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CHAPTER 10

FUNCTIONAL THROMBOMODULIN DEFICIENCY CAUSES ENHANCED THROMBUS GROWTH IN A MURINE MODEL OF CAROTID ARTERY THROMBOSIS

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Summary

Thrombomodulin (TM), a glycoprotein of the endothelium, binds to thrombin and initiates the anticoagulant protein C pathway subsequently decreasing thrombin formation. Recently, it has been suggested that TM polymorphisms may influence the risk of myocardial infarction. In order to study the effect of a functional TM deficiency on arterial thrombus growth we used mice with a 4th Glu-to-Pro mutation in the TM gene (TM<sup>pro/pro</sup>), their wildtype littermates, and C57Bl/6 mice in a model of chemically induced carotid artery thrombosis. Time-to-occlusion (TTO) within 30 minutes was assessed by arterial blood flow measurement, using a transonic flow probe. Complete occlusion occurred in 16/21 (76%) TM<sup>pro/pro</sup> mice, and in [4/21 (19%) controls (2/11 WT, 2/10 C57BL/6). Mean TTO [± SE] was 747.1 sec ± 127.7 in TM<sup>pro/pro</sup> mice versus 1507 sec ± 158.7 in WT and 1548 sec ± 167.7 in C57Bl/6 mice (p = 0.0044 Mann Whitney test). Histological transsection analysis confirmed presence of complete occlusion in cases interpreted as occluded by flow measurements. These data show that a functional deficiency in TM results in enhanced thrombus formation in a murine model of carotid artery thrombosis and indirectly support a role for TM in arterial thrombotic disease.
Introduction

In vivo thrombosis formation in mammalian arteries and veins is physiologically prevented by specific mechanisms involving the fibrinolytic and anticoagulant system. Among these, the anticoagulant protein C pathway provides a natural antithrombotic mechanism of the vascular endothelium interacting with the blood clotting system. Thereby, a membrane-bound glycoprotein of the endothelium, thrombomodulin (TM), binds to thrombin forming a complex. The TM-thrombin complex activates the endothelial receptor-bound protein C (PC)\textsuperscript{12}. After activation by the TM-thrombin complex, activated protein C (APC) proteolytically inactivates coagulation factors Va and VIIIa. This inactivation reaction only proceeds efficiently in the presence of Ca\textsuperscript{2+} ions and the co-factor protein S. APC may also inhibit plasminogen-activator inhibitor type-1\textsuperscript{3}.

The clinical relevance of defects of the PC pathway has been established by the identification of PC and protein S deficiency, and by the identification of inherited and acquired APC resistance, in patients suffering from venous thromboembolic disease\textsuperscript{4-6}. In contrast, there is little evidence for a similar association with arterial thrombosis. An increased risk for the early onset of myocardial infarction and stroke has been reported in small cohorts of individuals with a defect in the PC pathway, but this has not been confirmed by others\textsuperscript{7-13}. In humans, a number of mutations of the TM gene or gene promoter region have been reported to be associated with arterial or venous thromboembolic diseases\textsuperscript{14-17}; such an association, however, has also been contradicted by others\textsuperscript{18,19}. Therefore, the clinical relevance of TM-mutations has still to be demonstrated. Theoretically, a deficiency in TM might lead to a prothrombotic state and enhanced risks for arterial or venous thrombosis. As reported recently\textsuperscript{20}, such a prothrombotic state could be achieved in mice that were transgenically modified by a targeted point-mutation of the TM-gene, resulting in a loss of the capability of TM binding to thrombin. Under defined conditions, these mice manifested microvascular myocardial thrombosis\textsuperscript{21}. In the present study we show for the first time, that a functional TM-deficiency has a direct impact on arterial thrombus growth in a murine model of carotid artery thrombosis using TM-deficient mice presenting with a prothrombotic state.

Materials and Methods

Animal model

TM\textsuperscript{pro/pro} mice are homozygous with a targeted Glu-to-Pro mutation in the TM gene with an impaired capability of TM binding to thrombin and reduced capacity to activate protein C, as described in detail by Weiler-Guettler et al\textsuperscript{20}. The TM\textsuperscript{pro/pro} mice, which were generously provided
by Dr. RD Rosenberg (MIT, Boston, USA), were direct descendants from an F1 cross, and thus genetically 50% C57BL/6 and 50% 129Sv. In a pilot experiment we compared ferric chloride-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. Due to the mixed genetic background of these mice, which could represent a determining factor causing the observed difference in thrombus growth, we repeated the experiments in 21 age and sex matched mutant TM^{pro/pro} and wildtype littermates that were born from a cross between F2 heterozygotes. In order to avoid confusion between F1 descendents and the littermate generation, F1-descendents will furtheron be termed as 'F1-TM^{pro/pro}' and the descendents of the F2 heterozygotes 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 guidelines of the American Physiological Society and the Dutch Law for Animal Experiments.

Ferric chloride thrombosis model

Thrombus formation and growth was evaluated in a standardized model, whereby thrombosis in the carotid artery is induced by ferric chloride as reported before. Mice were anaesthetized 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). The mice were placed on a heating pad and if necessary exposed to a warming lamp. Body temperature was kept between 36.5 and 37 °C. Animals were tracheotomized and ventilated during the experiment. After a median section from the xiphoid to the neck, the left carotid artery was bluntly isolated, 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 transonic flow probe (Transonic Systems Inc., NY, USA) with a diameter of 0.5 mm was placed to the distal part of the 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 x 2 mm, soaked in a 10% solution of ferric chloride, was placed on the carotid artery, whereby care was taken to provide good contact between the filter paper and the vessel. 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-reversed flow stop, was the principal endpoint-parameter. Clot formation as analysed histologically from the excised carotid artery segment was a second parameter for vessel occlusion.
**Histological evaluation**

At the end of the acute experiment, the carotid artery segments from both sides were excised and processed for paraffin blocks. Transsectional slides of the proximal, medial, and distal part of the site, where the filterpaper had been placed, were prepared, and tissues were stained with haematoxylin and eosin. Thrombi and vessel walls were analysed by two independent blinded investigators under a light microscope.

**Data analysis**

Values are expressed as means ± SEM, with the numbers of experiments performed provided in the figure legends. The statistical significance of differences between groups was assessed using the Fisher's Exact test or the Mann-Whitney test where applicable. Data were considered significantly different when p < 0.05.

**Results**

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

Mean (+/- SE) blood flow at steady state before application of FeCl₃ in the different groups was as follows: for F1-TMₚ⁰/ₚ₀ 0.7 ± 0.04 mL/min, for F2- TMₚ₀/ₚ₀ 0.7 ± 0.06 mL/min, for WT 0.7 ± 0.04 mL/min, and for C57BL/6 mice 0.6 ± 0.06 mL/min (no significant difference). Heart rates ranging from 476 to 478 bpm did also not differ between groups.

Occlusion occurred in 4 of 21 (19%) mice, 2 in the WT and 2 in the C57BL/6 groups, considered as control mice presenting a normal PC pathway, while in 17 of the 21 (76%) animals in the F1-TMₚ₀/ₚ₀ and F2- TMₚ₀/ₚ₀ groups had vessel occlusion (flow < 0.1 mL/min) by the end of the observation time (p=0.0001). In those mice, in whom occlusion occurred, mean TTO was not different between groups (726 ± 358 sec for F1-TMₚ₀/ₚ₀ and F2- TMₚ₀/ₚ₀, 499 ± 248 sec for WT, 542 ± 276 sec for C57BL/6).

Among the animals with patent carotid arteries, there were five observed in the control group to have had occlusion for only one and two minutes with spontaneous recanalisation and re-established perfusion at the end of the observation time, whereas this occurred in two cases of the F1-TMₚ₀/ₚ₀ group with final occlusions at 870 and 720 seconds. Figure 1 shows TTO values in seconds as measured in TMₚ₀/ₚ₀, WT and C57BL/6 mice. Those animals with still measureable blood flow at the end of the observation time (30 minutes) were assigned to TTO 1800 sec.
Figure 2 Time-to-occlusion (TTO) was significantly different, when animals without an occlusion were included with TTO = 1800 seconds. Mean values for those animals, in which occlusion occurred, did not differ between groups.

An example of flow registration in a TM⁰⁄₄₀ mouse before and during occlusion is presented in Figure 2.

**Figure 2** Blood flow in a TM⁰⁄₄₀ mouse decreased to full stop at 5 minutes and 5 seconds after application of ferric chloride; no reflow occurred until the end of the observation time.
Histological assessment of thrombosis

Histological assessment of the arterial segments revealed fibrin rich thrombi in 16 TM\textsuperscript{pro/pro} mice with evidence of complete or partial occlusion; in one animal, in which a clear flow stop occurred, no clot was found. Consistency between flow stop registration and histological clot detection was found for 2 WT and 2 C57Bl/6 mice in the control group. Those animals, in which blood flow could still be registered at the end of the observation time, no clots were detected. Granulocytes were detected within the thrombi and surrounding vessel wall segments suggesting an inflammatory reaction to the chemical stimulus.

Discussion

Arterial thrombosis commonly forms on an atherosclerotic lesion, such as a ruptured plaque. In addition to local thrombogenic vascular factors, like enhanced tissue factor expression, circulating pro- and anticoagulant elements may be involved. Limited clinical data suggest a role for the thrombomodulin –protein C mechanism in influencing the risk of arterial thrombotic events, as for example myocardial infarction in young people. To determine the influence of a specific defect in the PC activation pathway in arterial thrombosis, we performed the present study. We showed for the first time, that in a murine model of a functional deficiency in TM, there is indeed evidence for a direct impact on enhanced thrombus growth in a well-established and reproducible model of arterial thrombosis.

The FeCl\textsubscript{3} thrombosis model has been applied to the carotid artery in mice before\textsuperscript{22}. We adopted the method with slight modifications such as using the optimal concentration of FeCl\textsubscript{3} to cause vessel occlusion as a sensitive endpoint parameter. In a pilot experiment, we applied a 25% solution in 10 mice (5 WT and 5 TM) by which thrombosis was induced in all animals, whereas reducing the concentration to 10% resulted in an 80% occlusion rate in TM mice and 20%, respectively, in WT and C57Bl/6. The induction of thrombosis in this model is probably due to the toxic challenge of the endothelial and muscular cell membranes to FeCl\textsubscript{3} causing inflammatory cells to produce oxygen radicals with subsequent endothelial and medial damage. Light microscopical analysis could not detect endothelial disrupture, but in two third of the cases, in which thrombi were detected, there was also granulocyte influx described in the vessel wall, independently from the mouse strain. This is suggestive of an inflammation mediated increase in tissue factor expression.

In order to exclude the possibility of a purely background related difference in thrombus induction, we investigated a group of C57Bl/6 mice, the background strain, to which TM\textsuperscript{pro/pro} had been backcrossed. Such putative strain-related differences in thrombus growth, basal blood flow and heart rates could be excluded completely. We also used both genders, a group of male
and female TM<sup>pro/pro</sup> mice and found that there was no difference, neither in TTO, basal flow, heart rates, nor in thrombus growth between genders within one group. This study represents the first demonstration derived from an experimental transgenic model pointing to a significant role of TM in arterial thrombus formation, a finding that gives further support to the hypothesis that TM gene variations might influence arterial thrombotic risk.

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