Acute and severe coagulopathy in adult mice following silencing of hepatic antithrombin and protein C production

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Key Points

- RNA interference of SerpinC1 and/or Proc allows for evaluation of the function of these genes, alone or in combination, in normal adult mice.
- RNA interference of SerpinC1 and Proc provides a novel, controlled mouse model for spontaneous venous thrombosis.

Mice deficient in the anticoagulants antithrombin (SerpinC1) or protein C (Proc) display premature death due to thrombosis-related coagulopathy, thereby precluding their use in gene function studies and thrombosis models. We used RNA interference to silence SerpinC1 and/or Proc in normal adult mice. The severe coagulopathy that followed combined “knockdown” of these genes is reported. Two days after siRNA injection, thrombi (occlusive) were observed in vessels (large and medium-sized) in multiple tissues, and hemorrhages were prominent in the ocular, mandibular, and maxillary areas. Tissue fibrin deposition and reduction of plasma fibrinogen accompanied this phenotype. The coagulopathy was prevented by dabigatran etexilate treatment. Silencing of SerpinC1 alone yielded a comparable but milder phenotype with later onset. The phenotype was absent when Proc was targeted alone. We conclude that RNA interference of SerpinC1 and/or Proc allows for evaluation of the function of these genes in vivo and provides a novel, controlled mouse model for spontaneous venous thrombosis. (Blood. 2013;121(21):4413-4416)

Introduction

Wild-type mice do not spontaneously develop venous thrombosis. Mice knockout 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 and as a model for thrombosis. To early death, we used RNA interference to silence SerpinC1 and/or Proc production, alone or in combination, in livers of wild-type mice. Here, we report the spontaneous thrombotic phenotype that was observed shortly after this treatment.

Study design

Effective synthetic siRNAs (Life Technologies, Carlsbad, CA) targeting SerpinC1 and Proc were identified using mouse hepatocytes as described previously.3 Selected siSerpinC1, siProc (sequences, see supplemental Methods), and control siNEG (Life Technologies) complexes with Invivofectamine (Life Technologies) were injected into the tail veins of female C57Black/6J mice (Charles River, Maastricht, the Netherlands) age 8-10 weeks, alone or in combination. Mice were euthanized 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, fibrinogen levels (prothrombin time [PT] and activated partial thromboplastin time [aPTT]), and tissue fibrin deposition were analyzed as described previously.3,5,6 Leiden University’s animal welfare committee approved all experimental procedures.

Results and discussion

Intravenous injection of siSerpinC1 or siProc (7 mg siRNA/kg body weight) resulted in a significant 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-treated animals, respectively; n = 3) after 2 days. It is important to note that consumptive loss could have contributed to these low plasma levels. Consumptive loss, if present, did not coincide with detectable plasma thrombin–antithrombin complexes in siSerpinC1-treated animals (<2 ng/mL). After 2 days the animals appeared 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 siSerpinC1 and siProc (7

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Figure 1. Phenotypic appearance of mice following silencing of hepatic antithrombin and protein C production. (A) Effectiveness of silencing Serpinc1 and Proc in mouse liver. siRNAs targeting Serpinc1 and Proc were complexed, mixed, and intravenously injected into C57Black/6J mice at a dose of 3.5 mg/siRNA/kg (hatched bars, n = 13) or 7 mg/siRNA/kg (black bars, n = 6). At 2 days post siRNA injection, mice were removed from the experiment and euthanized; livers were subjected to Serpinc1 (left) or Proc (right) transcript analysis by quantitative polymerase chain reaction. β-actin was used as the internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siNEG-treated, 7 mg/kg, open bars, n = 11). (B) Plasma antithrombin (left) and protein C levels (right) in siNEG-injected mice (open circles), siSerpinc1/siProc-injected mice at 3.5 mg/siRNA/kg (open squares), and 7 mg/siRNA/kg (black squares). (C) Right eye of an siSerpinc1/siProc-injected animal. Unilateral severe exophthalmos and periorcular hemorrhages are shown. (D) Multifocal hemorrhages in the mandibular and submandibular area and masseter muscle. (E) Severe multifocal hemorrhages within the eye (Hematoxylin and Eosin [HE]-stained 5-μm section, magnification 40×). (F) Eye region: presence of harderian glands with multifocal hemorrhages (h) and thrombus (t) (HE-stained 5-μm section, magnification 100×). (G) Cyanosis of the right hind leg. (H) Hemorrhages (arrow) and thrombi (arrowheads) were present in the subcutis but also among muscular fibers of the tibial and femoral areas (HE-stained 5-μm section, magnification 200×). (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 100×). Liver fibrin (K) and plasma fibrinogen (L) in siNEG-treated mice (open circles), siSerpinc1/siProc-injected animals at 3.5 mg/siRNA/kg (open squares), and 7 mg/siRNA/kg (black squares). (M) Treatment of siSerpinc1/siProc-injected mice (3.5-mg/kg dose) with DE. DE was administered by oral gavage of 3 mg per mouse at 7 AM, 3 PM, and 11 PM for 5 days starting on the day before siRNA injection. This resulted in an aPTT of 66.9 ± 6.8 seconds to 36.2 ± 4.4 seconds, as determined in a parallel treated control group at 2 hours after dosing and 1 hour before the next dose (vehicle treated animals aPTT of 26.7 ± 5.1 seconds and 24.5 ± 0.8 seconds, respectively). Presence of periorcular contusion (arrow heads) for vehicle-treated animals (left) and animals not treated with DE (right). (N) Liver fibrin deposition in siSerpinc1/siProc-treated mice (3.5 mg/siRNA/kg) treated with DE (open squares) or vehicle (filled squares). Data were analyzed using the Instat software (GraphPad, San Diego, CA). Statistical differences between control siNEG and siSerpinc1, siProc, siSerpinc1/siProc were evaluated using a Mann-Whitney rank sum test. P values < .05 were regarded as statistically significant. *P < .05, †P < .01, ‡P < .001.
mg/siRNA/kg; n = 6; Figure 1A-B). However, 2 days after injection, 1 animal died and the remaining 5 animals displayed severe weight loss (−15.6 ± 2.4%, P = .0011 compared with siNEG-treated animals [n = 6]), lethargy, unresponsiveness to stimuli, and hypothermia. Four of the 5 surviving animals exhibited unilateral lesions involving the eye (exophthalmos) as well as intraocular and periocular hemorrhages (Figure 1C). Maxillary, mandibular, and submandibular regions were severely swollen and showed focally extensive subcutaneous and intramuscular hemorrhages, especially involving the masseter muscle (Figure 1D). Hind leg cyanosis was observed in 2 animals (Figure 1G). Because of these severe and irreversible clinical conditions, animals were promptly euthanized, making further pathological and biochemical analyses possible. Collection of citrate blood from the caval vein of affected animals was difficult or impossible, likely because of circulatory failure and shock. At day 3 and day 4, the surviving siSerpinc1/siProc-treated and siSerpinc1-alone animals also showed the clinical signs (hind leg cyanosis was not observed). During 5 days of observation, none of the siProc-treated animals (n = 6) exhibited abnormalities.

Because of the severe symptoms seen in the siSerpinc1/siProc group, further experiments using the 7-mg/siRNA/kg dose were discontinued. Use of half the dose (3.5 mg/siRNA/kg) in the Serpinc1/siProc group reproduced most pathological 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 < .0001 compared with the siNEG group), immediate death and hind leg cyanosis were not observed, and appropriate collection of citrate blood was possible for most animals. In total, 19 of 19 siSerpinc1/siProc-treated animals (sum of animals for 3.5- and 7-mg/kg dose) compared with 0 of 11 siNEG-treated animals exhibited abnormalities (P < .0001; Fisher exact test).

Necropsy was performed on siSerpinc1/siProc-treated (7- and 3.5-mg/siRNA/kg dose, n = 5 and 12, respectively) and siSerpinc1-treated animals (7-mg/siRNA/kg dose, n = 6). Sagittal and coronal serial sections of the head showed severe multifocal hemorrhages within the eye (Figure 1E), surrounding muscles, and harderian glands (Figure 1E-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 as well as the submucosa of the palate, nasal turbinates, tongue, and subdural spaces. Lesions were observed in all animals and were comparable between the siSerpinc1/siProc and siSerpinc1-alone groups. Hind leg cyanosis, if present, coincided with hemorrhages and thrombi in the subcutis and tibia–femoral muscular fibers (Figure 1H).

Regarding the liver, in at least 6 specimens (high and low doses of siSerpinc1/siProc), multifocal areas of necrosis were grossly visible (Figure 1I). Microscopic liver abnormalities were found in all animals and ranged from minimal multifocal hepatic degeneration and presence of rare thrombi (4 of 12 animals receiving the low dose of siSerpinc1/siProc, 4 of 6 animals receiving siSerpinc1 alone) to multifocal, extensive areas of severe coagulative and lytic necrosis with thrombi (occlusive) in large and medium-sized vessels (Figure 1J; for 8 of 12 animals receiving low-dose siSerpinc1/siProc, all animals receiving high-dose siSerpinc1/siProc, and 2 of 6 animals receiving siSerpinc1 alone). In head, leg, and liver, thrombi were located in veins, characterized by organized fibrin layering and surrounding tissues devoid of inflammatory cells, indicative of an acute process. None of the animals subjected to microscopic analysis exhibited lesions, thrombi, and/or hemorrhages in kidney or gastrointestinal tract; minor incidences were observed in heart, lung, and brain.

Biochemical analysis of livers of siSerpinc1/siProc-treated animals (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 relationship with the siRNA dose. Liver fibrin deposition was at background levels in siNEG-treated animals (5.4 ± 2.7 ng/mg, n = 11) and siProc-treated animals (5.6 ± 2.7 ng/mg, n = 6). Remarkably, siSerpinc1-treated animals had lower fibrin deposition (10.7 ± 3.1 ng/mg, n = 6). The siSerpinc1-treated animals displayed a later onset of the phenotype (reproduced in additional experiments; at both 3.5 and 7 mg/siSerpinc1/kg, n = 5 per dose) and unaffected plasma fibrinogen (data not shown), suggesting that a combination of siSerpinc1 and siProc results in a more severe phenotype than the sum of siSerpinc1 or 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 siSerpinc1/siProc-treated mice demonstrated increased fibrin deposition (3.5-mg/siRNA/kg dose, 50 ± 38 ng/mg, n = 13 compared with 13 ± 7.3 ng/mg, n = 11 in siNEG; P = .0010), despite a minor incidence of microscopically visible thrombi in this tissue (1 of 12 animals).

The presence of thrombi (occlusive) and fibrin deposition in siSerpinc1/siProc-treated animals indicates that the observed coagulopathy is thrombotic in nature and that hemorrhages are likely secondary to consumption of fibrinogen and/or other coagulation factors. This is illustrated by prolonged PT and aPTT for 6 of 10 siSerpinc1/siProc-treated animals (>70 seconds and >120 seconds, respectively, compared with 11 ± 0.3 seconds and 27 ± 1.6 seconds for siNEG-treated animals; both P = .0039, Fisher test). To demonstrate that thrombin formation underlies this phenotype, siSerpinc1/siProc-treated mice (3.5 mg/siRNA/kg) were administered the thrombin inhibitor dabigatran etexilate (DE). DE prevented the clinical signs found in siSerpinc1/siProc-treated mice, including weight loss (−5.7 ± 5.2% compared with −11.2 ± 3.9%) in vehicle-treated siSerpinc1/siProc animals; n = 16–17, P = .0011), exophthalmos, periocular contusion (0/17 compared with 16/16, P < .0001, Fisher test; Figure 1M), and largely suppressed liver fibrin deposition (Figure 1N).

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

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Authorship

Contribution: H.S., B.J.M.v.V., and P.H.R. designed the experiments; H.S., K.L.C., B.J.M.v.V., E.H.L., G.T.M.W., H.H.V., and
D.S. performed experiments and analyzed data; B.J.M.v.V. wrote the paper; and all authors commented on manuscript drafts.

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References


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