C until next morning, and centrifuged at 4000 r/min for 5 min. The supernatant liquid was loaded into 2 Eppendorf tubes and frozen at −70 °C. Meanwhile, blood samples were collected from the ear vein in each rabbits before test after being decoagulated with 3.8% sodium citrate at the ratio of 9 to 1 (v/v, blood/anticoagulant), and the plasma samples were separated as control group.

Plasminogen activator inhibitor-1 (PAI-1) activity assay

The inhibitory activity of PAI-1 was determined using chromogenic substrate method (Mayer *et al*., 1990). Briefly, 200 μl tissue extracts that contained PAI-1 were added to a constant amount of two-chain tissue plasminogen activator (tPA) (0.05 IU/ml, 200 μl) diluted with TNT buffer (50 mmol/L Tris-HCl pH 7.4, 0.1 mol/L NaCl, 0.01% Tween 80) in the tubes. After 20 min at 25 °C, 100 μl mixtures mentioned above were subsequently drawn into the wells of microtiter plates, and then an equal volume of TNT buffer (100 μl) containing 0.5 μmol/L glu-plasminogen, 50 μg/ml des-AA-fibrinogen, and 1 mmol/L chromogenic plasmin substrate D-Val-Leu-Lys-pNA (S2251) was added (Sun Bio Corporation, Shanghai, China). Residual tPA activity was determined by measuring the increase in optical density at 405 nm after 1.5 h of incubation at 37 °C. Meanwhile, increasing volumes of PAI-1 were simultaneously measured to draw standard curve. The linear portion of the inhibition curve was extrapolated to obtain the concentration of PAI-1 necessary for complete inhibition of the tPA. One arbitrary unit (AU) is defined as the amount of PAI-1 required to inhibit 1 IU of tPA at 25 °C in 20 min.

Other activity assays

tPA, plasminogen (Plg), plasmin (Pl) and α_2 plasmin inhibitor (α_2PI) were also measured by colorimetric assay which adopted similar procedure as PAI-1 activity assay. All reagents were purchased from Sun Bio Corporation (Shanghai, China).

Statistical analysis

All data were expressed as mean±*SD*, and analyzed with *q* test and correlation method by SPSS 12.0 statistical package. *t*-test was used to compare the data of rabbit gastrointestinal mucosa and submucosa.

RESULTS

Levels of fibrinolytic factors and their inhibitors in rabbit tissues extract (Tables 1 and 2)

1. tPA level

The tPA levels in renal cortex and renal marrow were the highest, the second highest was in small intestine, brain, lung and spleen, and they were all higher than those in liver, cardiac muscle, stomach, large intestine and plasma, respectively ($P<0.01$), while the levels in skeletal muscle and tongue were the lowest among all tissues as well as plasma ($P<0.05$). Furthermore, the level in small intestine was higher than that in large intestine, and in cardiac muscle was higher than that in skeletal muscle ($P<0.01$).

2. PAI-1 level and the ratio of tPA to PAI-1

In contrast to tPA, the PAI-1 levels in renal cortex and renal marrow were the lowest among all tissues and plasma ($P<0.01$). The levels in skeletal muscle and stomach were the highest; the second highest was in liver and spleen; and they were all higher than those in lung and brain ($P<0.05$). The levels in small intestine, brain and lung were lower than that in plasma, respectively ($P<0.05$). Furthermore, the level in small intestine was lower than that in large intestine, and in cardiac muscle was lower than that in skeletal muscle ($P<0.01$). Also, the average ratio of tPA to PAI-1 in skeletal muscle, tongue and stomach was lower than that in plasma, whereas in other tissues, it was higher than that in plasma.

Table 1 Levels of fibrinolytic factors and their inhibitors in rabbit tissues extract ($\bar{\chi} \pm s$, n=10)

Groups	tPA (IU/L)	PAI-1 (AU/L)	Plg (IU/L)	Pl (%)	α_2PI (%)
Liver	362.3±39.3*	1312.9±122.5	7.3±1.7*	156.9±63.4*	230.0±103.9
Lung	553.5±91.5*	1066.5±215.8*	5.5±1.2*	99.1±32.8*	206.9±66.3
Spleen	661.7±122.4*	1307.1±99.2	3.8±1.9	83.9±28.3*	276.8±35.2
Brain	742.4±261.7*	863.0±325.6*	2.4±0.5	24.7±12.1	271.1±53.5
Cardiac muscle	277.5±81.7$^{\#\#}$	1172.9±253.1$^{\#\#}$	4.3±1.2$^{\#\#}$	80.5±51.3$^{*\#\#}$	213.8±50.2$^{\#\#}$
Skeletal muscle	110.6±23.6*	1453.2±80.1	2.4±0.6	20.5±19.3	314.0±77.1*
Tongue	133.0±27.7*	1229.5±92.4	2.6±0.2	33.5±12.4	266.8±70.2
Stomach	150.1±46.7	1455.7±181.2	2.5±0.6	20.9±12.7	298.9±42.8*
Small intestine	744.2±96.4$^{*\triangle}$	443.6±304.4$^{*\#}$	3.0±0.9	51.6±12.1$^{\triangle}$	223.2±76.7$^{\triangle}$
Large intestine	321.1±109.6*	1180.2±224.3	2.7±0.5	21.4±10.9	352.3±86.8*
Renal cortex	1272.3±290.7*	79.5±76.3$^{*\#}$	7.7±3.5*	165.5±88.9*	164.8±33.2
Renal marrow	1289.2±386.9*	58.3±53.8$^{*\#}$	3.2±1.0$^{\triangle\triangle}$	33.4±15.1$^{\triangle\triangle}$	240.2±93.1$^{\triangle\triangle}$
Plasma	195.6±95.6	1316.7±363.2	3.3±1.3	34.6±22.2	226.0±53.9

$^{*}P$<0.05 or P<0.01 vs plasma group; $^{\triangle}P$<0.01 vs large intestine group; $^{\#}P$<0.01 vs all other groups; $^{\#\#}P$<0.05 or P<0.01 vs skeletal muscle group; $^{\triangle\triangle}P$<0.05 or P<0.01 vs renal cortex group

Table 2 Average ratio of fibrinolytic factors to their inhibitors in rabbit tissues extract ($\bar{\chi} \pm s$, n=10)

Groups	tPA/PAI-1	Plg/Pl	Pl/α_2PI
Liver	0.27	0.06	0.87
Lung	0.53	0.06	0.51
Spleen	0.51	0.06	0.31
Brain	0.86	0.10	0.10
Cardiac muscle	0.25	0.07	0.38
Skeletal muscle	0.07	0.35	0.07
Tongue	0.11	0.08	0.14
Stomach	0.11	0.30	0.06
Small intestine	2.99	0.06	0.26
Large intestine	0.28	0.13	0.07
Renal cortex	30.60	0.06	1.01
Renal marrow	45.41	0.13	0.16
Plasma	0.18	0.12	0.16

3. Plg level

The Plg levels in liver and renal cortex were the highest; the second highest was in lung, and they were all higher than that in plasma (P<0.01). The levels in skeletal muscle, brain, tongue and stomach were lower than that in plasma; and also lower than those in cardiac muscle, lung, liver and renal cortex, respectively (P<0.05). Furthermore, the level in renal cortex was higher than that in renal marrow, and in cardiac muscle was higher than that in skeletal muscle and tongue (P<0.01).

4. Pl level

The Pl levels in liver and renal cortex were the highest; the second highest was in lung, spleen and cardiac muscle, and they were all higher than those in other tissues and plasma (P<0.05). The levels in skeletal muscle, brain, large intestine, renal marrow and stomach were relatively low. Furthermore, the level in renal cortex was higher than that in renal marrow, in cardiac muscle was higher than those in skeletal muscle and tongue, and in small intestine was higher than that in large intestine (P<0.01).

5. α_2PI level and the ratio of Pl to α_2PI

The α_2PI level in large intestine was the highest, the second highest was in stomach and skeletal muscle, and they were all higher than those in plasma, liver, lung, cardiac muscle, small intestine and renal cortex, respectively (P<0.05). The lowest level was in renal cortex and lung. Furthermore, the level in renal cortex was lower than that in renal marrow, in cardiac muscle was lower than that in skeletal muscle, and in small intestine was lower than that in large intestine (P<0.01). Meanwhile, the ratio of Pl to α_2PI was highest in renal cortex, and lowest in stomach and skeletal muscle among all tissues.

Levels of fibrinolytic factors and their inhibitors in rabbit gastrointestinal mucosa and submucosa tissues extracts (Table 3)

The tPA and Pl levels in mucosa of small intestine and large intestine were higher than those in submucosa, whereas PAI-1 level was lower than that in submucosa (P<0.05). Other indexes and the levels of fibrinolytic factors and their inhibitors in mucosa and submucosa of stomach showed no obvious statistical difference (P>0.05).

Relativity of fibrinolytic factors and their inhibitors in rabbit tissues extracts

Correlation analysis for the levels of fibrinolytic factors and their inhibitors in rabbit tissues extracts was carried out. A negative correlation existed between levels of tPA and PAI-1 (r=−0.8076, P<0.01), Pl and α_2PI (r=−0.3278, P<0.05). In contrast, a positive correlation showed between the levels of Plg and Pl (r=0.6811, P<0.01).

Table 3 Levels of fibrinolytic factors and their inhibitors in rabbit gastrointestinal mucosa and submucosa tissues extracts ($\bar{\chi} \pm s$, n=5)

Tissue		tPA (IU/L)	PAI-1 (AU/L)	Plg (IU/L)	Pl (%)	α_2PI (%)
Stomach	Mucosa	120.3±5.7	1174.0±257.1	2.2±0.4	17.8±6.0	267.6±113.5
	Submucosa	84.2±9.0	1402.6±120.8	2.4±0.4	14.0±5.1	267.2±77.2
Small intestine	Mucosa	685.8±87.3*	268.6±66.2*	3.4±0.8	92.6±30.3*	182.6±33.0
	Submucosa	561.7±78.6	324.2±61.6	3.2±0.7	41.0±22.4	203.0±48.9
Large intestine	Mucosa	411.2±141.7*	648.0±104.2*	2.9±0.4	43.2±17.8*	279.0±53.4
	Submucosa	147.7±39.8	1094.0±187.3	2.7±0.3	21.4±9.9	317.0±99.5

*P<0.05 vs submucosa tissue group

DISCUSSION

There may be some interrelationships between thrombosis or haemorrhage and the levels of fibrinolytic factors and their inhibitors in tissues. As far as clinical pathological mechanism was concerned, in addition to its role in degradation of extracellular matrix, fibrinolysis in tissues may participate in tumor invasion and be related with other diseases. For instance, PAI-1 in renal glomerulus induced by angiotensin IV in peritubular epithelial cells is related with occurrence of interstitial fibrosis in chronic nephritis (Gesualdo *et al*., 1999). Increased expression of PAI-1 had been detected in normal migrating cells and in metastasizing tumor cells (Sumiyoshi *et al*., 1991; Liu *et al*., 1994). Overexpression of PAI-1 protein was observed at the invasive front of breast cancer tissue specimens (Umeda *et al*., 1997). While in mucosa of peptic ulcer and ulcerative colitis, the activity of fibrinolysis evidently increased, with the latter also being pertinent to the degree of inflammation (Kurose *et al*., 1992). In addition, previous study had already shown that the gastrointestinal pathological changes may be ameliorated through therapy against fibrinolysis (Stenberg *et al*., 1983).

Our study found that the expression patterns of fibrinolytic factors and their inhibitors were different in various rabbit tissues extract. Some organs such as kidney, small intestine, lung, brain and spleen presented significantly higher levels of fibrinolytic factors (tPA, Plg and Pl), and lower fibrinolytic inhibitors (PAI-1 and α_2PI), while in skeletal muscle, tongue and stomach, the matter was just the reverse. So, the results indicated there was a diversity of fibrinolysis in these tissues. Furthermore, we found that a negative correlation existed in tPA and PAI-1, Pl and α_2PI, respectively, and a positive correlation between Plg and Pl. Meanwhile, analysis of the ratio of Pl to α_2PI, tPA to PAI-1 and Plg to Pl showed that a different intensity and proportion of fibrinolysis distributed in these tissues. For example, a higher ratio of tPA to PAI-1 appeared in renal cortex and renal marrow, whereas in skeletal muscle and tongue the ratio was lower. Those results reflected the fibrinolytic levels of rabbit in the normal condition and were helpful for evaluating the fibrinolysis condition of tissues and the susceptibility of haemorrhage during operation.

To our great surprise, there were many interesting direct ratios and inverse ratios of fibrinolytic factors to their inhibitors in some related tissues. For instance, the tPA level was higher and the PAI-1 level was lower in renal cortex and renal marrow; the Plg and Pl levels were higher in renal cortex than those in renal marrow, and the α_2PI was higher in renal marrow than that in renal cortex. The levels of fibrinolytic factors were higher in small intestine than those in large intestine, whereas the fibrinolytic inhibitors in large intestine were more numerous than those in small intestine. Analysis of levels of fibrinolytic factors and their inhibitors in gastrointestinal mucosa and submucosa revealed that higher activities of tPA and Pl are present in mucosa of large intestine and small intestine compared to in submucosa, which implied that the function of fibrinolysis in intestine is chiefly located in submucosa. Hence, we suggest that fibrinolytic factors and their inhibitors may participate in some gastrointestinal mucosa pathological changes in given condition, consistent with certain work in other clinical trials (Kurose *et al*., 1992; Stenberg *et al*., 1983). Kurose *et al*.(1992) also found the mucosa lesion in ulcerative colonitis was often linked to its aberrant fibrinolysis. Padro *et al*.(1994) investigated the relationship of the layers of rat aorta with fibrinolysis and subsequently confirmed that tPA activity and tPA antigen were detected in the intima-media and tunica adventitia, whereas in the tunica media only PAI-1 activity showed, no tPA activity was detected. This distribution pattern enabled us to obtain extraordinary knowledge about the fi-

brinolysis, coagulation and their inhibitors in tissue. In brain, our results indicated that only tPA level was higher whereas fibrinolytic inhibitors level was lower, thus, the brain showed relatively high activity of fibrinolysis. In addition, it may be an interesting phenomenon that the muscle tissues, such as skeletal muscle, cardiac muscle and tongue, showed lower fibrinolysis activity as well as higher inhibitors activity. However, with regard to muscle tissue, the activity of fibrinolysis in cardiac muscle is obviously stronger than that in skeletal and tongue muscles which also poses a worthwhile question. Consistent with our data, previous study also found the fibrinolytic and coagulant activity in rabbit tissues was similar to that in rat (Lu, 1990). As far as procoagulant active substances and their inhibitors in rabbit tissue extracts are concerned, we observe that a significant heterogeneity in the levels of fibrinolysis, coagulation and their inhibitors is shown in some tissues (Fang *et al*., 2005). For instance, the levels of fibrinolytic and anticoagulant factors were markedly lower but procoagulant activity increased significantly in muscle tissues, this vivid contrast against each other may be linked to the muscle tissues' toughness and resistance to autolysis. Although it was difficult to explain what physiological and pathological implications this considerably amazing phenomenon gave, it indeed deserved further investigation. Similarly, the obvious discrepancy between small intestine which showed the higher activities of fibrinolysis and coagulation and large intestine which showed the lower activities of fibrinolysis accorded with the symptoms that hemorrhagic infarct easily appeared in small intestine and hemorrhage in large intestine, suggesting that this discrepancy may be closely linked to their different physiological function. In summary, our interesting data suggest that further studies warrant a rethinking of unexpected properties of fibrinolysis, coagulation and their inhibitors in tissues other than their functions in the blood stream.

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