Life-Threatening Thrombosis in Mice With Targeted Arg48-to-Cys Mutation of the Heparin-Binding Domain of Antithrombin

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Abstract—Antithrombin (AT) inhibits thrombin and some other coagulation factors in a reaction that is dramatically accelerated by binding of a pentasaccharide sequence present in heparin/heparan-sulfate to a heparin-binding site on AT. Based on the involvement of R47 in the heparin/AT interaction and the frequent occurrence of R47 mutations in AT deficiency patients, targeted knock-in of the corresponding R48C substitution in AT in mice was performed to generate a murine model of spontaneous thrombosis. The mutation efficiently abolished the effect of heparin-like molecules on coagulation inhibition in vitro and in vivo. Mice homozygous for the mutation (ATm/m mice) developed spontaneous, life-threatening thrombosis, occurring as early as the day of birth. Only 60% of the ATm/m offspring reached weaning age, with further loss at different ages. Thrombotic events in adult homozygotes were most prominent in the heart, liver, and in ocular, placental, and penile vessels. In the neonate, spontaneous death invariably was associated with major thrombosis in the heart. This severe thrombotic phenotype underlines a critical function of the heparin-binding site of antithrombin and its interaction with heparin/heparan-sulfate moieties in health, reproduction, and survival, and represents an in vivo model for comparative analysis of heparin-derived and other antithrombotic molecules. (Circ Res. 2003;93:1120-1126.)

Key Words: coagulation ■ gene targeting ■ knock-in ■ heparin ■ antithrombin deficiency

Antithrombin (AT) is a major physiological coagulation inhibitor, primarily inhibiting thrombin and factor Xa. In the presence of heparin, a potent anticoagulant and antithrombotic agent, the moderate inactivation rate by AT is dramatically accelerated. Heparan-sulfate proteoglycans intercalated in the endothelial cell membrane are thought to have a similar effect in vivo. The acceleration depends on a specific pentasaccharide sequence within the heparin glycosaminoglycan chain, binding of which induces a conformational change in the AT molecule. The interaction with the pentasaccharide appears sufficient to enhance the inhibition of factor Xa. However, full enhancement of thrombin or factor IX inhibition requires heparin species with longer polysaccharide chains. Biochemical and structural studies with wild type and variants of AT have shown that the heparin/pentasaccharide-binding domain of AT involves two clusters of basic amino acids (residues 41 to 49 and 107 to 156).

The importance of AT in maintaining normal hemostasis is emphasized by the increased incidence of thromboembolism in individuals with inherited deficiency. Among these are several mutations affecting the heparin-binding site (type II HBS AT deficiency). Heterozygous type II HBS patients have a low incidence of thrombosis, whereas all homozygous patients display thrombotic disease that may include venous as well as arterial events. To date, 70 reports on 12 distinct HBS mutations have been published, of which affect R47 [substitution to cysteine (19 reports), histidine, or serine; see also16]. Heparin-based pentasaccharides or mimetics, which act, at least in part, by binding to the heparin-binding site in AT, have potent anticoagulant and antithrombotic properties. To develop a murine model of thrombosis suitable for evaluation of such anticoagulants, mutant mice with targeted Arg48-to-Cys substitution (R48C; corresponding to the human “Toyama” R47C mutation) were generated.

Materials and Methods

Generation of the AT Mutant Mice
A targeting vector, pPNT.APn−, was constructed to replace the wild-type exon 2 with a mutated exon 2 encoding the R48C substitution and an additional silent mutation creating a diagnostic SacI site (for details, see the online data supplement and online Figure S1, available at http://www.circresaha.org). Electroporation
of R1 embryonic stem cells, subsequent Cre-mediated excision of the loxP-flanked neo cassette, generation of mice, and genotyping and expression controls were performed as detailed in the online data supplement. All experiments were performed on littermates. Housing and procedures involving experimental animals were approved by the Institutional Animal Care and Research Advisory Committee of the KULeuven, Belgium.

Developmental, Histological, and Immunohistochemical Analysis

Animals and embryos were initially evaluated by visual inspection and stereomicroscopy. Organs of dead or anesthetized saline- and fixative-perfused mice or embryos were processed for immunohistochemical analysis as described.18 Fibrin(ogen) staining was as described.19 Histological staining for collagen (Sirius red) and reticulin (Gordon and Sweets silver staining) were performed using standard procedures.

Coagulation and Hematological Parameters

Whole blood was collected from anesthetized animals by cardiac puncture into 0.1 vol 3.2% trisodium citrate and centrifuged twice for 10 minutes at 3000 rpm to obtain plasma. All blood collections proceeded fast and with instantaneous mixing with the citrate solution. Measurements were performed immediately after sample preparation or after storage at −80°C without intermediate thawing. Antithrombin inhibitory activity was measured using the Coamatic amidolytic anti-Xa heparin cofactor assay (Chromogenix, Sweden). Progressive fXa inhibitory activity was measured analogously but using heparin-free buffer and longer reaction times. Coagulant activities were determined in one-stage clotting assays as described.19 A similar analysis was performed after preconditioning of the mice with lepirudin to temporarily neutralize a possible in vivo activation of the intrinsic coagulation factors (see online data supplement). Plasma fibrinogen was determined by a coagulation rate assay.20 Factor VIII plasma levels were additionally determined using the Coatest Factor VIII kit (Chromogenix, Sweden) and by ELISA.21 Plasma AT antigen levels were determined by rocket immunoelectrophoresis23 using a polyclonal rabbit anti-human AT antibody (Dako) that cross-reacts with murine AT. Blood cell counts were determined as described.18 Prothrombin time (PT) and activated partial thromboplastin time (aPTT) was measured using routine assays. Plasma TAT levels were determined using the Enzygnost TAT kit (Dade Behring). Blood was collected, processed, and assayed for FPA as described.24

Thrombin Generation in Plasma and Venous Thrombosis Model

Platelet poor plasma was pooled from at least 3 mice, defibrinated, and 20 μL-aliquots were used for continuous monitoring of tissue factor-induced thrombin generation25 (see online data supplement), with or without addition of the pentasaccharide fondaparinux (Arrixtra).17,26 the heparin mimetic SanOrg123781A,27 heparin, or hirudin (Sanofi-Synthelabo) as indicated. Thrombus formation in vivo was measured in a thromboplastin-induced venous cava model with or without administration of fondaparinux (see online data supplement).

Statistical Analysis

Data are mean±SD unless otherwise indicated. The statistical significance of differences between groups was determined by unpaired t test, unless mentioned otherwise. A value of P<0.05 was considered significant.

Results

Targeted R48C Mutation of the AT Gene

Mice with an Arg48-to-Cys (R48C) substitution were generated by targeted mutation using homologous recombination in embryonic stem cells (see online Figure S1a). This mutation corresponds to the most prevalent of the type II mutations affecting heparin binding in patients with hereditary AT deficiency, which have an increased risk of thrombosis (Toyama type II HBS AT deficiency).14,28 RNA analysis (semiquantitative RT-PCR on liver mRNA; not shown), and plasma antigen determination showed normal expression of the mutant protein in heterozygous (ATm/m) and homozygous (ATm/m) mutant mice (plasma antigen levels by rocket immunoelectrophoresis were 100±8% in ATm/m and 110±10% in ATm/m mice, n=3 to 4, P=NS; expressed in percent of wild-type control values). DNA sequencing of the entire cDNA prepared from ATm/m liver samples confirmed the integrity and correct mutation of the expressed message (not shown).

Viability and Survival

Among 167 progeny of heterozygous parents, ATm/m offspring were somewhat underrepresented at birth (P0: 16% instead of the expected 25%) and suffered frequent neonatal death, often within 24 hours after birth, with only 60% of them reaching weaning age (Table 1). Spontaneous death of ATm/m neonates was occasionally observed. Beyond weaning age, spontaneous death among wild-type and heterozygous animals was rare, whereas survival of ATm/m mice remained compromised with death at various ages and only about 30% survival beyond 6 months. No underrepresentation of ATm/m offspring was found at embryonal ages up to embryonal day (E) 18.5 (Table 1), suggesting that the early loss of ATm/m pups occurred during or immediately after birth, likely with immediate cannibalism by the mother (occasionally witnessed) and therefore without recovery of these dead pups.

Heparin Binding and Inhibitor Activity of the R48C AT Mutant Protein

ATm/m plasma samples displayed low heparin cofactor activity in the Coamatic fXa inhibition assay (96±4.3% residual fXa activity at 1.5 minutes versus 8.4±2% for wild type; mean±SD, n=6 to 8, P<0.001) but normal progressive fXa inhibitory activity (37±7.8% residual fXa activity at 30

| TABLE 1. Genotype Distribution Among Offspring of Heterozygous (ATm/m×ATm/m) or Mixed Breeding Pairs (ATm/m Male×ATm/m Female) at Different Embryonal (E) or Postnatal (P) Ages |
|-----------------|-----------------|-----------------|-----------------|
| Genotype        | +/+             | +/m             | m/m             | Total            |
| Breeding pairs  | E14.5           | 11              | 15              | 26               |
|                 |                 | 42%             | 58%             |                  |
| Mixed Breeding  | E18.5           | 13              | 26              | 13               |
|                 |                 | 25%             | 50%             | 25%              |
| P20             | 52              | 88              | 27              | 167              |
|                 | 31%             | 52%             | 16%             |                   |
| P21*            | 66              | 120             | 20              | 206              |
|                 | 32%             | 58%             | 10%             |                   |

*Difference from expected Mendelian distribution statistically significant by χ² analysis.
Impaired heparin cofactor activity of the mutant antithrombin in vitro and in vivo. A, In vitro thrombin generation in plasma from wild-type (top) or ATm/m mice (bottom) showing lack of effect on mutant antithrombin of heparin and heparin derivatives [pentasaccharide fondaparinux (penta), heparin mimetic SanOrg123781A] at concentrations up to 8-fold higher than those affecting wild-type antithrombin, whereas the effect of the direct thrombin inhibitor hirudin is not affected. Specific activity of heparin and of the heparin mimetic was 180 anti-IIa units/mg. Data represent mean±SD, n=3; *P<0.05 vs control; #P<0.05 vs same concentration of heparin or of SanOrg123781A. B, In vivo thrombus formation, showing 69% inhibition by pentasaccharide (100 nmol/kg) in wild-type mice, but only 5% inhibition in ATm/m mice. C indicates control; penta, pentasaccharide. Mean value±SD, n=4 to 5; *P<0.05 vs control.

Figure 1. Impaired heparin cofactor activity of the mutant antithrombin in vitro and in vivo. A, In vitro thrombin generation in plasma from wild-type (top) or ATm/m mice (bottom) showing lack of effect on mutant antithrombin of heparin and heparin derivatives [pentasaccharide fondaparinux (penta), heparin mimetic SanOrg123781A] at concentrations up to 8-fold higher than those affecting wild-type antithrombin, whereas the effect of the direct thrombin inhibitor hirudin is not affected. Specific activity of heparin and of the heparin mimetic was 180 anti-IIa units/mg. Data represent mean±SD, n=3; *P<0.05 vs control; #P<0.05 vs same concentration of heparin or of SanOrg123781A. B, In vivo thrombus formation, showing 69% inhibition by pentasaccharide (100 nmol/kg) in wild-type mice, but only 5% inhibition in ATm/m mice. C indicates control; penta, pentasaccharide. Mean value±SD, n=4 to 5; *P<0.05 vs control.

min and minutes versus 42±10% for wild type; mean±SD, n=6 to 8, P=NS). Tissue factor–induced thrombin generation measured in defibrinated plasma from ATm/m animals was comparable to that in wild-type samples (71±10 versus 63±3 mOD for wild type; mean±SD, n=3, P=NS) (Figure 1A). However, the R48C mutation totally prevented the action of AT-mediated inhibitors added at concentrations up to 8-fold higher than those affecting wild-type AT (heparin, pentasaccharide, or the heparin mimetic SanOrg123781A, which comprises both an AT and a thrombin-binding domain27), whereas the effect of direct inhibitors (hirudin) was not affected (Figure 1A).

In vivo, using a stasis-plus-tissue factor-induced thrombosis model in the caval vein, the pentasaccharide inhibited thrombus formation in wild-type mice (69% inhibition), but not in ATm/m mice (only 5% inhibition; P=NS) (Figure 1B). These results confirm effective abolition of heparin interaction by the R48C mutation.

Spontaneous Thrombosis in ATm/m Mice

Homozygosity for the R48C mutation was associated with spontaneous, often massive, thrombosis in the heart and, less frequently, lungs. Out of 16 adult ATm/m mice euthanized for histology at different ages (2 to 17 months), 6 showed massive thrombosis in the atria and/or ventricles, staining positive for fibrinogen/fibrin (Figures 2a and 2b), and often associated with leukocyte infiltration (Figure 2b). Out of 14 adult ATm/m mice, 3 showed fibrin deposition and/or vessel occlusion in the lungs (Figures 2c and 2d). In addition, although obstruction of hepatic blood flow was not directly observed, signs of portal hypertension were seen in the majority of adult ATm/m mice analyzed (12/15), characterized by nodular regenerative hyperplasia (Figures 2e and 2f), dilatation of the sinusoids and formation of shunt vessels (Figures 2g and 2h). Abnormalities at the cellular level included macrovesicular steatosis and the presence of neutrophil clusters in the sinusoids and of phagocytosing macrophages in the parenchyma indicating an inflammatory response (not shown). In 1-day-old ATm/m pups (2 of 4 analyzed), infarcted zones in the liver with coagulative necrosis were observed (Figures 3a through 3c), presumably illustrating acute impaired blood flow in the liver and contributing to the development of the liver pathology. Although liver/body weight ratios were normal in ATm/m animals, they showed enlarged spleens (see online data supplement), which is frequently seen in liver disease. Both males (8/31; 26%) and, more frequently, females (9/19; 47%) displayed severe degeneration of the eyes (Figures 2i and 2j), often with disruption of the retina and occasionally perforation of the cornea (not shown), likely due to ocular vein occlusion (Figure 2k). No obvious thrombosis was observed in other organs nor in the larger vessels (caval vein, femoral artery and vein, brachial vein; not shown). However, in animals used for breeding, severe thrombosis was observed in the placenta of pregnant females, and in the penile veins of sexually active males. Placental thrombosis in ATm/m females (Figures 2l and 2m) occurred irrespective of the genotype of the embryo (Table 1), and likely caused the decreased litter sizes observed for ATm/m mothers (3.9±2.8 pups per litter, n=7, versus 8.6±2.9 for AT+/m mothers, n=44; mean±SD, P<0.005). Fifty percent of all sexually active males developed irreversible priapism (9 of 18 mated males) due to occlusion of the dorsal penile vein and impaired drainage and thrombosis of the corpora cavernosa (not shown).

All ATm/m neonates [6 at postnatal day (P) 1 or P2, one at P7] recovered dead and analyzed histologically, showed
massive thrombosis in the heart (Figures 3d and 3e). Three succumbed heterozygous AT/H11001/m neonates (age P1) also revealed a small clot in the heart and an occluded lung vessel in one of them (not shown). Neonates euthanized immediately after birth (P0) revealed, apart from the liver infarction zones mentioned above, clots in the heart in 2 out of 4 ATm/m pups (not shown), whereas no abnormalities in the wild-type or AT/H11001/m neonates were observed.

Histological analysis of ATm/m offspring at E18.5 revealed no abnormalities (7 embryos analyzed; not shown). However, although rare, thrombosis was observed at earlier embryonal age: in 1 out of 16 E14.5 ATm/m embryos, a thrombus was found in the atrium, and a second embryo showed bleeding and fibrin deposition in the myocardial tissue, whereas all of 18 AT/H11001/m and 4 AT/H11001/+ embryos appeared normal (not shown).

Hemostasis Parameters in Adult Wild-Type and Mutant Mice

Hematological parameters (blood cell counts, hematocrit, platelets; not shown), plasma TAT, and fibrinogen levels (Table 2) in AT/H11001/m and ATm/m blood samples were similar to those in wild-type samples. When measured using one-stage clotting assays, which are sensitive to preactivation of the coagulation factors, intrinsic clotting factors were elevated (Table 2). However, at least for fVIII, this increase was not observed in a preactivation-insensitive two-stage activity assay (Table 2). Similarly, fVIII antigen levels by ELISA were comparable in AT/H11001/m and ATm/m mice (500±100 ng/mL for ATm/m versus 410±230 for AT/H11001/m mice; mean±SD, n=4 to 8, P=NS). These results suggested a low level continuous activation of the coagulation system in ATm/m mice, consistent with the observed thrombotic phenotype. This possibility was further verified by one-stage clotting assay measurements on samples from mice preconditioned with lepirudin to prevent in vivo activation. Levels of intrinsic factors in lepirudin-treated ATm/m mice were reduced to values close, although not entirely comparable, to those of wild-type mice (for details, see online data supplement). A similar increase in intrinsic factor levels is seen in mice with targeted truncation of tissue factor. Presumably, this mutation results in the generation of soluble tissue factor. These mice exhibit severe thrombosis, but have a normal antithrombin molecule (Melis E, Moons L, Arnout J, Collen D, Carmeliet P, Dewerchin M, unpublished data, 2003). Taken together, these data suggest that low level
and activated partial thromboplastin times (aPTT) also were normal in AT\textsuperscript{m/m} mice (PT, 9.8±0.9 versus 9.8±1.9 seconds in AT\textsuperscript{+/+} mice, mean±SD, n=9 to 11, P=NS; aPTT, 28±3.8 versus 32±4 seconds in AT\textsuperscript{+/+} mice, mean±SD, n=6, P=NS), although aPTT values tended to be slightly reduced.

**Discussion**

In this study, knock-in of the R48C mutation in the heparin-binding site (HBS) of murine AT was shown to result in life-threatening, spontaneous thrombosis during neonatal and adult life. The mutant AT was synthesized and present in the plasma at levels comparable to those of wild-type AT in wild-type mice. Similar to the corresponding human R47C mutation,\textsuperscript{29} plasma from the AT\textsuperscript{m/m} mice showed low heparin cofactor activity but normal progressive FXa inhibitory activity. The R48C mutation abolished the action of AT-mediated inhibitors (heparin, heparin mimetic, or AT-binding pentasaccharide) in plasma, whereas the effect of direct thrombin inhibitors was not affected. The less pronounced inhibition by the pentasaccharide as compared with heparin (mimetic) in wild-type plasma (Figure 1A) shows that the effect of the latter molecules on coagulation via AT not only results from inhibition of factor Xa but also from other coagulation enzymes such as thrombin. However, the lack of inhibition of thrombin generation by the pentasaccharide and heparin mimetic in AT\textsuperscript{m/m} plasma, indicates that the R48C mutation effectively abolishes the functional interaction that results in AT-mediated inhibition of factor Xa and/or thrombin by these molecules. The weak dose-dependent response observed with heparin may reflect an AT-independent effect, possibly related to heparin cofactor II activation. These in vitro and in vivo results, together with the spontaneous thrombotic phenotype of the AT\textsuperscript{m/m} mice, underline the importance of heparin binding of AT for efficient inhibition of coagulation and show that normally functioning AT plays a key role with regard to other “natural” inhibitors such as heparin cofactor II in particular.

Thrombosis in the adult AT\textsuperscript{m/m} mice involved the heart, lung, liver, and eyes, and the reproductive organs in sexually active animals. Unlike in adult AT\textsuperscript{+/+} animals, spontaneous death and thrombotic events did occur in AT\textsuperscript{m/m} neonates, although less frequently and less severely than in AT\textsuperscript{+/+} neonates. This might be due to a critical imbalance between pro- and anticoagulation in a fraction of the AT\textsuperscript{+/+} neonates, likely due to the presence of the mutation in one allele on top of presumed lower neonatal plasma AT levels.\textsuperscript{30} Spontaneous thrombosis was more frequent and more severe in homozygous neonates, accounting for the frequent early loss of AT\textsuperscript{m/m} offspring. As in humans, our R48C phenotype was less severe than the embryonic lethal phenotype of AT-null mice.\textsuperscript{31} However, we occasionally observed fibrin deposition in the myocardium in E14.5 AT\textsuperscript{m/m} embryos, reminiscent of the AT-null findings,\textsuperscript{32} although not in older embryos (E18.5). Whether such affected E14.5 AT\textsuperscript{m/m} embryos recover or do not survive remains unclear. Nevertheless, the normal genotype distribution at these embryonal ages, the absence of apparent abnormalities before birth (E18.5), and the obvious thrombotic problems from early neonatal life onwards, sug-
gest that the trauma of birth represents a major trigger of thrombosis in the mutant mice.

Unlike patients with homozygous type II HBS AT deficiency,\footnote{1,2} AT\textsuperscript{HBS} mice displayed no obvious large vessel thrombosis, but showed massive thrombosis in the heart, severe thrombotic eye disease, liver pathology consistent with portal hypertension, placental thrombosis, and priapism. However, ocular vein occlusion, portal vein thrombosis, placental thrombosis, and although rarely, priapism are observed in patients with Factor V Leiden, prothrombin mutation, or decreased protein S, protein C, or AT levels.\footnote{32–36} Thrombosis in the heart has been described in neonates with proved or suspected AT deficiency, in addition to thrombus formation in the large vessels and intracranial venous sinus (see review\footnote{37}).

The AT\textsuperscript{HBS} thrombotic phenotype showed signs of inflammatory response (leukocyte infiltration in thrombi, neutrophil clusters, and phagocytosing macrophages in the liver). This response might contain a direct AT-dependent component, not only through impaired thrombin inhibition and increased PAR-mediated cytokine production, but also by impaired binding to cell surface heparan-sulfate proteoglycans (HSPG). Indeed, AT, via interaction with HSPG present on endothelial cells or on neutrophils, promotes the release of anti-inflammatory prostacyclin and blocks the activation of the proinflammatory NF-\kappaB, thereby decreasing platelet and neutrophil activation, chemotaxis, and interaction with the endothelium,\footnote{38–41} effects that are lost after chemically blocking the heparin-binding domain of AT.\footnote{42,43}

No thrombotic phenotype was observed so far in mice with altered heparan-sulfate (HS) moieties or deficiencies in the HSPG core proteins. 3-O-sulfation of the pentasaccharide core sequence of heparin/heparan-sulfate is essential for interaction with AT.\footnote{44} This modification is thought to be catalyzed mainly by heparan-sulfate-3-O-sulfotransferase-1 (3-OST-1), which consequently plays a major role in the synthesis of AT-binding anticoagulant HS.\footnote{45} However, mice deficient in 3-OST-1 did not display a procoagulant phenotype, perhaps due to redundancy by other 3-OST isoforms.\footnote{46}

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Heterozygous AT knockout mice recently were reported to develop thrombosis only after challenge, largely analogous to heterozygous AT type I deficiency patients in which one AT allele is not expressed giving low functional and immunological AT.\footnote{47} Mice deficient in heparin cofactor II displayed a shorter time to thrombotic occlusion of the carotid artery after endothelial denudation, but otherwise did not show spontaneous thrombosis nor other morphological abnormalities.\footnote{48} In contrast, transgenic mice with inactivation or mutation of plasminogen system components\footnote{49} or of coagulation inhibitors\footnote{33,50,51} display spontaneous thrombotic phenotypes that, however, are either mild, or more severe with early, some-times embryonal, lethality, or characterized by additional nonthrombotic abnormalities.

In conclusion, knock-in of an R48C substitution in the heparin-binding site of antithrombin in mice effectively abolished the effect of heparin or heparin derivatives on coagulation inhibition in vitro and in vivo. Homozygous mutant mice displayed life-threatening thrombosis at different sites, most prominently in the heart, liver, and in ocular, placental, and penile vessels, and represent an in vivo model for spontaneous thrombosis suitable for the analysis of heparin-like and other antithrombotic molecules.

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**References**


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DATA SUPPLEMENT: Expanded Materials and methods and Results

Expanded Materials and methods

Construction of the targeting vector

Genomic fragments comprising the entire murine AT gene were isolated from BAC clones obtained by PCR screening of a mouse 129 BAC library (Research Genetics, Huntsville, AL) using primers annealing in exon 2 or exon 5 of the murine AT gene (exon 2 primers: sense primer 5’-ACGACATCTCGCATAAGCGAAGCCCCCAG-3’; antisense primer 5’-CTGCTTTAGAGTTGTTACAAGGCACC-3’, corresponding to nt 128-154 and 417-390 of the murine AT mRNA, Genbank nr S47225, respectively; exon 5 primers: sense primer 5’-AGGAACCGTTTCATATAAGGTCATGGGC-3’; antisense primer 5’-TTTCAGGGCTGAAGAGTAGCAATGAGG-3’, corresponding to nt 821-847 and 1157-1131 of the murine AT mRNA, respectively). Subclones were further characterized by restriction digest mapping and sequencing, and used for the construction of the mutation targeting vector. This vector, pPNT:ATneo (Figure S1), contained 3.35 kb of 5’ flanking homology comprising 1.6 kb of 5’UT sequence, exon 1 and the major part of intron 1; 5.6 kb of 3’ flanking homology comprising the end of intron 1 (ca 50 bp), exon 2 through exon 3 and the major part of intron 3. Exon 2 comprised in this flank was mutated by PCR to introduce the R48C mutation (corresponding to the R47C mutation in man) and an additional silent mutation creating a diagnostic SacI site, yielding the mutated sequence 5’-
CGGTGCGTCTGGGAGCTCTCC-3’ corresponding to nt 250-270 of the AT mRNA (mutated 47Cys underlined; silent SacI mutation italic with mutated bases in bold). A loxP-flanked neomycin resistance (neo) cassette was added within the homology regions for positive selection, and a thymidine kinase expression cassette outside the homology regions for counterselection against random integration events. Restriction digestion and DNA sequencing confirmed the correct mutation and integrity of the vector.

**Embryonic stem cell transfection and generation of transgenic animals**

R1 embryonic stem cells were cultured and electroporated with the NotI-linearized pPNT.AT\textsuperscript{mco} construct and targeted clones (AT\textsuperscript{+/neo}) were obtained as described previously \textsuperscript{1}. Transient transfection with a Cre recombinase expression plasmid (pOG231; \textsuperscript{2}) to excise the intronic loxP-flanked neo cassette, yielded clones harboring the desired R48C mutation and an intronic loxP site (AT\textsuperscript{+/m}; Figure S1).

Targeted AT\textsuperscript{+/m} ES cell clones were aggregated with Swiss morula-stage embryos as described previously \textsuperscript{1} to generate chimeric and transgenic animals. Heterozygous mutant offspring (AT\textsuperscript{+/m}) among the germline animals were identified by Southern blot or PCR analysis of tail tip genomic DNA, and were intercrossed to generate homozygous AT\textsuperscript{m/m} progeny with overall 50:50 R1 129:Swiss genetic background. DNA was isolated from ES cell clones, from mouse or embryo tail tips or from yolk sac fragments for genotype analysis by Southern blotting or by PCR. RNA expression and integrity was analysed by semi-quantitative RT-PCR and cDNA sequencing.

**Genotype analysis, RT-PCR, and sequence analysis**

**Genotype analysis by Southern blotting:** Correct homologous recombination of AT\textsuperscript{+/neo} clones at the 5’ and 3’ side was confirmed by Southern blot analysis using restriction digests and
external probes giving hybridization patterns discriminating the wild type and targeted allele (Figure S1a.b). The 5’ probe used for hybridisation was a 0.9-kb SpeI-XbaI fragment located immediately upstream of the 5’ homology; the 3’ probe was a 0.5-kb BlnI-NdeI fragment located downstream from the 3’ homology in intron 4. To exclude additional random integration of the targeting vector, a 0.6-kb PstI fragment of the neo gene was used to verify the presence of a single 6.2-kb BstXI fragment of the mutated allele. Correct Cre-mediated excision of the intronic neo cassette in AT<sup>+/m</sup> ES cell clones was confirmed by hybridization of SacI digested genomic DNA with the 5’ probe, detecting an 11-kb band for the wild type allele, a 9.5-kb band for non-Cre-excised AT<sup>neo</sup> targeted allele, and a 7.5-kb band for the correctly neo-excised AT<sup>m</sup> allele (Figure S1a,c).

**PCR-genotyping:** To genotype the AT<sup>m</sup> colony, a combined PCR/SacI digestion protocol discriminating between the wild type allele and the AT<sup>m</sup> allele was used (Figure S1d). Briefly, primers annealing in exon 2 and spanning the mutated region are used in a PCR reaction (Ready-to-go PCR Beads; Amersham Pharmacia Biotech, Roosendaal, The Netherlands) amplifying a 345-bp fragment for both the wild type and mutated allele (sense primer 5’-CTCATCGGTGCTTGGGCTGTC-3’ and antisense primer 5’-CTCCATCAGCTGCTTGGAGTGC-3’ corresponding to nt 82-104 and 426-403 of the murine AT mRNA, respectively). With the mutated but not with the wild type allele, subsequent SacI digestion of this amplification product yields a double band of about 180 and 160 bp due to the presence of the novel diagnostic SacI site.

**RT-PCR and sequence analysis:** Poly-adenylated RNA was extracted from liver using the Quick Prep mRNA purification kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), and was submitted to first-strand cDNA synthesis by oligo(dT) priming using the Ready-to-Go T-primed First strand kit (Amersham Pharmacia Biotech). For expression analysis, the first-strand cDNA was used for PCR amplification with an exon 2 primer set
(primers 5’-ACGACATCTGCATAGCGAAGCCCGAG-3’ and 5’-CTGCTTGAGAG-TGTCGTTACAGGCACCC-3’ corresponding to nt 128-154 and 416-390 of the AT mRNA, respectively). The RT-PCR products were analyzed by electrophoretic separation on a 1% agarose gel and relative intensities were determined by densitometry of computer images of the agarose gel (Stratagene Eagle EyeII Still Video System, Stratagene) using the NIH Image 1.62 program. The signal obtained for the HPRT gene was used as the reference control. For sequence analysis, the first-strand cDNA was amplified by PCR using the primers 5’-CTTGGAGCATCGGCCATGTATTCCCC-3’ and 5’-TTCACAAGGATTAGCCACCTCTCCCC-3’ (corresponding to nt 1-26 and 1409-1383 of the AT mRNA, respectively) and sequenced on both strands using forward and reverse primers evenly distributed over the entire cDNA.

**Preconditioning of mice with lepirudin**

Preconditioning of mice with lepirudin to temporarily neutralize a possible *in vivo* activation of the intrinsic coagulation factors, was done by subcutaneous injection of 200 µg lepirudin (Refludan; Schering AG, Berlin, Germany) in 200 µl saline, twice, respectively 6 and 2 hours prior to blood collection. This lepirudin regimen was confirmed to prevent activation of the coagulation system by a sublethal dose of LPS (E. coli serotype 055:B5, Sigma Chemical Co; i.p. injection of 20 µg LPS/g body weight administered 2 hours after the first lepirudin injection), as monitored by plasma TAT levels (data not shown).

**Thrombin generation in plasma**

Defibrinated platelet poor plasma (PPP) was prepared by incubation of an aliquot of PPP, pooled from at least 3 mice, in the presence of ancrod (50 U/ml) (Sigma, L’isille d’Abeau, France) for 10 minutes at 37°C, further incubation of the clotted plasma for 10 minutes at
0°C, and removal of the fibrin formed. For continuous registration of thrombin generation \textsuperscript{4,5}, 10-μl aliquots of the chromogenic substrate Bz-Ile-Glu-Gly-Arg-pNA (1.6 mmol/L S2222, Biogenic, Montpellier, France), which can be converted by thrombin sufficiently slow and yet sufficiently specific for thrombin, were pipetted in a 96-well microplate. Then 10 μl of 0.2 mmol/L rabbit brain tissue factor (Sigma, L’isle d’Abeau, France) and 100 μl of buffer C (Tris.HCl 0.05 mol/L, NaCl 0.1 mol/L, 100 mmol/L CaCl₂, pH 7.35, 0.05% ovalbumin) were added, followed by 10 μl of buffer A (Tris.HCl 0.05 mol/L, NaCl 0.1 mol/L, pH 7.35, 0.05% ovalbumin) with or without the compounds. The reaction was started at time zero by addition of 20 μl defibrinated plasma. The reagents were prewarmed and the microplate was thermostated at 37°C during the measurement. The optical density at 405 nm was recorded at 15 s intervals using a spectrophotometer. From the curve obtained, Endogenous Thrombin Potential (ETP) was calculated using the method described previously \textsuperscript{4}. The percentage of inhibition was then calculated using ETP with the formula: (ETP control – ETP in presence of compound) / ETP control.

**Thromboplastin-induced thrombosis in a vena cava stasis model in mice**

Mice were anaesthetized by intra-peritoneal injection of 0.1% xylasine and 0.1% ketamine. The abdominal vena cava (AVC) was isolated and a venous ligature was prepared at the renal bifurcation. At T0, the mice received saline solution or the pentasaccharide fondaparinux (100 nmol/kg) via a catheter inserted into the jugular vein in a volume of 5mL/kg, and 1 ng/kg of recombinant human tissue thromboplastin (DADE, Baxter, Germany) was injected at T+5 min via the same catheter into the jugular vein in a volume of 5mL/kg. Immediately after the injection, the AVC was tightened to induce stasis. Stasis was maintained for 15 minutes. The segments were then opened longitudinally and any existing thrombus was removed and weighed.
Expanded Results

Spontaneous thrombosis: signs of liver disease

In relation to the signs of liver disease observed in AT\(^{n/m}\) animals, no difference in liver/body weight ratio was observed between AT\(^{m/m}\) animals and their healthy heterozygous littermates (49 ± 5.2 mg/g in AT\(^{m/m}\) mice, n=5, \textit{versus} 46 ± 4.4 mg/g in AT\(^{r/m}\) mice, n=3; mean ± SD, p=NS). However, AT\(^{m/m}\) mice had enlarged spleens (spleen/body weight in mg/g 4.4 ± 0.78 for AT\(^{m/m}\) mice, n=5, \textit{versus} 2.7 ± 0.36 for AT\(^{r/m}\) mice, n=3; mean ± SD, p=0.023), which is frequently seen in liver disease.

Plasma coagulant levels after lepirudin preconditioning

Results of plasma coagulant level measurements using one-stage clotting assays, two-stage activity assays and ELISA, indicated an increase in the intrinsic factor activities and suggested a low level activation of the coagulation system in AT\(^{m/m}\) mice. To further verify this possibility, the one-stage clotting assays for the intrinsic factors were performed on plasma samples of mice preconditioned with lepirudin as described above to prevent \textit{in vivo} activation.

The values in AT\(^{r/+}\) plasma were comparable before and after lepirudin treatment (46 ± 18\%, 65 ± 18\%, 55 ± 30\%, and 61 ± 22\% \textit{versus} 49 ± 26\%, 74 ± 23\%, 76 ± 31\%, and 80 ± 24\% before lepirudin for fVIII, fIX, fXI and fXII, resp.; mean ± SD, n=6, p=NS), indicating that the lepirudin possibly still present in the collected plasma did not significantly interfere with the assay. In the AT\(^{m/m}\) mice however, the lepirudin-treatment resulted in a decrease, although not entirely to the wild type levels, of up to 2.3-fold as compared to non-treated mice (74 ± 30\%, 85 ± 29\%, 78 ± 50\%, and 97 ± 41\% \textit{versus} 150 ± 46\%, 140 ± 39\%, 180 ± 68\%, and 200 ± 47\% before lepirudin for fVIII, fIX, fXI and fXII, resp.; mean ± SD,
n=3, p<0.05 for the fXII levels). These findings are in accordance with a possible in vivo activation of coagulation. Although all blood collections proceeded fast and without difficulties, we can however not exclude that the increased levels might result from activation during collection itself. However, a similar increase in the intrinsic factor levels was also observed in mice in which targeted truncation of tissue factor presumably resulted in a soluble molecule and which show severe thrombosis, but have a normal antithrombin molecule (Melis et al, unpublished data), further suggesting that low level activation of coagulation may be a feature of thrombotic-prone mice.

References


2. O'Gorman S, Dagenais NA, Qian M, Marchuk Y. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. Proc Natl Acad Sci U S A. 1997;94:14602-14607


![Figure S1](image)

(a) Targeting strategy for the R48C knock-in mutation of the murine *antithrombin (AT)* gene. The targeting vector pPNT.*AT*<sup>neo</sup>, the wild type *AT* allele and the targeted allele before (*AT*<sup>neo</sup>) and after Cre-mediated excision of the *loxP* flanked *neo* cassette (*AT*<sup>m</sup>) are schematically represented. The targeting vector also contains the *thymidine kinase (tk)* gene outside the flanking homologies to allow for negative selection against random integration events. Black boxes in the genomic structures represent exon sequences; the asterisk on exon 2 denotes the R48C mutation; the underlined *SacI* the extra diagnostic restriction site created by silent mutation. The expected restriction fragments are indicated with their relative size by underlining. Black boxes under the genes represent the probes used for Southern blot analysis. The arrowheads under the genes represent the exon 2 primers used for PCR genotyping. (b-d) Confirmation at the DNA level of correct targeting of the *AT* gene. Correct homologous recombination as identified by the additional >12-kb band in Southern blot analysis of BstXI digested genomic DNA with the 3’ probe (b). Correct Cre-mediated excision of the *loxP*-flanked *neo* cassette as confirmed by the appearance of a 7.5-kb recombined instead of the 9.5-kb targeted fragment in Southern blot analysis of *SacI* digested DNA using the 5’ probe (c). Genotyping of genomic DNA from tail biopsies using a combined PCR/SacI-digestion protocol yielding a 345-bp amplicon for the wild type allele (panel d,
lanes 2 and 3) and a double amplicon of about 180/160-bp for the mutant $AT^m$
allele (panel d, lanes 3 and 4). Lane 1 represents the result of a control (C) PCR
without template.