Int J Legal Med (2012) 126:421–425
DOI 10.1007/s00414-012-0680-z

ORIGINAL ARTICLE

Immunohistochemical detection of uPA, tPA, and PAI-1 in a stasis-induced deep vein thrombosis model and its application to thrombus age estimation

Mizuho Nosaka · Yuko Ishida · Yumi Kuninaka · Akihiko Kimura · Toshikazu Kondo

Received: 24 September 2011 / Accepted: 14 February 2012 / Published online: 24 February 2012

Abstract We immunohistochemically examined the expression of urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and plasminogen-activator inhibitor type-1 (PAI-1) using venous thrombi developed by ligation of the inferior vena cava (IVC) in mice. The uPA-, tPA- and PAI-1-positive cells could be firstly detected 5, 7, and 3 days, respectively, after IVC ligation. Morphometrically, the number of PAI-1-positive cells was significantly higher than those of uPA- and tPA-positive cells at later than 7 days. In all of the thrombus samples aged 10–21 days, the uPA/PAI-1 and tPA/PAI-1 ratios were >0.1 and >0.2, respectively. In contrast, all of the thrombus samples aged 1–7 days had uPA/PAI-1 of <0.1 and tPA/PAI-1 ratios of <0.2. These findings implied that uPA/PAI-1 of >0.1 and tPA/PAI-1 of >0.2 indicated an age of 10 days or more. Moreover, in four of five samples aged 10 days, uPA/PAI-1 ratios were <0.3, and the remaining one had uPA/PAI-1 of 0.32. All thrombi aged 14–21 days showed values greater than 0.3. Thus, uPA/PAI-1 ratios, markedly exceeding 0.3, strongly indicated an age of more than 14 days. The present study demonstrated that the immunohistochemical detection of uPA, tPA, and PAI-1 was suitable to estimate the age of venous thrombi.

Keywords Forensic pathology · Thrombus age estimation · Immunohistochemistry · Urokinase-type plasminogen activator · Tissue-type plasminogen activator · Plasminogen-activator inhibitor type 1

M. Nosaka · Y. Ishida · Y. Kuninaka · A. Kimura · T. Kondo (✉)
Department of Forensic Medicine, Wakayama Medical University,
811-1 Kimiidera,
Wakayama 641-8509, Japan
e-mail: kondot@wakayama-med.ac.jp

Introduction

It is well known that pulmonary thromboembolism (PTE) is one of the major diseases in sudden unexpected natural death. Most cases of PTE are caused by the embolization of deep vein thrombus (DVT) in lower extremity. In cases of DVT-related PTE, forensic pathologists are always required to find the origin of DVT and estimate how old the venous thrombi are. Immunohistochemical techniques are widely used in the forensic research fields to estimate the age of skin wounds and brain contusions, and to diagnose early ischemic heart disease or drowning [1–23]; however, there have been only a few forensic studies on thrombus age estimation [24–27].

Urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) are serine proteases that are members of the trypsin family, and they are essential to the intrinsic coagulation system. During the resolution of venous thrombi, tPA is primarily involved in fibrinolysis; whereas, uPA principally mediates cell migration and tissue-remodeling processes. Both uPA and tPA are responsible for cleaving plasminogen, a large serum β-globulin that is deposited on the fibrin strands within a thrombus. On the other hand, plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor family, has been shown to regulate uPA and tPA, resulting in the inhibition of proteolytic activity [28–31].

In the present study, we immunohistochemically examined the intrathrombotic expression of uPA, tPA, and PAI-1 in a murine venous thrombus model and discussed the availability of these molecules for thrombus age estimation.

Materials and methods

Antibodies

In the present study, the following polyclonal antibodies (pAbs) were used for immunohistochemical analysis: rabbit anti-uPA, -tPA, and -PAI-1 (active type) pAbs (Santa Cruz Biotechnology, Inc., CA, USA).

Mice

Specific pathogen-free 8- to 10-week-old male BALB/c mice were obtained from SLC (Shizuoka, Japan). All mice were housed individually in cages under specific pathogen-free conditions during the experiments. All animal experiments were approved by the Committee on Animal Care and Use of Wakayama Medical University.

Stasis-induced deep vein thrombus model

Intravenous thrombus was induced as described previously [25–27]. Briefly, after deep anesthesia with intraperitoneal injection of pentobarbital (50 mg/kg body weight), a 2-cm incision was made along the abdominal midline, and the inferior vena cava (IVC) was exposed and ligated with 3-0 silk suture. The abdominal wall was closed, and 1 ml of phosphate buffered saline (PBS) was injected subcutaneously. At the indicated time intervals after the IVC ligation, mice were euthanized by an overdose of diethyl ether, and thrombi with vessel walls were harvested and subjected to further histological analyses. At each time point, five mice were used.

Immunohistochemical analyses

Intravenous thrombi were fixed in 4% formaldehyde buffered with PBS (pH 7.2), transversely cut at the central part of the thrombi, and paraffin-embedded sections (4 μm thick) were made. The primary Abs were diluted with the blocking buffer (PBS containing 1% normal serum corresponding to the secondary Abs and 1% bovine serum albumin) to reduce nonspecific reactions. Thereafter, the sections were reacted with rabbit anti-uPA pAbs (1.3 μg/ml, 60 min), anti-tPA pAbs (1.3 μg/ml, 60 min) or anti-PAI-1 pAbs (0.7 μg/ml, 48 min) at 37°C. After incubation with biotinylated rabbit anti-rabbit IgG pAbs (0.7 μg/ml) at 37°C for 60 min for the anti-uPA or -tPA sections and 24 min for the anti-PAI-1 sections, the DAB Map kit (Ventana Medical Systems, Inc., AZ, USA) was used to visualize the antigens for all stains.

Semiquantitative evaluation of intrathrombotic uPA, tPA, and PAI-1 expression

Intrathrombotic uPA, tPA, and PAI-1 levels were evaluated semiquantitatively, as described previously [25–27]. Briefly, in each section, five microscopic fields (two central and three peripheral fields) were randomly selected within the thrombi (magnification, ×1,000), and the numbers of positive cells were counted and summed from the five microscopic fields. All measurements were performed by two examiners without prior knowledge of the experimental procedures.

Statistical analysis

All data were presented as the mean±SEM. Statistical significance was evaluated using Mann–Whitney's U test. $P<0.05$ was accepted as significant.

Results

Intrathrombotic appearance of uPA, tPA, and PAI-1

We immunohistochemically examined the intrathrombotic appearance of uPA, tPA, and PAI-1. The earliest appearance of uPA-positive cells and that of tPA-positive cells could be observed at 5 and 7 days, respectively. Both could be constantly detected at 10 days or more after the IVC ligation. On the other hand, PAI-1-positive cells could first be detected at 3 days after IVC ligation and all samples with the postligation intervals of 7 days or more had PAI-1 positive cells in IVC (Table 1). These positive cells were mainly observed in macrophages at the marginal region of thrombi (Fig. 1).

Morphometrical analyses

There were no significant differences in the positive cell number between uPA and tPA at the postligation intervals of 7 days or more. The number of PAI-1-positive cells was

Table 1 The earliest and routine appearance

Markers	Earliest (positive case)	Routine
uPA	5 Days (1/5)	≥10 days
tPA	7 Days (3/5)	≥10 days
PAI-1	3 Days (3/5)	≥7 days

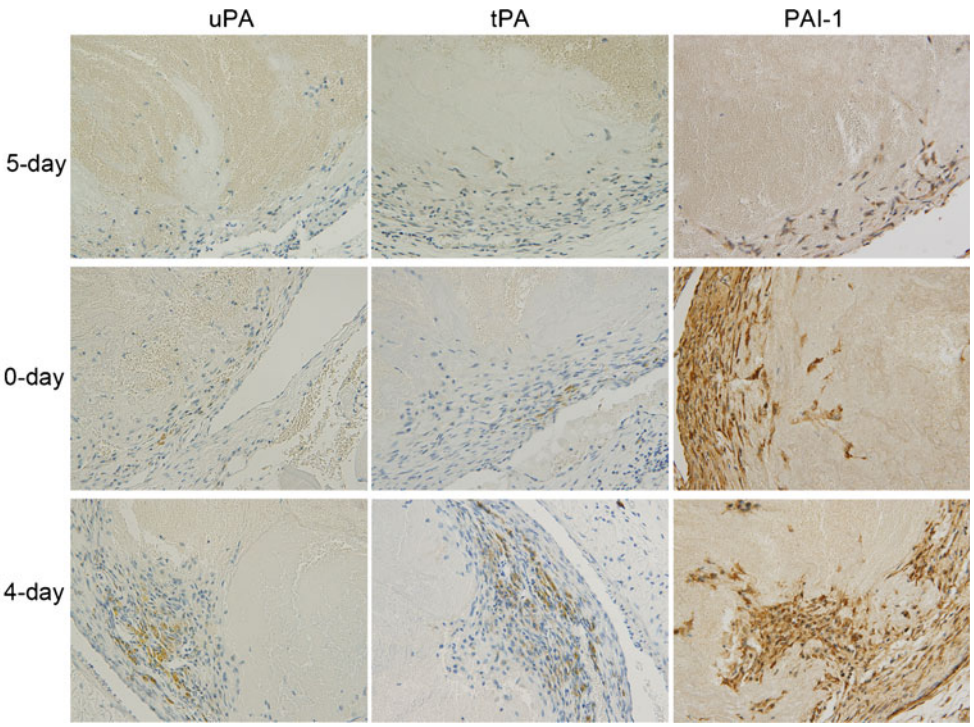

Fig. 1 Immunohistochemical detection of uPA, tPA, and PAI-1 (5, 10, and 14 days after IVC ligation). Representative results are shown. Original magnification, ×400

significantly higher than those of uPA- and tPA-positive cells after 7 days (Fig. 2). In thrombus samples with the postligation intervals of 1–7 days, the average ratio of uPA to PAI-1 (uPA/PAI-1 ratio) and that of tPA to PAI-1 (tPA/PAI-1 ratio) were less than 0.1 and 0.2, respectively. In contrast, uPA/PAI-1 and tPA/PAI-1 ratios were greater than 0.1 and 0.2 at ≥10 days after the IVC ligation, respectively (Fig. 3).

Discussion

In the formation and resolution of thrombus, the interaction of the vessel walls and the blood cells is indispensable.

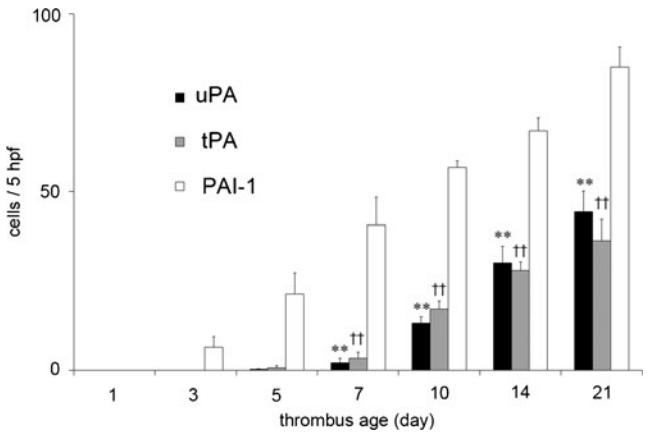

Fig. 2 Semiquantitative analyses of the number of uPA-, tPA-, and PAI-1-positive cells (n=5 in each group). **$p \leq 0.01$, uPA vs. PAI-1; $^{\#\#}p \leq 0.01$, tPA vs. PAI-1

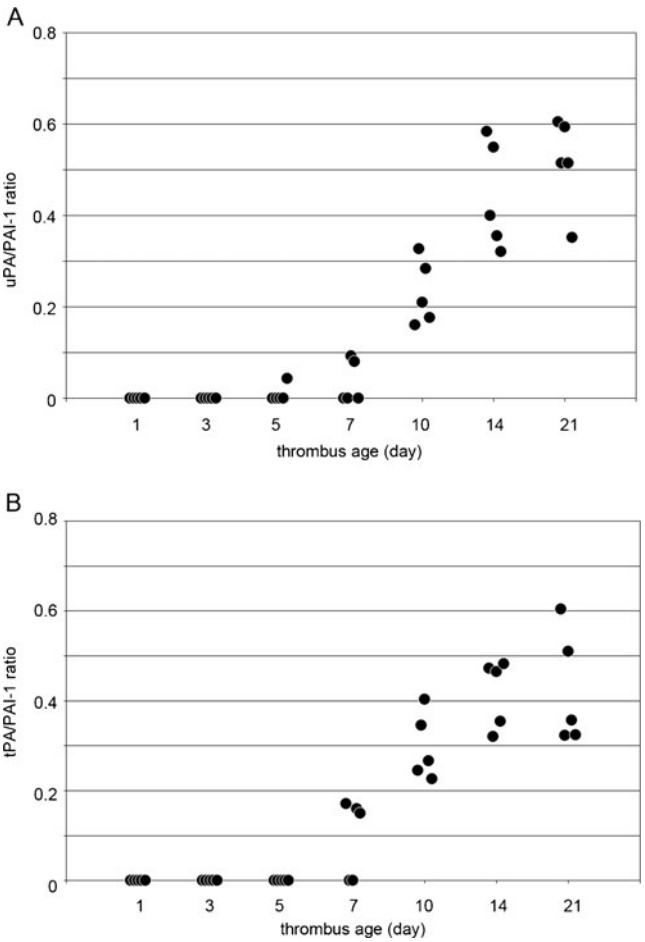

Fig. 3 **a** The distribution of uPA/PAI-1 ratios and **b** tPA/PAI-1 ratios in relation to thrombus age (n=5 in each group)

There are pathophysiological similarities between thrombus formation/resolution and skin wound healing. In both processes, various cells such as red blood cells, leukocytes, vascular endothelial cells, smooth muscle cells, and platelets are involved [32–35]. We demonstrated the time-dependent reciprocal changes between the numbers of macrophages and neutrophils in the venous thrombus [25]. Biofunctional molecules derived from leukocytes and endothelial cells are involved in the process of thrombus resolution [36, 37]. Actually, the intrathrombotic expression of matrix metalloproteinase (MMP)-2 and MMP-9 also seemed useful for thrombus age estimation [27].

In the fibrinolysis process, the essential enzyme is plasmin, a serine protease generated by the proteolytic cleavage of the proenzyme plasminogen. Its main substrates include fibrin, fibrinogen, and other coagulation factors. Plasminogen activators are serine proteases that activate plasminogen, by cleavage of a single arginine–valine peptide bond, to the enzyme plasmin [38–40]. There exist tPA and uPA, both of which are produced by macrophages. The migration and activity of monocytes are important components of the process of thrombus resolution because these cells are known to produce a variety of growth factors, chemotaxins, and matrix-degrading enzymes [41, 42]. The formation of plasmin from plasminogen by tPA and uPA is presumed to be essential for cell migration through the regulation of fibrin deposition or pericellular fibrinolysis [43–45].

In contrast, PAI-1 is the primary inhibitor of plasminogen activators. It is secreted in an active form from liver and endothelial cells and stabilized by binding to vitronectin. PAI-1 levels are elevated by hyperlipidemia, and PAI-1 elevation appears to synergize with factor V Leiden genetic abnormalities. It is plausible that elevated PAI-1 could suppress fibrinolysis and increase thrombosis, hence increasing the clinical manifestations of DVT, although studies on the role of elevated levels of PAI-1 in venous thrombosis have been contradictory [46, 47]. The balance of tPA/uPA and PAI-1 determine the fate of thrombus.

Our study implies that tPA, uPA, and PAI-1 may be useful for thrombus age estimation. Considering from the first appearance of each molecule, uPA- and tPA- positive cells would indicate thrombus ages of ≥5 and ≥7 days, respectively. Intrathrombotic PAI-1 expression indicates the thrombus age of ≥3 days. In the previous studies, we demonstrated that neutrophil/macrophage and MMP-9/MMP-2 ratios in the thrombi could give significant information to estimate the thrombus age [21, 22]. In line with these previous studies, uPA/PAI-1 and tPA/PAI-1 were presumed to be more useful for the age estimation of venous thrombi. In all of the thrombus samples aged 10–24 days, the uPA/PAI-1 and tPA/PAI-1 ratios were >0.1 and >0.2, respectively. In contrast, all of the thrombus samples aged 1–7 days had uPA/PAI-1 of <0.1 and tPA/PAI-1 ratios of <0.2. These findings implied that uPA/PAI-1 of >0.1 and tPA/PAI-1 of >0.2 indicate an age of 10 days or more. Moreover, in four out of five samples aged 10 days, uPA/PAI-1 ratios were less than 0.3 and the remaining one had uPA/PAI-1 of 0.32. All thrombi aged 14–21 days showed values greater than 0.3. These observations implied that uPA/PAI-1 ratios, markedly exceeding 0.3, strongly indicated an age of more than 14 days.

For forensic safety, it is needless to say that combined analyses of several different markers are important. Our previous study showed that the neutrophil/macrophage ratio (N/M) in thrombi was useful for thrombus age estimation [25]. Actually, a thrombus with both N/M of ≤1.0 and uPA/PAI-1 of 0.1–0.3 may be a thrombus age of 10 days.

Finally, because the present results were obtained from well-controlled animal experiments, it is, of course, necessary to perform further study by the use of human thrombus samples with variety ages. Herewith, we provide the experimental evidence that immunohistochemical detection of intrathrombotic uPA, tPA, and PAI-1 would be applicable for thrombus age estimation.

Acknowledgments We thank Ms. Mariko Kawaguchi for her excellent assistance in the preparation of this manuscript. This study was financially supported in part by Grants-in-Aid for Scientific Research (A) and (C) from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

References

1. Betz P, Nerlich A, Wilske J, Tübel J, Penning R, Eisenmenger W (1993) Analysis of the immunohistochemical localization of collagen type III and V for the time-estimation of human skin wounds. Int J Legal Med 105:329–332
2. Betz P, Nerlich A, Wilske J, Tübel J, Wiest I, Penning R, Eisenmenger W (1992) Immunohistochemical localization of fibronectin as a tool for the age determination of human skin wounds. Int J Legal Med 105:21–26
3. Betz P (1994) Histological and enzyme histochemical parameters for the age estimation of human skin wounds. Int J Legal Med 107:60–68
4. Betz P, Nerlich A, Tübel J, Wiest I, Hausmann R (1997) Detection of cell death in human skin wounds of various ages by an in situ end labeling of nuclear DNA fragments. Int J Legal Med 110:240–243
5. Dressler J, Bachmann L, Koch R, Müller E (1999) Estimation of wound age and VCAM-1 in human skin. Int J Legal Med 112:159–162
6. Hayashi T, Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2004) Forensic application of VEGF expression to skin wound age determination. Int J Legal Med 118:320–325
7. Kondo T, Ohshima T, Eisenmenger W (1999) Immunohistochemical and morphometrical study on the temporal expression of interleukin-1alpha (IL-1α) in human skin wounds for forensic wound age determination. Int J Legal Med 112:249–252
8. Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2008) Expression of oxygen-regulated protein 150 (ORP150) in skin

wound healing and its application for wound age determination. Int J Legal Med 122:409–414
9. Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2009) Detection of fibrocytes in human skin wounds and its application for wound age determination. Int J Legal Med 123:299–304
10. Hausmann R, Betz P (2000) The time course of the vascular response to human brain injury—an immunohistochemical study. Int J Legal Med 113:288–292
11. Hausmann R, Betz P (2001) Course of glial immunoreactivity for vimentin, tenascin and alpha1-antichymotrypsin after traumatic injury to human brain. Int J Legal Med 114:338–342
12. Hausmann R, Riess R, Fieguth A, Betz P (2000) Immunohistochemical investigations on the course of astroglial GFAP expression following human brain injury. Int J Legal Med 113:70–75
13. Hausmann R, Kaiser A, Lang C, Bohnert M, Betz P (1999) A quantitative immunohistochemical study on the time-dependent course of acute inflammatory cellular response to human brain injury. Int J Legal Med 112:227–232
14. Dressler J, Hanisch U, Kuhlisch E, Geiger KD (2007) Neuronal and glial apoptosis in human traumatic brain injury. Int J Legal Med 121:365–375
15. Hausmann R, Seidl S, Betz P (2007) Hypoxic changes in Purkinje cells of the human cerebellum. Int J Legal Med 121:175–183
16. Hausmann R, Biermann T, Wiest I, Tübel J, Betz P (2004) Neuronal apoptosis following human brain injury. Int J Legal Med 118:32–36
17. Oehmichen M, Walter T, Meissner C, Friedrich HJ (2003) Time course of cortical hemorrhages after closed traumatic brain injury: statistical analysis of posttraumatic histomorphological alterations. J Neurotrauma 20:87–103
18. Dressler J, Bachmann L, Koch R, Müller E (1999) Enhanced expression of selectins in human skin wounds. Int J Legal Med 112:39–44
19. Cecchi R (2010) Estimating wound age: looking into the future. Int J Legal Med 124:523–536
20. An JL, Ishida Y, Kimura A, Kondo T (2011) Immunohistochemical examination of intracerebral aquaporin-4 expression and its application for differential diagnosis between freshwater and saltwater drowning. Int J Legal Med 125:59–65
21. An JL, Ishida Y, Kimura A, Kondo T (2010) Forensic application of intrarenal aquaporin-2 expression for differential diagnosis between freshwater and saltwater drowning. Int J Legal Med 124:99–104
22. Fracasso T, Pfeiffer H, Michaud K, Köhler H, Sauerland C, Schmeling A (2011) Immunohistochemical expression of fibronectin and C5b-9 in the myocardium in cases of carbon monoxide poisoning. Int J Legal Med 125:377–384
23. Yoshida C, Ishikawa T, Michiue T, Quan L, Maeda H (2011) Postmortem biochemistry and immunohistochemistry of chromogranin A as a stress marker with special regard to fatal hypothermia and hyperthermia. Int J Legal Med 125:11–20
24. Fineschi V, Turillazzi E, Neri M, Pomara C, Riezzo I (2009) Histological age determination of venous thrombosis: a neglected forensic task in fatal pulmonary thrombo-embolism. Forensic Sci Int 186:22–28
25. Nosaka M, Ishida Y, Kimura A, Kondo T (2009) Time-dependent appearance of intrathrombus neutrophils and macrophages in a stasis-induced deep vein thrombosis model and its application to thrombus age determination. Int J Legal Med 123:235–240
26. Nosaka M, Ishida Y, Kimura A, Kondo T (2010) Time-dependent organic changes of intravenous thrombi in stasis-induced deep vein thrombosis model and its application to thrombus age determination. Forensic Sci Int 195:143–147
27. Nosaka M, Ishida Y, Kimura A, Kondo T (2010) Immunohistochemical detection of MMP-2 and MMP-9 in a stasis-induced deep vein thrombosis model and its application to thrombus age estimation. Int J Legal Med 124:439–444
28. Singh I, Burnand KG, Collins M, Luttun A, Collen D, Boelhouwer B, Smith A (2003) Failure of thrombus to resolve in urokinase-type plasminogen activator gene-knockout mice: rescue by normal bone marrow-derived cells. Circulation 107:869–875
29. Humphries J, Gossage JA, Modarai B, Burnand KG, Sisson TH, Murdoch C, Smith A (2009) Monocyte urokinase-type plasminogen activator up-regulation reduces thrombus size in a model of venous thrombosis. J Vasc Surg 50:1127–1134
30. Lund LR, Green KA, Stoop AA, Ploug M, Almholt K, Lilla J, Nielsen BS, Christensen IJ, Craik CS, Werb Z, Dano K, Romer J (2006) Plasminogen activation independent of uPA and tPA maintains wound healing in gene-deficient mice. EMBO J 25:2686–2697
31. Jankun J, Aleem AM, Struniawski R, Lysiak-Szydlowska W, Selman SH, Skrzypczak-Jankun E (2009) Accelerated thrombus lysis in the blood of plasminogen activator inhibitor deficient mice is inhibited by PAI-1 with a very long half-life. Pharmacol Rep 61:673–680
32. Nagai R, Suzuki T, Aizawa K, Miyamoto S, Amaki T, Kawai-Kowase K, Sekiguchi KI, Kurabayashi M (2001) Phenotypic modulation of vascular smooth muscle cells: dissection of transcriptional regulatory mechanisms. Ann N Y Acad Sci 947:56–66
33. Takeya H, Gabazza EC, Aoki S, Ueno H, Suzuki K (2003) Synergistic effect of sphingosine 1-phosphate on thrombin-induced tissue factor expression in endothelial cells. Blood 102:1693–1700
34. Furie B, Furie BC (2004) Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. Trends Mol Med 10:171–178
35. Lambert MP, Sachais BS, Kowalska MA (2007) Chemokines and thrombogenicity. Thromb Haemost 97:722–729
36. Wakefield TW, Myers DD, Henke PK (2008) Mechanisms of venous thrombosis and resolution. Arterioscler Thromb Vasc Biol 28:387–391
37. Nosaka M, Ishida Y, Kimura A, Kuninaka Y, Inui M, Mukaida N, Kondo T (2011) Absence of IFN-γ accelerates thrombus resolution through enhanced MMP-9 and VEGF expression in mice. J Clin Invest 121:2911–2920
38. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L (1985) Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44:139–266
39. Vassalli JD, Sappino AP, Belin D (1991) The plasminogen activator/plasmin system. J Clin Invest 88:1067–1072
40. Kohler HP, Grant PJ (2000) Plasminogen-activator inhibitor type 1 and coronary artery disease. N Engl J Med 342:1792–1801
41. Leibovich SJ, Wiseman DM (1988) Macrophages, wound repair and angiogenesis. Prog Clin Biol Res 266:131–145
42. Lijnen HR (2001) Elements of the fibrinolytic system. Ann N Y Acad Sci 936:226–236
43. Tabrizi P, Wang L, Seeds N, McComb JG, Yamada S, Griffin JH, Carmeliet P, Weiss MH, Zlokovic BV (1999) Tissue plasminogen activator (tPA) deficiency exacerbates cerebrovascular fibrin deposition and brain injury in a murine stroke model: studies in tPA-deficient mice and wild-type mice on a matched genetic background. Arterioscler Thromb Vasc Biol 19:2801–2806
44. Bugge TH, Flick MJ, Danton MJ, Daugherty CC, Romer J, Dano K, Carmeliet P, Collen D, Degen JL (1996) Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899–5904
45. Ploplis VA, French EL, Carmeliet P, Collen D, Plow EF (1998) Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. Blood 91:2005–2009
46. Schulman S, Wiman B (1996) The significance of hypofibrinolysis for the risk of recurrence of venous thromboembolism. Duration of Anticoagulation (DURAC) Trial Study Group. Thromb Haemost 75:607–611
47. Crowther MA, Roberts J, Roberts R, Johnston M, Stevens P, Skingley P, Patrassi GM, Sartori MT, Hirsh J, Prandoni P, Weitz JI, Gent M, Ginsberg JS (2001) Fibrinolytic variables in patients with recurrent venous thrombosis: a prospective cohort study. Thromb Haemost 85:390–394