Characterization of mouse coagulation (regulatory) genes with use of RNAi

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Chapter 5

Acute and Severe Coagulopathy in Adult Mice Following Silencing of Hepatic Antithrombin and Protein C Production

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Summary

Mice deficient in the anticoagulants antithrombin (*Serpinc1*) or protein C (*Proc*) display early lethality due to thrombosis-related coagulopathy, thereby precluding their use in gene function studies or thrombosis models. Here, RNA interference was used to silence *Serpinc1* and/or *Proc* in normal adult mice, and the severe coagulopathy following combined 'knockdown' of these genes is reported. Two days after siRNA injection, (occlusive) thrombi were observed in (large and medium-sized) vessels in multiple tissues, and hemorrhages were prominent in the ocular, mandibular, and maxillary areas. Tissue fibrin deposition and plasma fibrinogen lowering accompanied this phenotype. The coagulopathy was prevented by dabigatran treatment. Silencing *Serpinc1* alone yielded a comparable but milder phenotype with later onset. The phenotype was absent when targeting *Proc* alone. We conclude that RNA interference of *Serpinc1* and/or *Proc* allows studying the function of these genes *in vivo*, and provides a novel, controlled mouse model for spontaneous venous thrombosis.

INTRODUCTION

Wild type mice do not spontaneously develop venous thrombosis. Knock-out mice for the natural anticoagulants antithrombin (*Serpinc1*) and protein C (*Proc*) feature spontaneous thrombosis-related coagulopathy[1;2], and succumb during embryogenesis and/or perinatally, precluding their use in studies on the function of these anticoagulants, or as a model for thrombosis. To overcome early lethality we employed RNA interference to silence antithrombin and protein C production, alone or in combination, in livers of wild type mice. Here, we report the spontaneous thrombotic phenotype observed shortly after this treatment.

METHODS

Effective synthetic siRNAs (Life Technologies, Carlsbad, USA) targeting *Serpinc1* and *Proc* were identified using mouse hepatocytes as described previously[3]. Selected si*Serpinc1*, si*Proc* (sequences, see supplemental methods) and control siNEG (Life Technologies), complexed with Invivofectamine® (Life Technologies), were tail vein-injected in 8-10 weeks old female C57Black/6J mice (Charles River, Maastricht, The Netherlands), alone or in combination. Mice were sacrificed at different time points and subjected to necropsy according to international pathology guidelines[4]. Liver *Serpinc1* and *Proc* transcript, plasma antithrombin, protein C, thrombin-antithrombin complexes, and fibrinogen levels, PT and aPTT, and tissue fibrin deposition were analyzed as described previously[3;5;6].

RESULTS AND DISCUSSION

Intravenous injection of si*Serpinc1* or si*Proc* (7 mg siRNA/kg body weight) resulted in a strong reduction in *Serpinc1* or *Proc* hepatic transcript (4±0.3% and 11±0.5% of siNEG-treated animals, respectively (n=3)) and plasma antigen level (1±2.4% and 2±1.8% of siNEG, respectively (n=3)) after 2 days. In si*Serpinc1*-injected mice, plasma thrombin-antithrombin

complexes were not detectable (<2 ng/ml), indicating that low plasma antigen was not due to consumptive loss, at least for antithrombin. At 2 days the animals appeared fully normal (observation in 6 animals per siRNA). A similar reduction in transcript and plasma antigen level was observed when animals were treated with a combination of si*Serpinc1* and si*Proc* (7 mg/siRNA/kg; n=6, Figure 1A, B). However, here, 2 days after injection, one animal died and the remaining 5 animals displayed severe weight loss (-15.6±2.4%, *P*=0.0011 vs siNEG-treated animals (n=6)), lethargy, unresponsiveness to stimuli, and hypothermia. Four of the 5 surviving animals featured unilateral lesions involving the eye (exophthalmos), intraocular and periocular hemorrhages (Figure1C). Maxillary, mandibular and submandibular regions were severely swollen and showed focally extensive subcutaneous and intramuscular hemorrhages especially involving the masseter muscle (Figure1D). In 2 animals hind leg cyanosis was observed (Figure 1G). Because of the severe and irreversible clinical conditions animals were promptly sacrificed also allowing further pathological and biochemical analyses. In affected animals, collecting citrate-blood from the caval vein was difficult or not possible, likely because of circulatory failure and shock. At day 3 and 4, the surviving si*Serpinc1*/si*Proc* animal and si*Serpinc1*-alone animals also featured the clinical signs (hind leg cyanosis not observed). During 5 days of observation none of the si*Proc*-treated animals (n=6) featured abnormalities.

Because of the severe symptoms in the *Serpinc1*/si*Proc* group further experiments using the 7 mg/siRNA/kg dose were discontinued. Using half the dose of *Serpinc1*/si*Proc* (3.5 mg/siRNA/kg) reproduced most pathology findings (n=13), and all animals showed the described clinical signs within 72 hours after siRNA injection. However, weight loss was less severe (-12.1±5.1%, *P*<0.0001 vs siNEG), immediate death and hind leg cyanosis

were not observed, and appropriate collection of citrate-blood was possible for most animals. In total, 19 out of 19 si*Serpinc1*/si*Proc* (sum of animals for 3.5 and 7 mg/kg dose) versus 0 out of 11 siNEG-treated animals featured abnormalities (P<0.0001, Fisher's exact test).

Necropsy was performed on si*Serpinc1/siProc* (7 and 3.5 mg/siRNA/kg dose, n=5 and 12, respectively) and si*Serpinc1* animals (7 mg/siRNA/kg, n=6). Sagittal and coronal serial sections of the head showed severe multifocal hemorrhages within the eye (Figure 1E), surrounding muscles and harderian glands (Figure1E, F). The masseter and temporal muscles of affected eyes consistently displayed severe hemorrhages, mild muscle degeneration and necrosis. Hemorrhages and vascular thrombi were observed in the above-mentioned areas and submucosa of the palate, nasal turbinates, tongue and subdural spaces. Lesions were observed in all animals and were comparable between the si*Serpinc1/siProc* and si*Serpinc1*-alone groups. Hind leg cyanosis, if present, coincided with hemorrhages and thrombi in the subcutis and tibia-femoral muscular fibers (Figure1H). Regarding the liver, in at least six specimens (high and low dose of si*Serpinc1*/si*Proc*) multifocal areas of necrosis were grossly visible (Figure 1I). Microscopic abnormalities in the liver were found for all animals and ranged from minimal multifocal hepatic degeneration and presence of rare thrombi (4 out of 12 animals receiving the low dose of si*Serpinc1*/si*Proc*, 4 out of 6 animals receiving si*Serpinc1* alone*)* to multifocal, extensive areas of severe coagulative and lytic necrosis with (occlusive) thrombi in large and medium-sized vessels (Figure 1J, for 8 out of 12 animals receiving low dose si*Serpinc1*/si*Proc*, all animals receiving high dose si*Serpinc1*/si*Proc*, 2 out of 6 animals for si*Serpinc1*-alone). In head, leg and liver, thrombi were located in veins, characterized by organized fibrin layering, and surrounding tissues devoid of inflammatory cells, indicative for an acute process. Neither animal featured lesions,

thrombi and/or hemorrhages in kidney or gastrointestinal tract. Minor incidence was observed in heart, lung, and brain.

Biochemical analysis of livers of si*Serpinc1*/si*Proc*-treated animals at 3.5 mg/siRNA/kg demonstrated fibrin deposition (Figure 1K), coinciding with reduced plasma fibrinogen levels (Figure 1L). For the 7 mg/siRNA/kg dose, liver fibrin deposition was massive and plasma fibrinogen was virtually absent (Figure 1L), indicating a relation with the siRNA dose. Liver fibrin deposition was at background level in siNEG (5.4±2.7 ng/mg, n=11) and si*Proc*-treated animals (5.6±2.7 ng/mg, n=6). Remarkably, si*Serpinc1*-treated animals had low liver fibrin deposition (10.7±3.1 ng/mg, n=6). As si*Serpinc1* animals displayed a later onset of the phenotype (reproduced in additional experiments; both 3.5 and 7 mg/si*Serpinc1*/kg, n=5 per dose), and unaffected plasma fibrinogen (data not shown), this suggests that a combination of si*Serpinc1* and si*Proc* results in a more severe phenotype than the sum of si*Serpinc1* and *siProc* alone. Likely, combined loss of Serpinc1 and Proc pushes the animals over a thrombotic threshold that cannot be reached with diminution of either one.

Tissue fibrin deposition was not restricted to the liver; lungs of si*Serpinc1*/si*Proc*-treated mice demonstrated increased fibrin deposition (3.5 mg/siRNA/kg dose; 50±38 (n=13) vs 13±7.3 ng/mg (n=11) in siNEG, *P*=0.0010), despite minor incidence of microscopically visible thrombi in this tissue (1 animal out of 12).

The presence of (occlusive) thrombi and fibrin deposition in si*Serpinc1*/si*Proc*-treated animals indicates that the observed coagulopathy is thrombotic in nature and hemorrhages are likely secondary to consumption of fibrinogen and/or other coagulation factors as illustrated by prolonged PT and aPTT for 6 out of 10 si*Serpinc1*/si*Proc*-treated animals (>70 and >120 s, respectively vs 11±0.3 and 27±1.6 s for siNEG-treated animals, both *P*=0.0039, Fisher test). To demonstrate that thrombin forma-

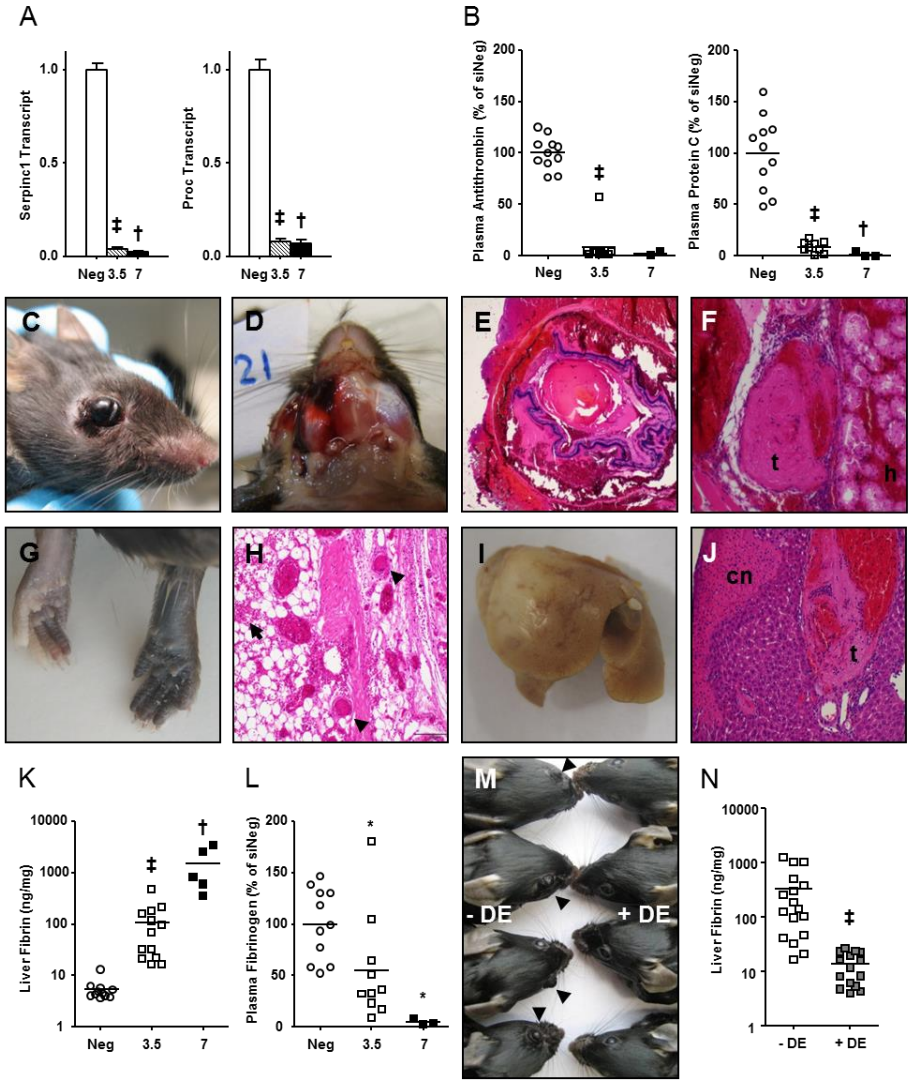

Figure 1: Phenotypic Appearance of Mice Following Silencing of Hepatic Antithrombin and Protein C Production. **A**. Effectiveness of silencing of *Serpinc1* and *Proc* in mouse liver. siRNAs targeting *Serpinc1* and *Proc* were complexed, mixed and intravenously injected in C57Black/6J mice at a dose of 3.5 (hatched bars, n=13) or 7 (black bars, n=6) mg per siRNA per kg mouse. At two days post siRNA injection mice were removed from the

experiment, sacrificed and livers were subjected to *Serpinc1* (left) or *Proc* (right) transcript analysis by qPCR. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siNEG, 7 mg per kg, open bars, n=11); **B**. Plasma antithrombin (left) and protein C levels (right) in siNEG (open circles), si*Serpinc1*/si*Proc*-injected animals at 3.5 (open squares) or 7 (black squares) mg siRNA per kg mouse; **C**. Right eye of a si*Serpinc1*/si*Proc* injected animal. Unilateral severe exophthalmos and periocular hemorrhages; **D**. Multifocal hemorrhages in the mandibular, submandibular area, and masseter muscle; **E**. Severe multifocal hemorrhages within the eye (HE-stained 5-µm section, magnification 40x); **F**. Eye region: harderian glands with multifocal hemorrhages (h) and thrombus (t) presence (HE-stained 5-µm section, magnification 100x); **G**. Cyanosis of the right hind leg; **H**. Hemorrhages (arrow) and thrombi (arrow heads) were present in the subcutis but also among muscular fibers of the tibial and femoral areas (HE-stained 5-µm section, magnification 200x); **I**. Liver (formalin-fixed specimen) presenting focally extensive areas of necrosis (asterisk); **J**. Liver section presenting severe multifocal to coalescing coagulative necrosis (cn) and thrombosis (t) in hepatic vein (HE-stained 5-µm section, magnification 100x); **K**. Liver fibrin, and **L**. plasma fibrinogen in siNEG (open circles), si*Serpinc1*/si*Proc* injected animals at 3.5 (open squares), or 7 (black squares) mg siRNA per kg mouse; **M**. Treatment of si*Serpinc1*/si*Proc*-injected treated mice (3.5 mg/kg dose) with dabigatran etexilate (DE). DE was administered by oral gavage of 3 mg per mouse at 7 am, 3 and 11 pm for five days, starting the day before siRNA injection. This results in an aPTT of 66.9±6.8 (s) to 36.2±4.4 (s) as determined in a parallel treated control group at two hours after dosing and one hour before the next dose (vehicle treated animals aPTT of 26.7±5.1 and 24.5±0.8 (s), respectively). Presence of periocular contusion (arrow heads) for vehicle treated animals (left) and not DE-treated animals (right); **N**. Liver fibrin deposition in si*Serpinc1*/si*Proc* (3.5 mg per siRNA per kg) treated with DE (open squares) or vehicle (filled squares). Data were analyzed with the Instat software (GraphPad, San Diego, USA). Statistical differences between control siNEG and si*Serpinc1*, si*Proc*, si*Serpinc1*/si*Proc* were evaluated using a Mann-Whitney Rank sum test. P-values<0.05 were regarded as statistically significant. *P<0.05, †P<0.01, ‡P<0.001

tion underlied this phenotype, si*Serpinc1*/si*Proc*-treated mice (3.5 mg/siRNA/kg) were administered the thrombin inhibitor dabigatran etexilate. Dabigatran prevented the clinical signs of si*Serpinc1*/si*Proc*-treated mice, including weight loss (-5.7±5.2 vs -11.2±3.9% in vehicle-

treated si*Serpinc1*/si*Proc* animals, n=16-17, *P*=0.0011), exophthalmos and periocular contusion (Figure 1M, 0/17 versus 16/ 16, *P*<0.0001, Fisher test), and largely suppressed liver fibrin deposition (Figure 1N).

We conclude that silencing hepatic *Serpinc1*/*Proc* or *Serpinc1* alone acutely induces thrombotic coagulopathy. This study a) highlights the importance of protein C and antithrombin in animals under challenge-free conditions, b) points to synergism between these anticoagulant systems c) will help to further unravel the *in vivo* function of these anticoagulants and d) provides a novel, controlled model for venous thrombosis research.

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SUPPLEMENTAL METHODS

Structural information on reagents and negative control

We used pre-designed siRNAs for mouse Serpinc1 and Proc mRNAs that were purchased from Life Technologies, Carlsbad, California, USA (Ambion Silencer® Select Pre-designed siRNA) catalogue number 4404014. For mouse Serpinc1 with sense sequence UCCUGGUUCUUAUAAGGGATT and antisense sequence UCCCUUAUAAGAACCAGGAAG and for mouse Proc with sense sequence GCAAGAUCCUCAAACGAGATT and antisense sequence UCUCGUUUGAGGAUCUUGCTGT. Ambion's siNEG (catalogue number 4404020) was used as control siRNA. This negative control siRNA was selected using a modified blast to account for short sequence length and demonstrated to exclude significant homology to any known gene targets in RefSeq and MirBase (more detailed documentation on this negative control siRNA is available on the manufacturer's website;http://products.invitrogen.com/ivgn/product/4457289?ICID==%3D%3D%3Dsearch-product).