Basic Res Cardiol 98: 347 – 352 (2003)
DOI 10.1007/s00395-003-0416-9

ORIGINAL CONTRIBUTION

Janine Dörffler-Melly
Martijn de Kruif
Lothar A. Schwarte
Rendrik F. Franco
Sandrine Florquin
C. Arnold Spek
Can Ince
Pieter H. Reitsma
Hugo ten Cate

Functional thrombomodulin deficiency causes enhanced thrombus growth in a murine model of carotid artery thrombosis

Received: 3 June 2002
Returned for 1. revision: 1 July 2002
1. Revision received: 10 March 2003
Returned for 2. revision: 24 March 2003
2. Revision received: 14 April 2003
Accepted: 17 April 2003
Published online: 12 May 2003

J. Dörffler-Melly, MD, PhD
M. de Kruif, MD · R. F. Franco, MD, PhD
C. A. Spek, PhD · C. Ince, PhD
P. H. Reitsma, PhD
Lab. for Experimental Internal Medicine
Academic Medical Center
Amsterdam, The Netherlands

H. ten Cate, MD (✉)
Dept. of Internal Medicine and
Cardiovascular Research Institute Maastricht
University of Maastricht
P.O. Box 616, UNS50/box 8
6200 MD Maastricht, The Netherlands
Tel.: +31 43 3884262
Fax: +31 43 3884159
E-Mail: h.tencate@BIOCH.Unimaas.nl

J. Dörffler-Melly, MD, PhD
Department of Vascular Medicine
Academic Medical Center
Amsterdam, The Netherlands

L. A. Schwarte, MD
Department of Experimental Anaesthesiology
Academic Medical Center
Amsterdam, The Netherlands

S. Florquin, MD, PhD
Department of Pathology
Academic Medical Center
Amsterdam, The Netherlands

J. Dörffler-Melly, MD, PhD
Present address:
Division of Angiology
University Hospital
Berne, Switzerland

Abstract Thrombomodulin (TM) bound thrombin initiates the protein C anticoagulant pathway and defects in TM result in enhanced coagulation. Recent studies suggest a role for TM in arterial vascular disease. In order to corroborate this association we studied arterial thrombus formation in mice with a functional TM defect. We used mice homozygous for a ^{404}Glu-to-Pro mutation in the TM gene ($TM^{pro/pro}$) and compared these with wildtype littermates in a model of $FeCl_3$ induced carotid artery thrombosis. Time-to-occlusion (TTO) was assessed by arterial blood flow measurement, using a Doppler flow probe. Complete occlusion occurred in 8/10 (80%) $TM^{pro/pro}$ mice and in 3/11 (27%) littermate controls. Mean time to occlusion (TTO) [± SE] was 767 ± 196 s in the F2-$TM^{pro/pro}$ mice, versus 1507 ± 159 s in controls ($p = 0.007$, Mann Whitney U test). Histology and immunostaining for tissue factor did not reveal any differences in thrombus morphology or thrombogenicity between the two groups.

These data confirm and extend the finding that a functional deficiency in TM results in enhanced thrombus formation in a murine model of carotid artery thrombosis and support a role for TM defects in arterial thrombotic disease.

Key words Arteries – thrombosis/embolism – thrombomodulin – defects – transgenic mice

Introduction

Thrombus formation in mammalian arteries and veins is physiologically prevented by specific mechanisms involving fibrinolytic and anticoagulant systems. Among these, the protein C (PC) anticoagulant pathway provides a natural antithrombotic mechanism of the vascular endothelium that interacts with the blood clotting system. The initial step in this pathway consists of the binding of thrombin to the endothelial membrane protein thrombomodulin (TM). The formed TM-thrombin complex activates endothelial receptor-bound PC (8, 9). Activated PC (APC) proteolytically inactivates coagulation factors Va and VIIIa, thereby inhibiting coagulation. This inactivation reaction only proceeds efficiently in the presence of Ca^{2+} ions and the co-factor protein S.

Defects of the PC pathway, in particular protein C deficiency, protein S deficiency, and factor V Leiden, are important risk factors for venous thrombosis (1, 5). In contrast, there is only limited evidence for a similar association of these abnormalities with arterial thrombotic complications such as myocardial infarction and stroke (2, 4, 14, 20, 24, 28).

The relationship with thrombosis is much less clear for TM deficiency. Several missense mutations have been discovered in patients with venous thrombotic disease (10, 22, 23, 25, 26), but it is uncertain whether these mutations result in diminished TM function (6, 10, 16, 22, 23). Several promoter polymorphisms with unknown effect on TM function have been implicated in arterial thrombotic disease, but this claim was not confirmed in a second survey. Recently, a true TM mutation was discovered in an individual with myocardial infarction, vitalizing the relationship with arterial thrombotic disease (5, 16, 18, 20).

In mice, complete ablation of the TM gene results in embryonic lethality. Mice heterozygous for null mutations are apparently normal and do not display an overt venous or arterial thrombotic phenotype. As reported recently, a prothrombotic phenotype could be achieved in mice that were carriers of a targeted point mutation of the TM-gene, resulting in a loss of the capability of TM binding to thrombin (33). Under defined conditions, these mice manifested microvascular myocardial thrombosis (3). In the present study we show that this functional TM-deficiency has a direct impact on murine carotid artery thrombosis.

Materials and methods

Animals

Homozygous $TM^{pro/pro}$ mice carrying Glu404Pro mutations resulting in impaired TM-thrombin binding and reduced capacity to activate PC were described in detail by Weiler-Guettler et al (33). The $TM^{pro/pro}$ mice, which were generously provided by Dr. Robert D. Rosenberg (Massachusetts Institute of Technology, and Harvard Medical School, Cambridge and Boston, MA, USA), were direct descendants from an F1-cross, and thus genetically 50% C57bL/6 and 50% 129Sv. In a pilot experiment we compared $FeCl_3$ induced thrombus growth in the carotid artery in 10 $TM^{pro/pro}$ mice with 10 C57bL/6 mice (Harlan, The Netherlands). In this experiment enhanced thrombus growth was observed in $TM^{pro/pro}$ mice (data not shown). Differences in genetic background of these mice could have caused the observed difference in thrombus growth, and therefore we repeated the experiments in age and sex matched mutant $TM^{pro/pro}$ and wildtype littermates that were born from a cross between F2-heterozygotes, indicated as 'F2- $TM^{pro/pro}$' mice.

Animal care and use were approved by the Institutional Review Board for Animal Experiments at the Academic Medical Center, Amsterdam, The Netherlands, and the experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85–23, revised 1996) as well as the Dutch Law for Animal Experiments.

$FeCl_3$ thrombosis model

Thrombus formation and growth was evaluated in a standardized model, whereby thrombosis in the carotid artery is induced by $FeCl_3$ as reported previously (11). Mice were anesthetized by FFM (fentanyl citrate 0.079 mg/mL, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H_2O; of this mixture 7.0 ml/kg intraperitoneally initially and 1.0 ml/kg every 30 minutes). Body temperature was kept between 36.5 and 37 °C. Animals were tracheotomized and ventilated during the experiment. After a median section from the xyphoid to the neck, the left carotid artery was bluntly isolated, and a plastic protection sheet was slipped under the vessel. Care was taken to separate the vagal nerve and vasa vasorum from the artery, and twisting of the vessel was avoided. A Doppler flow probe (Transonic Systems Inc., NY, USA) with a diameter of 0.5 mm was placed at the distal part of the exposed carotid artery and blood flow was recorded until steady state was reached for at least 10 minutes. For heart rate assessment, blood flow registration was shortly switched from the average to the pulsatile flow chart. After the steady-state phase, a filter paper of 2 × 2 mm, soaked in a 10% solution of $FeCl_3$, was placed on the carotid artery. In a pilot experiment we had previously assessed the optimal $FeCl_3$ concentration; a 25% solution induced thrombosis in all 10 mice (5 WT and 5 TM), whereas a concentration of 10% resulted in an occlusion rate of 80% in TM mice and 20% in WT and C57BL/6 mice, respectively.

After exactly 3 minutes the filter paper was removed and blood flow was continuously registered for 30 minutes (observation time). Time to occlusion (TTO), i.e. time elapsed between placing the filter paper on the artery until complete, non-reversible loss of flow measured by Doppler flow probe, was the principal endpoint-parameter.

Histological evaluation

At the end of the acute experiment, the carotid artery segments from both sides were excised, embedded in paraffin, and sectioned transsectionally over the entire length (4 μm). Fifteen to twenty-five sections from each artery were stained with haematoxylin and eosin and examined by two blinded investigators. Artery segments positive for the presence of a thrombus were further stained by a polyclonal antibody against murine tissue factor peptide P5 raised in rabbits as previously described (31), used in a dilution of 1:500.

Statistical analysis

We assumed that outcome measurements were not normally distributed. For this reason we compared the groups with an unpaired, non-parametric test, i.e. Mann-Whitney test. A p value of 0.05 was considered a limit of statistical significance.

Results

Flow assessment, heart rates, and time-to-occlusion

Mean (± SE) blood flow at steady state before application of $FeCl_3$ was for F2-$TM^{pro/pro}$ 0.78 ± 0.23 ml/min and for wildtype littermates 0.80 ± 0.35 ml/min (not significantly different). Mean heart rates and standard deviations were 478 ± 61 for $TM^{pro/pro}$ and 476 ± 72 beats per minute for wildtype littermates (p = 0.9, not significant). From previous experiments it is known that mean arterial blood pressure remains fairly stable at around 78 ± 5 mm Hg during FFM anesthesia (17).

Complete occlusion (flow < 0.1 mL/min) occurred in 8 of the 10 (80%) animals in the F2-$TM^{pro/pro}$ groups by the end of the observation time, while this occurred in 3 of 11 (27%) wildtype mice. Mean TTO [± SE] differed significantly between the groups with 767 ± 196 s in the F2-$TM^{pro/pro}$ mice and 1507 ± 159 s in littermate control mice (p = 0.007) (Fig. 1). Since recording of blood flow was maximized at 30 minutes (1800 s), the median values were 433 s in the F2-$TM^{pro/pro}$ and 1800 s in control mice.

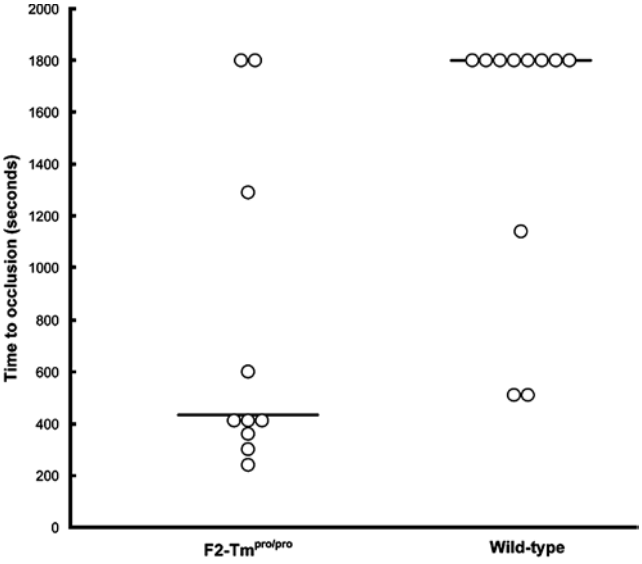
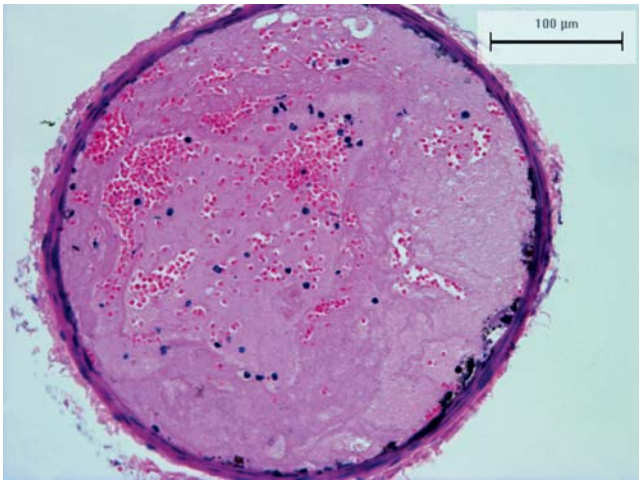


Fig. 1 Time to complete occlusion of the carotid artery after application of $FeCl_3$ in seconds, indicated for each individual mouse and as mean value per group. F2-$TM^{Pro/Pro}$ indicates TMGlu404Pro mutated mice from intercross of heterozygotes (littermates). Wildtypes are obtained from the same intercross of heterozygotes

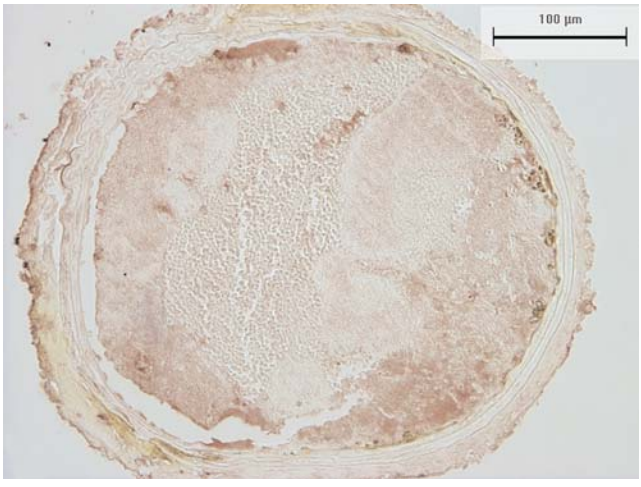
Histology

Histological assessment of the arterial segments showed fibrin-rich thrombi in 9 $TM^{pro/pro}$ mice and in 3 control mice corresponding with evidence of vessel occlusion; in one animal, in which a clear flow stop had been registered, a clot was missed and could not be evaluated. In those animals, in which blood flow could still be registered at the end of the observation time, no clots were detected.

The presence of vessel wall changes was primarily scored on HE stained sections (see methods). The typical pattern observed is a thin layer of an iron-rich deposit lining the luminal part of the artery. There is no indication of morphological endothelial cell or intima damage and/or inflammation induced by ferrochloride, nor is there any difference among the groups in the histological appearance of the artery or thrombus formed. In general the thrombus is located intraluminally, without apparent attachment site to the vessel wall, and completely occludes the vessel lumen. A representative specimen is shown in Fig. 2a. A specific anti-murine tissue factor staining shows on occasional sections the presence of tissue factor-rich material adjacent to the ferrochloride deposits on the vascular inner surface (Fig. 2b); however among the series of sections from this study there were no differences in tissue factor intensity explaining the apparent difference in the rates of thrombus formation (data not shown). As to the thrombotic mechanism it appears that in the absence of morphological damage



a



b

Fig. 2 **a** Cross sectional HE staining of carotid artery at the level of ferrochloride induced thrombosis (magnification x 20). The dark staining indicates the ferrochloride layer covering part of the luminal area. The lumen is filled by a fibrin-rich thrombus. **b** Cross sectional staining with anti-murine tissue factor antibody. A positive signal is indicated by purple or dark purple intensity which is most obvious in regions where most ferrochloride has accumulated. The adventitia layer also shows moderate intensity staining for tissue factor (magnification x 20)

to the arterial wall, tissue factor positive cells and clotting factors accumulate at ferrochloride covered sides of the artery.

Discussion

Preliminary clinical investigations suggest an association between defects in the TM-PC mechanism and arterial vascular disease, but the data are inconsistent and confined to selected groups of patients. There also appears to be an inverse association between soluble TM concentrations in plasma and atherosclerosis (29), but the relationship with an anticoagulant function or defect is uncertain. To investigate the importance of the protein C system in arterial thrombosis we performed the present study.

Our data clearly indicate that mice with a functional deficiency of TM more rapidly develop arterial thrombosis as compared to wildtype littermates. In this model the actual trigger of thrombosis, induced by local application of $FeCl_3$ to the carotid artery is unknown, but in some of our experiments a clear indication of tissue factor-positive material deposited adjacent to the arterial wall is evident. The latter may be an indication of accretion of so-called blood borne tissue factor positive cells (13) on top of endothelium that is covered by ferrochloride. This way, the ferrochloride may mask and impair the natural anticoagulant capacity of the vascular endothelium. Histologically, no apparent differences in the vessel wall or thrombus were noted by HE and tissue factor staining. Although occasional granulocytes were observed this was not associated with an apparent inflammatory lesion in the arterial wall. There was also no difference in thrombus histology among the study groups, suggesting that the initiation of thrombosis was qualitatively comparable in the $TM^{Pro/Pro}$ and the control mice. Since the main functional difference between the tested mice is their capacity to activate protein C we must assume that this step is critical in arterial thrombus formation under these conditions. The results from our study confirm the published data from Weiler and colleagues. In their studies in the same $TM^{pro/pro}$ mutant mice, carotid artery thrombosis induced by either stasis or ferrochloride was accelerated as compared to wildtype controls (34). Histologically, there were no apparent differences between thrombi of mutant versus wildtype animals in their study (34). One important limitation in our study is the lack of data on blood pressure during the experiments, since it may be possible that this factor interfered with thrombus formation, although we do not have any indication that pressures would be different in the tested groups.

The results of the latter and our study suggest an important role for TM in regulating arterial blood coagulation, most likely via an impaired effect on PC activation. In contrast to a recent study revealing a role for the PC-TM system in inflammatory effects associated with arterial thrombosis induced by a combined trauma and stasis procedure (32), our study did not show evidence of vessel wall inflammation. Other studies have shown specific contributions of TM in modulating inflammation and theoretically such a mechanism may play a role in vascular inflammation associated with atherosclerosis (15, 30). Indeed, in advanced atherosclerosis a down regulation of TM and other anticoagulant molecules at the endothelium may play a role in impaired protection against inflammation and thrombogenicity (21).

A final limitation of our study is the absence of atherosclerosis. In man, arterial thrombosis forms on an atherosclerotic lesion, probably after exposure of tissue factor to the circulating blood. Although the trigger of thrombosis in our model is unknown, and not mediated by atherosclerosis, the observed effect on blood flow and thrombus formation may encourage further studies of the interactions between an atherosclerotic background and thrombosis in order to reveal the nature of thrombogenicity *in vivo*.

Acknowledgments We acknowledge the expert help of Theo Dirksen and colleagues from the Department of Pathology, AMC, for reliable and skillful processing of the tissue specimens, as well as to Adri Maas and Joost Daalhuisen for expert animal care. Dirkje W. Sommeijer, MD is acknowledged for assistance with TF staining. H. ten Cate is a Clinical Established Investigator of the Netherlands Heart Foundation. J. Dörffler-Melly is supported by a research scholarship from the Novartis Foundation, Basel, Switzerland, and the Department of Clinical Investigation, University Hospital Berne, Switzerland. R. F. Franco is supported by a grant from FAPESP, Brazil.

References

1. Allaart CF, Poort SR, Rosendaal FR, Reitsma PH, Bertina RM, Briet E (1993) Increased risk of venous thrombosis in carriers of hereditary protein C deficiency defect. Lancet 341: 134–138
2. Bom van der JG, Bots ML, Haverkate F, Slagboom E, Meijer P, de Jong PT, Hofman A, Grobbee DE, Kluft C (1996) Reduced response to activated protein C is associated with increased risk for cerebrovascular disease. Ann Intern Med 125: 265–269
3. Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W, Weiler-Guettler H, Rubin RH, Gilbert P, Rosenberg RD (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104: 533–539
4. Dacosta A, Tardy-Poncet B, Isaaz K, Cerisier A, Mismetti P, Simitsidis S, Reynaud J, Tardy B, Piot M, Decousus H, Guyotat D (2000) Prevalence of factor V Leiden (APCR) and other inherited thrombophiliacs in young patients with myocardial infarction and normal coronary arteries. Heart 80: 338–340
5. Dahlback B (1997) Resistance to activated protein C as risk factor for thrombosis: molecular mechanisms, laboratory investigation, and clinical management. Semin Hematol 34: 217–234
6. Doggen CJ, Kunz G, Kyriakoulis K, Stubbs PJ, Lane DA (1998) A mutation in the thrombomodulin gene, 127G to A coding for Ala25Thr, and the risk of myocardial infarction in men. Thromb Haemost 80: 743–748
7. Dorffler-Melly J, Schwarte LA, Ince C, Levi M (2000) Mouse models of focal arterial and venous thrombosis. Basic Res Cardiol 95: 503–509
8. Esmon CT (1987) The regulation of natural anticoagulant pathways. Science 235: 1348–1352
9. Esmon CT (1992) The protein C anticoagulant pathway. Arterioscler Thromb Vasc Biol 12: 135–145
10. Faioni EM, Merati G (1997) The G1456 to T mutation in the thrombomodulin gene is not frequent in patients with venous thrombosis. Blood 89: 1467
11. Farrehi PM, Ozaki CK (1998) Regulation of arterial thrombolysis by plasminogen activator inhibitor-1 in mice. Circulation 97: 1002–1008
12. Franco RF, Reitsma PH (2001) Review. Gene polymorphisms of the haemostatic system and the risk of arterial thrombotic disease. Br J Haematol 115: 491–506
13. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Falton JT et al. (1999) Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci USA 96: 2311–2315
14. Gowda MS, Zucker ML, Vacek JL, Carriger WL, van Laeys DL, Rachel JM, Strope BD (2000) Incidence of factor V Leiden in patients with acute myocardial infarction. J Thromb Thrombolysis 9: 43–45
15. Hirose K, Okajima K, Taoka Y, Uchiba M, Tagami H, Nakano K, Utoh J, Okabe H, Kitamura N (2000) Activated protein C reduces the ischemia-induced spinal cord injury in rats by inhibiting neutrophil activation. Ann Surg 232: 272–280
16. Ireland H, Kunz G, Kyriakoulis K, Stubbs PJ, Lane DA (1997) Thrombomodulin gene mutations associated with myocardial infarction. Circulation 96: 15–18
17. Jong WMC, Zuurbier CJ, de Winter RJ, van den Heuvel DAF, Reitsma PH, ten Cate H, Ince C (2002) Fentanyl-fluanisone-midazolam combination results in more stable hemodynamics than does urethane-α-chloralose and 2,2,2,-tribromoethanol in mice. Contemp Topics Lab Anim Sci 41: 28–32
18. Kiechl S, Muigg A, Santer P, Mitterer M, Egger G, Oberhollenzer M, Oberhollenzer F, Mayr A, Gasperi A, Poewe W, Willeit J (1999) Poor response to activated protein C as a prominent risk predictor of advanced atherosclerosis and arterial disease. Circulation 99: 614–619
19. Kunz G, Ireland HA (2000) Identification and characterization of a thrombomodulin gene mutation coding for an elongated protein with reduced expression in a kindred with myocardial infarction. Blood 95: 569–576
20. Lane DA, Grant PJ (2000) Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. Blood 95: 1517–1532
21. Laszik ZG, Zhou XJ, Ferrell GL, Silva FG, Esmon CT (2001) Down-regulation of endothelial expression of endothelial cell protein C receptor and thrombomodulin in coronary atherosclerosis. Am J Pathol 159: 797–802
22. Le Flem L, Picard V (1999) Mutations in promoter region of thrombomodulin and venous thromboembolic disease. Arterioscler Thromb Vasc Biol 19: 1098–1104
23. Magdelaine A, Verdy E (2000) Deep vein thrombosis during enoxaparin prophylactic treatment in a young pregnant woman homozygous for factor V Leiden and heterozygous for the G127 → a mutation in the thrombomodulin gene. Blood Coagul Fibrinolysis 11: 761–765
24. Makris TK, Krespi PG, Hatzizacharias AN, Gialeraki AE, Anastasiadis G, Triposkiadis FK, Mandalaki T, Kyriakidis MK (2000) Resistance to activated protein C and FV Leiden mutation in patients with a history of acute myocardial infarction or primary hypertension. Am J Hypertens 13 (1 Pt 1): 61–65
25. Norlund L, Zoller B, Ohlin AK (1997) A novel thrombomodulin gene mutation in a patient suffering from sagittal sinus thrombosis. Thromb Haemost 78: 1164–1166
26. Ohlin AK, Marlar RA (1999) Thrombomodulin gene defects in families with thromboembolic disease – a report on four families. Thromb Haemost 81: 338–344

▶

27. Ronde de H, Bertina RM (1994) Laboratory diagnosis of APC-resistance: a critical evaluation of the test and the development of diagnostic criteria. Thromb Haemost 72: 880–886
28. Sakata T, Kario K, Katayama Y, Matsuyama T, Kato H, Miyata T (2000) Studies on congenital protein C deficiency in Japanese: prevalence, genetic analysis, and relevance to the onset of arterial occlusive diseases. Semin Thromb Hemost 26: 11–16
29. Salomaa V, Matei C, Aleksic N, Sonsores-Garcia L, Folsom AR, Juneja H, Chambless LE, Wu KK (1999) Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. Lancet 353:1729–1734
30. Taoka Y, Okajima K, Uchiba M, Johno M (2000) Neuroprotection by recombinant thrombomodulin. Thromb Haemostas 83: 462–468
31. de Waard V, Timmerman JJ, Pannekoek H, Reitsma PH, ten Cate H (2002) Cellular divergence of tissue factor mRNA and protein expression in murine sepsis: the role of the granulocyte revisited. In: de Waard V (Academic thesis) University of Amsterdam May 2002
32. Waugh JM, Yuksel E, Li J, Kuo MD, Kattash M, Saxena R, Geske R, Thung SN, Shenaq SM, Woo SL (1999) Local overexpression of thrombomodulin for in vivo prevention of arterial thrombosis in a rabbit model. Circ Res 84: 84–92
33. Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW, Rayburn H, Edelberg JM, Rosenberg RD (1998) A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 101: 1983–1991
34. Weiler H, Lindner V, Kerlin B, Isermann BH, Hendrickson SB, Coolet BC, Meh DA, Mosesson MW, Shworka NW, Post MJ, Conway EM, Ulfman LH, von Adrian UH, Weitz JI (2001) Characterization of a mouse model for thrombomodulin deficiency. Arterioscler Thromb Vasc Biol 21:1531–1537