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Introduction to Antithrombin

Antithrombin (AT) is a plasma proteinase inhibitor that inactivates not only thrombin, but also factor Xa and coagulation enzymes (fVIIa-TF, fXIIa, fXIa, fIXa) that collectively mediate the generation of thrombin. Inherited and acquired antithrombin deficiencies cause thrombosis, and the homozygous deletion of AT is lethal. Rates of AT target enzyme inhibition and the associated anticoagulant activities can be accelerated as much 500- to 39,000-fold by cofactor HSPGs (heparin sulfate proteoglycans) and heparins, which are respectively present naturally in the vessel wall, and clinically via the administration of pharmaceutical heparins. The broad coagulation enzyme target specificity of antithrombin and its potentiation by HSPG/heparin enable AT to play a key role in maintaining circulatory system patency and providing protection against thrombosis.

The first section of this chapter reviews current understanding of the mechanisms underlying AT inhibition of coagulation enzymes, heparin/HSPG cofactor activation of AT, and inflamma-

tory inactivation of antithrombin. Then, building on this foundation, AT dysregulation in the context of trauma, tissue injury, blood loss, and resuscitation is addressed. Finally, the outcomes of several AT supplementation studies in trauma patients are reviewed.

AT is a Serpin and Uses the Serpin Proteinase Inhibitor Mechanism to Inactivate Its Coagulation Enzyme Targets

AT is a serpin (*serine proteinase inhibitor*) and exhibits sequence, structural, and functional homology to other members of this large gene family [1–3]. AT uses the canonical serpin suicide-substrate inhibition mechanism illustrated on the left side of Fig. 2.1 to form stable, covalent inhibitory complexes with its coagulation enzyme targets. Native AT (Fig. 2.1a) is a globular molecule with a protruding reactive center loop (RCL) (*purple*) containing a substrate-like sequence that serves as “bait” for its target enzymes. Inhibitory complex formation begins when a target enzyme cleaves the scissile P1–P1' bond (nomenclature of Schechter and Berger [4]) of the RCL to generate an acyl–enzyme complex in which the P1 residue (*purple sphere*) is covalently linked to the target enzyme active site serine, and in which the polypeptides generated by reactive loop cleavage become mobile and are rearranged [5, 6].

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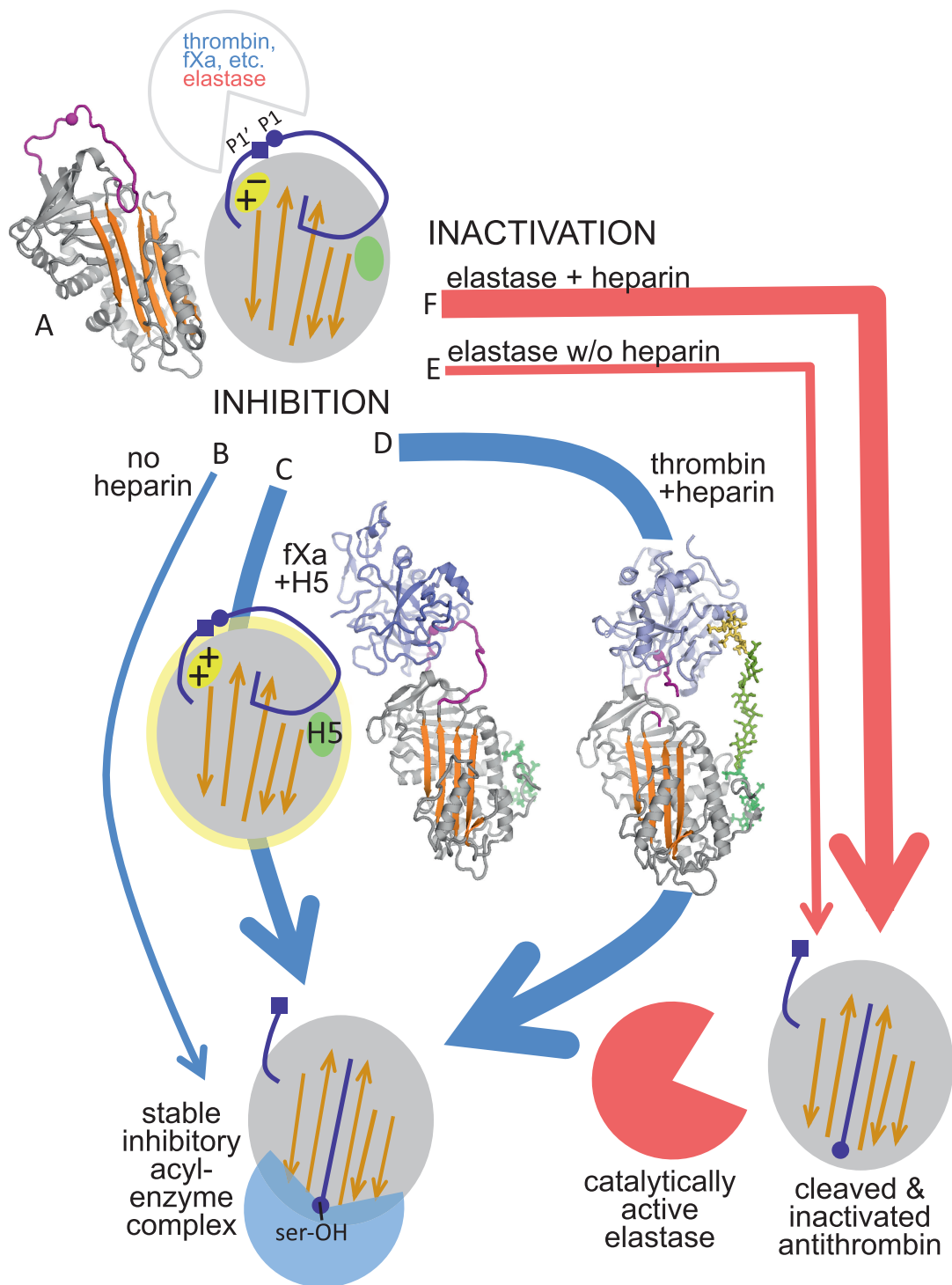


Fig.2.1 Structural basis of AT inhibition and inactivation mechanisms (A) *Native AT*. Left shows the 2.6 Å 1E04i structure of the native conformation human antithrombin which circulates in blood. Cartoon on the right illustrates functionally important structural features. Reactive center loop (purple) is bound by the active sites of target enzymes which cleave the RCL at its P1 residue (purple sphere). AT central β -sheet A is orange. Note that in contrast to

other serpins, the N terminus of the AT RCL is partially incorporated into β -sheet A of the native conformation. The yellow and green patches mark locations of AT fXa exosite and heparin pentasaccharide binding sites, respectively. (B) *Cofactor-independent, progressive AT inhibition of its target enzymes*. In the absence of heparin and HSPG cofactors, AT inhibits its targets via the formation of stable, covalent AT-target enzyme complexes like the

As illustrated in the cartoon representation of the stable inhibitor acyl-enzyme complex on the bottom left of Fig. 2.1, the P1-containing polypeptide incorporates into central β -sheet A (orange) of the inhibitor, translocating the covalently bound target enzyme approximately 70 Å to the opposite pole of the serpin, and initiating critical protein conformational changes in both molecules. The translocated target enzyme molecule is crushed against the hyperstable serpin molecule in a way that distorts its catalytic triad geometry and prevents deacylation and regeneration of active enzyme.

Although AT uses the canonical serpin inhibition mechanism, it is distinguished from other

serpins in several notable ways. A first unusual characteristic at the structural level is the partial insertion of the AT native conformation reactive center loop as a sixth strand at end of the central A β -sheet that is nearest to the RCL (see Fig. 2.1a; most other serpins have entirely external RCLs and the A sheet is 5-stranded at the reactive loop end). Additional distinguishing features of AT relate to functional characteristics: its native conformation inhibits target enzymes at rates that are at least 1000-fold slower than typical serpin inhibition rates, and AT requires cofactor heparin or HSPG (heparan sulfate proteoglycan) binding to increase its target enzyme inhibition rates into the range that is typical for other serpins.

Fig. 2.1 (continued) one depicted in the cartoon at the arrow head end of pathway B. Inhibitory complexes form upon target enzyme cleavage of P1–P1' peptide bond of native AT (A), which initiates a large protein conformation change wherein the N-terminal polypeptide of the RCL rapidly inserts into AT central β -sheet A as a sixth strand. This translocates the acylated target enzyme to “south pole” of AT, and produces inhibitor and target proteinase conformational changes which distort the enzyme’s catalytic triad and prevent deacylation of the covalent linkage between the AT P1 residue and the target enzyme active site serine. Heparin cofactor-independent, progressive rates for AT inhibition of its clotting factor target enzymes are modest compared to rates obtained in the presence of heparin/HSPG cofactors. These differences are symbolized by the widths of blue arrows in pathways B, C, and D, and illustrated quantitatively in Fig. 2.2b. (C) *Conformational activation of fXa inhibition by heparin pentasaccharide.* A specific heparin pentasaccharide (H5) component of endogenous HSPGs and pharmaceutical heparins accelerates the rate of AT inhibition of fXa by ~300-fold using the mechanism illustrated in pathway C. In the native conformation of AT (A), a fXa recognition exosite (yellow) underlying the RCL contains elements mediating favorable and unfavorable (+ –) interactions with fXa. Binding of H5 pentasaccharide to the green heparin binding site induces AT conformational changes that are transmitted across the serpin, and convert the fXa exosite (yellow) to a form (+ +) that is highly favorable for interaction with fXa. The 3.3 Å 2GD4 structure to the right of the C pathway H5-activated AT cartoon image shows the enzyme active site of fXa (blue) interacting with the P1 residue (purple sphere) of the AT RCL loop, and recognition of the H5-exposed fXa exosite on AT by the enzyme. P1–P1' cleavage, acylation of fXa, and translocation to form a stable inhibitory complex then proceed as described

for progressive AT inhibition in pathway B. In summary, heparin pentasaccharide activates AT for inhibition of fXa by inducing a conformational change that leads to favorable interactions between the AT fXa exosite and factor Xa. (D) *Heparin approximation-mediated acceleration of AT thrombin inhibition.* H5 pentasaccharide binding to AT increases its rate of thrombin inhibition by less than two-fold. In contrast to the conformational activation mechanism described in pathway C for fXa, heparin activation of thrombin inhibition requires pentasaccharide-containing long chain heparins, which accelerate the AT thrombin inhibition rates by ~6000-fold via the bridging mechanism illustrated in pathway D. The 2.5 Å 1TB6 AT-thrombin-heparin ternary complex is shown. One end of the long heparin molecule (light green) binds to the pentasaccharide binding site of antithrombin, while its other end (yellow) binds to a heparin-binding exosite on thrombin (blue). Thus, cofactor bridging of AT and thrombin promotes interaction of thrombin’s active site and the AT RCL, cleavage of the P1–P1' bond, initiation of the serpin inhibitory conformational change and formation of a stable inhibitory complex as previously described. (E, F) *Neutrophil elastase-mediated inflammatory inactivation of AT in the absence and presence of heparin.* Neutrophil elastase cleaves at the P5–P4 peptide bond of the AT RCL with out forming a stable E–I complex. Instead, AT is converted to a thermodynamically stable 6-stranded cleaved conformation that is devoid of inhibitory activity because the RCL has been cleaved. Relative arrow widths of the E and F pathways indicate that elastase inactivation of antithrombin occurs more rapidly in the presence of heparin. Neutrophil elastase has a heparin binding site which bridges elastase and AT according to an approximation mechanism that is similar to the one illustrated in pathway D for long chain heparin activation of AT thrombin inhibition.

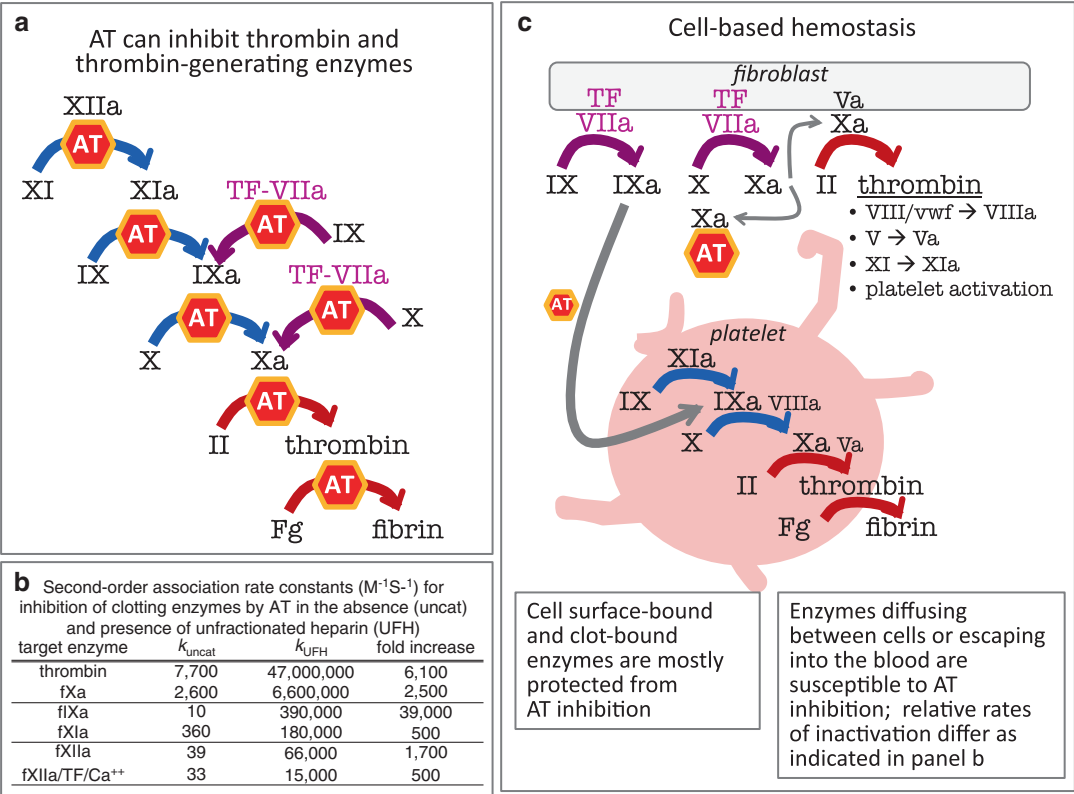


Fig. 2.2 AT inhibition of clotting enzymes in vitro and in a cell-based model of hemostasis (a) In vitro inhibition of coagulation enzymes by AT. Studies using plasma-based coagulation assays and purified proteins have established the in vitro inhibition of thrombin—and the extrinsic, intrinsic, and common pathway coagulation enzymes that generate thrombin—by AT. (b) Uncatalyzed and heparin cofactor-dependent rates for AT inhibition of thrombin, factors Xa, IXa, XIa, XIIa, and TF-VIIa. Note inactivation rates for different enzymes vary over ~3 orders of magnitude, both in absence and in the presence of heparin, and that there are also large differences in the magnitude of acceleration by heparin. Data from Olson et al. [19]. (c) AT in the regulation of cell-based hemostasis. The drawing highlights features of the Hoffman cell-

based model of hemostasis exhibiting various degrees of sensitivity to AT inhibition. *Left side*, Factor IXa generated on TF-bearing cells during the initiation phase is inefficiently inhibited by AT during diffusion to platelets and accordingly available to contribute to the formation of additional tenase (fIXa-VIIIa) complexes on the platelet surface. *Center*, In contrast, AT (and TFPI) effectively inactivate fXa produced by and released from fibroblasts. *Right side*, Factor Xa remaining on fibroblasts associates with Va to form prothrombinase (fXa-Va). The prothrombinase generates thrombin for the amplification phase, wherein platelets are activated and tenase and prothrombinase complexes assemble on their surfaces. Robust generation of thrombin and fibrin follows during the propagation phase

AT Inhibits a Broad Spectrum of Coagulation Enzymes

The name “antithrombin” emphasizes that AT inhibits thrombin but is somewhat misleading in implying that AT is primarily an inhibitor of thrombin. In fact, AT has broad inhibitory activity against a broad range of coagulation enzymes

including thrombin, fXa, TF-VIIa, fIXa, fXIa, and fXIIa (see Fig. 2.2a). Therefore AT regulation of hemostasis may occur at several levels, including thrombin-mediated fibrin clot formation, common pathway fXa-mediated thrombin generation, and effects on coagulation factors that are higher up in the intrinsic and extrinsic pathways. AT inhibition of thrombin may also

serve to regulate thrombin's non-coagulant functions, including platelet activation and vascular cell signaling and proliferation.

AT Inhibition of Its Target Enzymes is Accelerated by HSPGs and Heparin

As discussed earlier, the native conformation of AT is a considerably less efficient proteinase inhibitor than are most other members of serpin family. Rate constants for target proteinase inhibition by most serpins are in the 10^6 – 10^7 M⁻¹ s⁻¹ range. However, AT inhibits its target enzymes at rates that are about three orders of magnitude slower than typical serpins. Unlike other serpins, which have fully exposed reactive center loops, native ATIII circulates with the amino-terminal end of its reactive center loop partially inserted into its central A β -sheet [7] (see Fig. 2.1a). RCL insertion was originally thought to reduce AT inhibition rates by limiting target enzyme access to the arginine-393 P1–P1' scissile bond [8]. However, it is now appreciated that the slow, cofactor-independent, "progressive" inhibition rates of native antithrombin in the absence of heparin (pathway **B** in Fig. 2.1) reflect the unfavorable presentation of the fXa-binding exosite (yellow in Fig. 2.1a). In native AT, the fXa exosite is comprised of elements that engage positively and negatively with fXa, (+ –) and therefore the rate for inhibition of fXa is low [9, 10].

Conformational Activation of fXa Inhibition by Heparin

Pathway **C** of Fig. 2.1 illustrates the mechanism for heparin activation of AT fXa inhibition. The native conformation of AT (**A**) bears a fXa recognition exosite (yellow) which is located under the RCL. This native conformation exosite contains elements mediating favorable and unfavorable (+ –) interactions with fXa. Binding of heparin pentasaccharide (H5) to the AT heparin binding site (green) [11] induces long range allosteric conformational changes that are transmitted across the serpin [12, 13] and expose favorable exosite features (+ +) which promote interaction with fXa by minimizing the repulsive interactions which

were present in the native conformation exosite [10, 14–16]. Therefore, heparin pentasaccharide activates AT inhibition of fXa by inducing a conformational change that leads to favorable interactions between the AT fXa exosite and factor Xa, and accelerates the rate of inhibition by ~300 fold.

Heparin Approximation-Mediated Acceleration of AT Thrombin Inhibition

Pathway **D** of Fig. 2.1 illustrates the bridging mechanism responsible for heparin activation of AT thrombin inhibition. H5 pentasaccharide binding to AT increases its rate of thrombin inhibition insignificantly (<twofold). In contrast to the conformational activation mechanism described in pathway **C** for fXa, heparin activation of thrombin inhibition requires pentasaccharide-containing heparins of greater than 18 sugar units in length, which accelerate the rate of inhibitory complex formation by several thousand fold using an approximation mechanism wherein different regions of the same extended heparin chain bind to the pentasaccharide binding site on ATIII and to anion binding exosite II on thrombin [17, 18] as is illustrated in pathway **D** of Fig. 2.1, using the 2.5 Å 1TB6 structure of the AT-thrombin-heparin ternary complex. The bottom end of the long heparin molecule (light green) binds to the pentasaccharide binding site of antithrombin, while its top end (yellow) binds to anion-binding exosite II on thrombin (blue).

Progressive and Heparin Cofactor Activated Inhibition of Coagulation Enzymes by AT

Figure 2.2b presents a comparison of the rates at which AT inhibits six different coagulation enzymes under cofactor-independent, progressive (uncat) conditions, and heparin cofactor-activated conditions (UFH) [19]. As previously noted, AT uncatalyzed progressive inhibition rates for all of its coagulation enzyme targets are slow in comparison to those measured for other members of the serpin inhibitor family. However, the AT inhibition rates are significantly accelerated by heparin cofactor binding, and approach

the theoretical limits for serpins in the cases of thrombin and fXa.

AT Activation by Endogenous HSPGs

Heparin pentasaccharide-bearing HSPGs are the naturally occurring endogenous cofactor for AT activation. Small amounts of AT-binding HSPGs are present in the glycocalyx on the luminal side of the endothelium. On the abluminal side, the subendothelial basement membrane and extracellular matrix contain much greater concentrations of these cofactor molecules [20]. The observed asymmetrical distribution suggests that AT molecules bound and activated on luminal HSPGs may provide a basal level of scavenger inhibition activity against activated coagulation enzymes exposed to the endothelium, and that the magnitude of anticoagulant activity will be dramatically increased in the proximity of injured regions where endothelial damage liberates larger underlying pool of abluminal HSPGs and their associated bound and activated ATs.

The generation of increased anticoagulant activity via HSPG release and AT activation at sites of endothelium injury seems initially surprising in view of the need for hemostasis in this context. However, this mechanism for release of anticoagulant activity into the fluid phase of the blood may contribute to keeping injured vessels patent and capable of sustained systemic circulation, while platelet-based hemostatic mechanisms proceed in the vessel wall with little anticoagulant interference from AT as discussed in the section on cell-based hemostasis and Fig. 2.2, panel c. The physiologic importance of AT interactions with endogenous HSPGs is supported by the occurrence of lethal thrombosis in mice homozygous for an AT mutation that reduces binding to heparin/HSPG [21].

AT Role in Cell-Based Hemostasis

It is now recognized that in vivo hemostasis is more complex than the simple convergence of extrinsic and intrinsic enzymatic cascades into the common pathway (Fig. 2.2a). In a living organism, the individual enzymatic steps take

place on distinct cell surfaces, which allows for regulation of hemostasis via spatial compartmentalization. Hoffman et al. have proposed a cell-based model of in vivo hemostasis that is organized into three phases: an early *initiation* phase that takes place on the surface of tissue factor (TF) bearing cells, an *amplification* phase that takes place on unactivated platelet surfaces, and a final *propagation* phase that takes place on the surface of activated platelets (for further details please refer to Chap. 1). The essentials of cell-based hemostasis [22–24] are reviewed below with special focus on AT contributions to regulation of the process.

As depicted at the top of Fig. 2.2c, injury exposes circulating fVII to tissue factor (TF) on fibroblasts, which results in bound and activated TF-VIIa that converts fX to fXa (central position on fibroblast). The activity of the generated fXa is restricted to the surface of the TF-bearing fibroblast; fXa molecules that dissociate and diffuse into the blood are rapidly inhibited by AT. As shown on the right side of the fibroblast, fXa molecules remaining on the fibroblast surface combine with fVa to produce prothrombinase and small amounts of enzymatically active thrombin, which plays several important roles during the ensuing amplification phase. Before proceeding to discuss amplification, it is additionally noted that fibroblast surface TF-VIIa also converts fIX to fIXa (left side of fibroblast), and that this source of fIXa will provide an important boost to thrombin and fibrin generation during the propagation phase.

During the amplification phase of cell-based hemostasis, platelets and factors V, VIII, and XI are activated by a small number of thrombin molecules, which were produced on TF-bearing fibroblasts and were able to escape AT inhibition before encountering their substrates. Active tenase (fIXa/VIIIa) and prothrombinase (fXa/Va) complexes assemble on the activated platelets (Fig. 2.2c, center).

Upon assembly of the tenase and prothrombinase complexes, activated platelets bound to the exposed fibroblasts and collagen are now primed for the propagation phase of hemostasis, during which efficient and robust generation of throm-

bin occurs and catalyzes the formation of a fibrin network that physically stabilizes the clot. Factor Xa generated by the fIXa-VIIIa tenase on the activated platelet is protected from inhibitors, and can move directly into prothrombinase complexes with fVa. Prothrombinase production on the surface of activated platelets is further boosted by additional tenase complexes formed from fIXa produced by fibroblast TF-VIIIa [25]. Due to relatively inefficient AT inhibition of fIXa (Fig. 2.2b), active fIXa molecules generated during the initiation phase are able to escape inhibition during diffusion from the fibroblast to the platelet. The additional fIXa assembles into “extra” tenase complexes on the activated platelets, boosts downstream prothrombinase production, and contributes to robust thrombin and fibrin network generation and to growth as well as stabilization of the clot.

Propagation continues in the proximity of the clot whilst thrombin and procoagulant factor concentrations remain sufficient to activate additional platelets and to support tenase and prothrombinase assembly and thrombin and fibrin generation at sufficient levels.

Thrombin and fIXa and fXa molecules not trapped within the clot or binding to platelet surfaces may escape into the blood circulation where they will be inhibited by plasma AT at progressive (heparin-independent) rates. Progressive inhibition by plasma AT ($\sim 2.4 \mu\text{M}$ ($140 \mu\text{g/mL}$) [26]) is usually sufficient to prevent the non-productive, systemic activation and consumption of coagulation factors and thrombosis. However, as discussed below, the AT and other endogenous anticoagulant systems may be overwhelmed in the contexts of severe trauma or inflammation.

Inflammatory Inactivation of AT

As discussed previously and illustrated on the *left* side of Fig. 2.1, the serpin inhibition mechanism used by AT begins with target enzyme recognition and cleavage of the P1–P1' linkage in the exposed reactive center loop. However, instead of being released from the enzyme upon cleavage of the RCL polypeptide, large protein conforma-

tional changes in both the inhibitor and its target proteinase occur, and lead to formation of a stable covalent AT-target proteinase inhibitor complex. Accordingly, an *intact reactive center loop* is absolutely necessary for the serpin protease inhibitor mechanism to work, and non-target proteinase cleavages of the RCL, which cause the conformational change to occur prematurely, destroy the protease inhibitor functionality of serpin [27].

In the contexts of injury and inflammation, neutrophil elastase is released into the tissues systemically and cleaves the P5–P4 peptide bond of the AT RCL [28, 29]. For further details on neutrophils and inflammation in trauma, please refer to Chap. 10. Elastase cleavage converts AT to the inactivated conformation illustrated at the bottom of pathways **E** and **F** in Fig. 2.1. The inactivation arrow of pathway **F** is larger than that for pathway **E** because heparin accelerates the inactivation of AT using a heparin bridging mechanism. Neutrophil elastase has a heparin binding site, therefore as in the case of thrombin (pathway **D**), heparin approximation increases the enzyme and inhibitor association rate, but in this case results in AT cleavage and inactivation, instead of inhibition [30].

AT and Thrombosis

AT inhibits thrombin and the enzymes that generate thrombin. The complete absence of antithrombin is lethal [31], while partial reductions of AT permit over-activity of the coagulation system, and the increase the risk of thrombosis. Heterozygous AT deficiencies in mice [32] and in humans [33, 34] increase risk for renal and venous thrombosis, respectively. For further description of the clinical presentation, diagnosis and management of AT deficiencies, please refer to Chap. 27.

Acquired AT reductions may also occur as a consequence of trauma, sepsis, burns, malignancies, extracorporeal circulation, and surgery. In these contexts, which are frequently accompanied by inflammation, AT deficiency is associated with consumptive coagulopathies.

Functional AT activity declines because of stoichiometric consumption during neutralization of activated coagulation enzymes and inflammatory cleavage and inactivation of AT. The remainder of this chapter focuses on AT dysregulation in the settings of trauma and trauma induced coagulopathy.

Dysregulation of Antithrombin in Trauma

Trauma disrupts the physiological equilibrium between endogenous procoagulant and anticoagulant systems, including antithrombin. AT reduction in trauma is due to its stoichiometric consumption during the inhibition of activated clotting enzymes, its cleavage and inactivation by neutrophil elastase, and auto-heparinization secondary to endothelial glycocalyx shedding. For further description of endothelial glycocalyx shedding seen in trauma, please refer to Chap. 7. The immediate post-trauma drop in plasma AT activity may be followed by a gradual restoration, which is associated with better outcomes, or by persisting low levels of AT, which are associated with poor prognosis.

Reduction of Plasma AT Activity During Trauma

The first studies of trauma induced acquired AT deficiencies were conducted in the 1980s and early 1990s and used chromogenic substrate assays to measure plasma antithrombin activity [35–38]. These studies noted greater reductions in plasma AT activity (into the range of 50–60 % of normal human plasma values) for more severe cases of trauma. The gradual restoration of AT activity was also observed to associate with better outcomes and survival. In contrast, persisting low AT was associated with unfavorable outcomes, including acute respiratory distress syndrome (ARDS), sepsis and death, [35, 36] as well as limb loss after reconstructive vascular surgery due to thrombosis [38].

Kinetics of Plasma AT Activity Loss and Recovery Following Trauma

A variety of different blood sampling schedules have been used for studies of AT in traumatic injury. The first sample may be obtained at the accident scene, or not collected until up to 12 h after hospital admission. During the first 24 h of hospitalization, blood may be drawn multiple times, or only once, and after that, at daily or several-day intervals during follow up periods ranging from 1 to 3 weeks. In addition to sampling-time protocol differences, critical care interventions, including resuscitation, hemodilution, and heparin administration, may also affect measurements of plasma AT activity. Nevertheless, despite the above caveats, a general overall pattern of AT depletion and recovery emerges.

A study of 30 patients in which blood samples were drawn before primary resuscitation at the scene of the accident [39] showed that plasma AT activity begins to drop immediately upon injury. In patients with minor injuries [injury severity score (ISS) 9–17], AT activity did not fall below 70 % for the first sample, however, for severe (ISS >18) and very severe (ISS >32) injuries, the reduction in AT activity was more pronounced, and fell into the 60 % or lower range.

At 6 h after hospital admission, AT activity increased in all groups and reached into the normal range (80–120 % of NHP) for patients with ISS <32. AT activity for the severely injured group remained in the 50–70 % range. Other studies with extensive serial sampling of trauma patients are in general agreement with the pattern described above, [35, 40–43] although the inflection point when plasma AT activity begins to increase again may occur on day 1 or day 2.

Temporal Correlation of Plasma Thrombin-Antithrombin and Thrombelastography (TEG) Hypercoagulability with Plasma AT Activity Reductions are Evidence for Trauma Induced Consumption of AT by Activated Coagulation Factors

Theoretically, the reduction of plasma AT inhibitory activity that is observed early after traumatic

injury could be due to its stoichiometric consumption as inhibitory complexes with activated coagulation enzymes, or to cleavage and inactivation of AT by neutrophil elastase, or to both (see Fig. 2.1). Three studies have reported serial measurements of thrombin-antithrombin (T-AT) complexes in plasma from trauma patients [40, 44, 45]. All found T-AT complex levels to be markedly elevated on day 1, corresponding to the period when plasma AT activity is most reduced and there has been recent massive tissue factor liberation by the trauma. T-AT levels then decreased on days 2–4, during the period when plasma AT activity rebounds. One of the studies also measured hypercoagulability by TEG and determined that the prevalence of a hypercoagulable state was 62 % on day 1 and then decreased to 26 % on day 4 [45].

In summary, T-AT and hypercoagulability data provide direct evidence that AT participates in the regulation of activated clotting factors released after trauma, and that consumptive coagulation plays a role in the trauma induced depletion of plasma AT activity.

Plasma AT Activity as a Predictor of Deep Vein Thrombosis (DVT) and Disseminated Intravascular Coagulation (DIC) in Trauma Patients

A large study on the effect of critical injury on plasma antithrombin activity enrolled 157 trauma patients, of which 77 % had blunt and 23 % penetrating injuries [41]. The mean ISS was 23 (SD \pm 11, range 4–66). Blood samples were drawn upon emergency room arrival, at hours 8, 16, 24, and 48, and on days 3, 4, 5, and 6. Plasma antithrombin activity was measured by chromogenic anti-fXa assay.

Low AT levels were predictive of DVT (diagnosed by ultrasound based on clinical suspicion) and DIC thromboembolic complications by logistic regression analysis. Both nadir and average plasma AT activity predicted DVT (diagnosed using color flow duplex ultrasound) with p values of 0.030 and 0.042, respectively. The nadir levels usually occurred within the first 24 h after admission. Logistic regression analysis also demonstrated that nadir, maximum and average

AT levels were also predictive of DIC (all $p < 0.0001$). AT was lower in the DIC group overall and at each time point by repeated measures ANOVA.

Inflammatory Inactivation of Antithrombin

As discussed in introductory Section “Inflammatory Inactivation of AT” on mechanisms of AT inhibition and inactivation, in vitro cleavage of the AT reactive center loop by neutrophil elastase converts the serpin to a stable inactive conformation (Fig. 2.1e) that is devoid of protease inhibitor and anticoagulant function [28, 29]. Moreover, elastase inactivation of AT is accelerated by the AT cofactor heparin (Fig. 2.1f), to which neutrophil elastase can also bind with high affinity [30].

Collectively the above in vitro solution phase experimental results suggest that in vivo, inflammatory responses in tissues where injury has exposed blood to HSPGs may rapidly switch off AT anticoagulant function, and promote intravascular coagulation and thrombus formation. Accordingly, AT activity reductions that are typically attributed to its massive stoichiometric consumption during coagulation may—at least partially—result from neutrophil elastase cleavage and inactivation of the inhibitor, which occurs independently of inhibitory complex formation type consumption with target enzymes. Several studies with serial measurements of plasma elastase and AT levels in trauma patients with DIC or organ failure support this concept.

A study examined elastase and AT with respect to DIC [46]; plasma elastase was assayed in complexes with its inhibitor α 1-antitrypsin by ELISA, and plasma antithrombin activity determined using standard methods. At the first blood sampling (within 12 h after admission, day 1), but not on days 3 or 5, significant increases in plasma elastase and significant decreases in plasma antithrombin activity were observed for DIC patients in comparison with non-DIC patients. These data indicate that higher levels of elastase are present in patients who have lower antithrombin levels

and develop DIC, and are supportive of an elastase contribution to reduced AT levels in some trauma patients.

Also of relevance to the *in vivo* question of elastase inactivation of AT is a study of multiple organ failure (MOF) in severe trauma patients. This study [43, 47] divided patients into MOF non-survivors, MOF survivors, and survivors without MOF groups. Plasma elastase and antithrombin levels were determined over a 2-week course. Plasma elastase was significantly higher ($p < .01$) for MOF patients (survivors and non-survivors) vs patients without MOF over the entire observation period. Beginning on day 3, non-surviving MOF patients had significantly higher plasma elastase than did surviving MOF patients ($p < .05$). Plasma antithrombin levels were significantly lower for non-surviving and surviving MOF patients vs. patients without MOF ($p < .01$) through out the study, and the values for non-surviving MOF patients were significantly decreased compared to survivor MOF patients ($p < .05$) after the first week. Thus, there was a significant (and temporally logical) inverse relationship between antithrombin activity and elastase levels, and antithrombin levels also demonstrated a negative relationship with the severity of organ failure. A later study from the same group reported similar results [48].

In summary, the above clinical studies suggest that inflammatory, elastase-mediated cleavage and inactivation of AT contributes to the pathogenesis of coagulopathies in trauma patients with DIC and organ failure.

Glycocalyx Shedding and Auto-heparinization

As illustrated in the introductory section, heparins dramatically increase rates of AT target enzyme inhibition (Fig. 2.1b, c, d and Fig. 2.2b), and also the rate of its cleavage and inactivation by neutrophil elastase (Fig. 2.1e, f). Therefore, the cofactor interaction with heparins can cause anticoagulant—as well as procoagulant—effects.

In the body endogenous heparin cofactor activity is provided by heparan sulfate glycos-

aminoglycan molecules. Some of them are attached to syndecan-1 proteins and form the HSPGs (heparan sulfate proteoglycans) of the endothelial glycocalyx [49]. For further description of the endothelial glycocalyx, please refer to Chap. 7. The glycocalyx is shed into the circulation during hemorrhagic shock [50], and ensuing “auto-heparinization” has been proposed to exert an anticoagulating effect by way of heparan sulfate potentiation of AT clotting factor inhibition [51]. In a study of 77 trauma patients, differences in kaolin-TEG vs heparinase-TEG were used to identify the subset ($n=4$, 5 %) with the highest degree of endogenous heparinization. These patients had fourfold increased syndecan-1 levels, higher ISS, INR, thrombomodulin, and IL-6, and lower protein C. Thus, they had profound endothelial damage, coagulopathy, and inflammation. A trend towards plasma AT activity deficiency, which did not reach statistical significance ($p=0.055$), was also observed [51].

The AT that was present may have been activated by auto-heparinization to be more efficient in procoagulant enzyme neutralization. Alternatively, given the inflammatory status of these patients, auto-heparinization may have contributed to reduction of the potential anticoagulant effect of AT by increasing the rate at which it is cleaved and inactivated by neutrophil elastase [30]. In either case, it is reasonable to believe that the effects of auto-heparinization on AT inhibition and/or inactivation contribute to the overall coagulopathic state in these trauma patients.

AT Supplementation in Trauma

Early Trials Focusing on DIC and Organ Failure Prevention in Trauma Patients

As discussed in Section “Reduction of Plasma AT Activity During Trauma”, multiple studies have demonstrated that plasma AT activity begins to decrease immediately after trauma. Patients who have less severe reductions—and whose plasma AT activity deficits improves during the first week—experience better outcomes and survival than do patients who develop severe AT activity

deficiencies that do not resolve, and predict non-survival.

Although it has been recognized that antithrombin is just one of the several endogenous anticoagulants that becomes dysregulated in trauma and critically ill patients, early trials of antithrombin supplementation [52–54] were conducted to explore whether correction of AT deficiency might nonetheless reduce mortality. The straightforward premise of these early trials was that normalization of AT activity by concentrate administration would be helpful for reducing post-traumatic intravascular coagulation and the subsequent development of DIC and organ failure. Furthermore, additional favorable advantages might accrue from AT interruption of thrombin non-coagulant functions that contribute to trauma pathogenesis (e.g., platelet activation, signaling, leukocyte recruitment). For further details on the pathophysiology, diagnosis, and management of DIC, please refer to Chap. 13.

None of the early trials of AT supplementation demonstrated improvements in survival of the treated patients. However, based these studies it was noted that AT supplementation permitted better control of alterations of the clotting system that are responsible for DIC [52], that AT replacement may reduce the incidence of renal impairment [54], and that infusion of high doses of AT might benefit certain severe DIC patients in whom rapid and strong thrombin generation was the main problem [53].

A final common conclusion from all of these early studies was that a large, randomized, double-blinded study would be required to properly assess the value of AT substitution therapy in trauma patients.

High-Dose AT for Attenuation of Inflammation and Prevention of Organ Failure in Trauma Patients

A prospective, placebo-controlled, double-blinded study of antithrombin supplementation in severely injured patients was reported in 1998 [42]. It is relevant to note that high-dose antithrombin was utilized in this trial, and that there was a new focus and efficacy objective, which was distinct from those of previous AT replace-

ment studies in trauma and critical care patients. Specifically, “the primary intention of the [high-dose antithrombin III treatment of severely injured patients] study was not the treatment of DIC but the attenuation of the inflammatory response and the decrease in organ dysfunction” [42].

During the period when this AT supplementation in trauma study, as well as a large clinical trial on AT supplementation for sepsis [55], were being designed and conducted there was great excitement about anti-inflammatory properties of antithrombin. AT anti-inflammatory properties were attributed to endothelial prostacyclin release and attenuated cytokine liberation and leukocyte activation secondary to antithrombin binding to vascular HSPGs. A series of papers published between 1995 and 2007 reported that AT induction of prostacyclin attenuated endotoxin-induced pulmonary vascular injury and hypotension, ischemia-reperfusion (I/R)-induced renal and hepatic injury, crush and I/R-induced spinal cord injury, and hepatic metastasis of colon cancer cells in experimental rat models. Thus, the rationale for expecting AT to exert protective anti-inflammatory effects against organ failure in humans seemed solid, and was a motivating factor for clinical studies of AT supplementation in trauma and sepsis.

Recently, however, the animal work forming the basis for anti-inflammatory properties of AT has come into question, and some papers have been officially retracted [56]. Nevertheless, at the time the trauma and sepsis AT supplementation clinical trials were conducted, the anti-inflammatory potential of AT constituted a large part of the justification for these studies.

Therefore, the primary objective of the High-Dose Antithrombin III Treatment of Severely Injured Patients trial was to evaluate the effects of early and high-dose administration of AT on clinical measures of the inflammatory response, and its secondary objective was to evaluate the effects of AT on plasma indicators of DIC and the systemic inflammatory response. Forty consecutive patients with severe blunt trauma (ISS ≥ 29) were enrolled into the double-blind study, and randomized to receive plasma-derived high dose

AT (Pharmacia & Upjohn) (20,000 IU per patient during a 4 day period) or placebo (human serum albumin) within 6 h after trauma. Additional AT or an equivalent protein weight of placebo was infused at 6 hourly intervals over the first 4 days to obtain plasma AT activity at 140 % of normal concentrations. During this period, AT activity averaged ~70 % in placebo-treated patients.

The primary efficacy response variables for the study were the incidence and severity of multiple organ dysfunction. Durations of liver and multi organ failure were significantly shorter for the AT supplemented group, but exhibited no significant differences with respect to respiratory failure and ARDS. Mortality, respiratory failure incidence, mechanical ventilation duration, and length of ICU stay were not significantly different for the control and AT groups.

The secondary efficacy objective of the study was to evaluate AT supplementation effects on DIC and the systemic inflammatory response. In the AT group plasma prothrombin tended to be elevated, and prothrombin fragment F1+2 and thrombin-AT complex tended to be lower on the first day. No differences between groups were observed for PTT, PT, platelets, PAI-1, sTNF receptor II, neutrophil elastase, IL-1 receptor antagonist, IL-6, and IL-8. DIC occurred in six patients (30 %) of each group.

In summary, the overall conclusions of the trial were that early and high-dose administration of antithrombin to patients with severe blunt trauma does not attenuate the posttraumatic inflammatory response or disseminated intravascular coagulation, or significantly improve outcome.

It is noted that a larger trial of high-dose antithrombin for sepsis was also conducted at about the same time as the trauma trial, and obtained similar negative results [55]. As in the trauma trial, the sepsis trial did not exclude concomitant heparin, which a large subset of the patients received. In retrospect heparin was speculated to have interfered with potential therapeutic benefits of AT. Reanalysis of the sepsis trial data showed that for patients without concomitant

heparin, 28-day mortality was lower for the AT group compared to the placebo group, and that this trend continued through 90 days, whereas it was not observed with groups receiving heparin [57].

Accordingly, it is pertinent to address the question of concomitant heparin administration in the trial of high-dose AT in trauma patients [42]. It is reported that “many” of the trauma study patients received heparin as prophylaxis for thromboembolic complications. Therefore, similar caveats about the negative results from the trauma trial can be raised, and lead the authors to conclude that “further study of high-dose antithrombin III therapy without the use of heparin in patients with sepsis and trauma is warranted.”

This view was based on the idea that heparin interfered with anti-inflammatory functions of AT by competitively inhibiting AT binding to the endothelium and subsequent prostacyclin production. However, the recent retraction of work which formed the foundation for proposal of the AT-prostacyclin anti-inflammatory pathway has cast some doubt on antithrombin anti-inflammatory properties [56].

Trauma induced coagulopathy is a complex pathology in which multiple components of endogenous procoagulant and anticoagulant systems are dysregulated and also undergo consumption. Unlike hereditary factor and inhibitor disorders which are mostly due to deficiency of a single isolated component, the multifactorial nature of TIC may cause it to be inherently more difficult to correct using simple replacement therapies.

Conclusion

The causes of trauma induced coagulopathy are multifactorial and the course for each patient is complex and distinct in its own way. A better understanding of the basic physiological functions of antithrombin and its dysregulation will support efforts to develop strategies to mitigate TIC.

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