A comprehensive review of the physiology of hemostasis and antithrombotic agents

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Tremendous strides have been made in the development of antithrombotic agents. The strategies of these developments aim at interrupting platelet activity, inactivating thrombin, inhibiting thrombin production, inhibiting fibrin formation, and destroying fibrin substrate (fibrinogen). Different antithrombotic agents and their sites of action are presented.

Antithrombotic agents are used in both the prevention and treatment of active vascular thrombosis. Currently, a number of antithrombotic agents are used in clinical practice, including aspirin, standard heparin, coumarin, ticlopidine, and low-molecular-weight (LMW) heparins. However, these agents have significant limitations, because an arterial thrombosis occurring at the site of atherosclerotic stenosis or plaque rupture generally is thrombin mediated, platelet dependent, and incompletely responsive to these agents (1). The procurement of more effective agents that secure full antithrombotic benefits while minimizing the antihemostatic outcome is being actively pursued.

The current trend in antithrombotic therapy is the identification of effective and safe pharmacological agents that have the ability to modulate specific sites in the hemostatic trail to circumvent the thrombotic process and maintain hemostasis. This article presents 1) an overview of platelet activation and blood coagulation that points out these specific sites, 2) a brief review of thrombogenesis, and 3) a discussion of the different classes of antithrombotic agents and their mechanisms of action.

OVERVIEW OF HEMOSTASIS

The human body has an intricate system designed to keep the blood in a fluid state under physiologic conditions. This system also is primed to stem blood loss when the integrity of the vascular system is interrupted. The normal vascular endothelium maintains blood fluidity by inhibiting blood coagulation and platelet aggregation while promoting fibrinolysis. The endothelium also provides a protective barrier that separates the blood cells and plasma factors from the highly reactive and thrombogenic elements of the matrix in the deeper layers of the vessel wall. The thrombogenic elements of the matrix include adhesive proteins, such as collagen and von Willebrand factor (vWF) (both of which promote platelet adhesion), and tissue factor (TF) (a membrane protein located in fibroblasts and macrophages) that triggers blood coagulation. When a vessel is severed, it constricts to divert blood from the site of injury. However, the extravasated
blood comes into contact with the exposed subendothelial matrix, which stimulates the formation of the hemostatic plug by promoting activation of platelets and blood coagulation (2).

**Platelet activation**

Platelets play a fundamental role in hemostasis. When a blood vessel injury occurs, platelets exhibit a sequence of events. These events include 1) adhesion of platelets to the injury site, 2) spreading of adherent platelets over the exposed subendothelial surface, 3) secretion of platelet granule constituents, 4) platelet aggregation, and 5) platelet coagulant activity (2, 3).

Platelets do not adhere to normal vascular endothelium. However, an area of endothelial disruption provides binding sites for adhesive proteins such as vWF in the subendothelial matrix (which binds to the platelet glycoprotein Ib/IX complex) and fibrinogen, as well as fibronectin through integrin receptors. These adhesive proteins are thought to form a bridge between platelets and subendothelial connective tissue. The importance of this event is illustrated by the occurrence of hemorrhage in Bernard-Soulier disease, in which there is a lack of glycoprotein Ib/IX, or in von Willebrand disease, in which vWF is decreased or defective. Other adhesive events include interaction of collagen with platelet receptor glycoprotein IV and the integrin Ia-IIa complex. Abnormalities in both of these collagen platelet receptors cause bleeding defects (2).

Once they adhere to the subendothelium, platelets spread out on the exposed surface and additional platelets from the circulation adhere, first to the basal layer of adherent platelets and eventually to one another, forming a mass of aggregated platelets. A critical event in platelet aggregation is the expression of surface membrane receptor glycoprotein IIb/IIIa (GPIIb/IIIa) that has the capacity to bind fibrinogen as well as vWF, fibronectin, and vitronectin. Fibrinogen appears to be the most important in aggregation by virtue of its divergent structure that allows it to form a bridge from platelet to platelet, thereby mediating aggregation. While vWF and collagen can interact with resting platelets, fibrinogen forms a high-affinity bond only with the integrin GPIIb/IIIa on activated platelets. In the congenital disorder Glanzmann’s thrombasthenia, the GPIIb/IIIa complex is deficient, and the associated defect in fibrinogen binding results in a bleeding tendency (2).

Many agonists, such as thrombin, adenosine diphosphate (ADP), collagen, arachidonic acid, and epinephrine, have the ability to induce platelet aggregation and secretion. Specific receptors exist on the platelet surface for these agonists. Many of the receptor-agonist complexes interact in the platelet membrane with coupling proteins, called G proteins, that trigger biochemical reactions. These biochemical reactions, resulting from the stimulatory agonists, lead to platelet activation and intracellular liberation of arachidonic acid from membrane phospholipids by the enzyme phospholipase A2. Arachidonic acid is converted by the enzyme cyclooxygenase (COX) into prostaglandin endoperoxides. Ultimately, it is converted into the potent platelet agonist thromboxane A2, as well as into the stable prostaglandins, such as PGD2, that inhibit platelet activation. Throm-boxane A2 is a potent mediator of platelet aggregation and secretion (2) (*Figure 1*).

During platelet activation, platelets expose surface receptors for specific plasma clotting factors, particularly active factor V (Va), which may be either secreted and expressed by the platelet or bound from plasma. This receptor, with bound factor Va and in conjunction with anionic membrane phospholipids exposed on activated platelets, functions as a binding site for active factor X (Xa). An analogous system exists for binding factor IXa (2) (*Figure 2*). Platelet activation and its effects are modulated by regulatory substances, one of which is cyclic 3’5’-adenosine
monophosphate (cAMP). Like virtually all other cells except human red blood cells, platelets contain adenylate cyclase, the enzyme that converts ATP into cAMP. Its action is stimulated by arachidonic acid products, prostaglandin D\textsubscript{2} (PGD\textsubscript{2}) in platelets and prostacyclin (PGI\textsubscript{2}) in endothelial cells. Platelets also contain cyclic phosphodiesterases that cleave cAMP to inactive 5'AMP, hence modulating intracellular cAMP concentration. In sufficient concentration, cAMP inhibits platelet aggregation (2) (see Figure 4).

**Coagulation**

The coagulation glycoproteins are called factors. There are at least 12 distinct plasma glycoproteins designated with roman numerals I to XIII (numerals III, IV, and VI are not used). The order of the numerals does not reflect the reaction sequence. Although there may be other sites of synthesis, the liver cells probably synthesize and secrete all the proteins involved in coagulation, including the coagulant portion of factor VIII complex (VIII:C). Endothelial cells synthesize and secrete factor VIII:vWF polymers that form ionic bonds with factor VIII:C molecules (VIII:C/VIII:vWF) in the circulation. Hepatic synthesis of prothrombin (factor II) and factors VII, IX, and X is vitamin K dependent (4).

Traditionally, the coagulation system is divided into extrinsic and intrinsic pathways. The principal initiating pathway of in vivo blood coagulation is the extrinsic system (Figure 3). The central precipitating event is considered to involve TF, which is not exposed to the circulating blood under physiologic conditions (2). Human TF is located in the adventitia, comes into contact with blood after vascular injury, and is a membrane glycoprotein tightly associated with phospholipids (5, 6). The intrusion into the circulation of the TF–phospholipid surface membranes and organelle membranes from the disrupted cells initiates the extrinsic pathway of blood coagulation. These membranes normally are extrinsic to the circulation (4). The vitamin K–dependent proenzyme factor VII, which is a major plasma component of the extrinsic pathway, binds via its g-carboxyglutamic acid residues and calcium bridges to phospholipids in these membranes and is activated by exposure of its protease sites to active factor VII (VIIa). Tissue factor acts in concert with factor VIIa and membrane phospholipids to activate factor IX to active factor IX (IXa) and factor X to active factor X (Xa) (2, 4). Factor Xa is the active catalytic ingredient of the prothrombinase complex, which also includes factor Va and phospholipids. The prothrombinase complex converts prothrombin to thrombin. Thrombin cleaves fibrinogen (factor I) to fibrin and also converts factor XIII to active factor XIII (XIIIa), which cross-links the fibrin clot and stabilizes it (2) (see Figure 4).

The intrinsic system (contact activation) refers to reactions that occur following adsorption of contact factors to a highly negatively charged surface (3). All necessary components for this pathway are present (intrinsic) in the circulating blood. Subendothelial adsorption of factor XII (Hageman factor) and kininogen (with bound prekallikrein and factor XI) alters and partially activates factor XII to active factor XII (XIIa). Factor XIIa then cleaves nearby factor XI and prekallikrein, both of which are bound to kininogen, and converts them into active kallikrein and factor XIa (4). Factor XIa, in the presence of Ca\textsuperscript{2+}, activates factor IX to IXa on the surface of adherent and aggregated platelets. Factor IXa, in concert with factor VIIIa (cleaved by thrombin from circulating VIII:C/VIII:vWF complex), activates platelet-bound factor X to Xa. Factor Xa remains bound to platelets by attaching to factor Va. This complex of factors Xa and Va on the platelet surface cleaves the prothrombin molecules into 2 portions (see Figure 3). One part contains
the γ-carboxyglutamic acid residues; the other part is freed into the circulation as thrombin. Thrombin induces local platelet aggregation and produces fibrin monomers from plasma fibrinogen (4).

The explosive cellular and molecular reactions that occur when the hemostatic process is triggered are modulated by endothelial cell elaboration of the antithrombotic compounds prostacyclin (PGI₂), thrombomodulin, nitric oxide, heparan, antithrombin III (ATIII) (which inhibits factors IXa, Xa, and thrombin), C1 inhibitor (which inhibits the contact system enzyme factors XIIa and kallikrein), and α₁-antitrypsin (which inhibits factor Xa). Throm-bomodulin binds thrombin and inhibits its ability to cleave fibrinogen and activate platelets, while markedly enhancing its ability to activate protein C. Protein C, in turn, inactivates factors Va and VIIIa and enhances fibrinolysis by binding to an inhibitor of plasminogen activators. Thrombin that is bound to thrombomodulin is also inactivated by circulating ATIII, a step accelerated by heparan sulfate. Protein C activity is controlled by protein C inhibitor as well as α₂-proteinase inhibitor. Protein C is stimulated by a cofactor, protein S. Protein S is controlled by C4b which joins and forms a complex, thus preventing protein S’s action. The enhancement of fibrinolysis by protein C may also be dependent on protein S. Binding of thrombin to thrombomodulin results in the loss of the coagulant effect of thrombin and in the enhancement of its ability to activate protein C and, therefore, to inhibit thrombo-genesis (2).

THROMBOGENESIS

Thrombosis may occur if the hemostatic stimulus becomes unregulated because the capacity of the inhibitory pathways is impaired, or, more commonly, the capacity of the natural anticoagulant mechanism is overwhelmed by the intensity of the stimulus (2). Important predisposing conditions to thrombosis are low flow state (stasis), disturbed flow (turbulence), and altered endothelial coverage (ulceration or endarterectomy). Injury of the vessel wall plays a major role in vascular thrombosis. However, it is more important in the pathogenesis of arterial thrombosis than its venous counterpart. In regions of high arterial shear rate, endothelial cells may sustain injury and become denuded, an event that promotes platelet adhesion. Blood flow rate is a key determinant of shear rate. Platelet adhesion increases with wall shear rate because of the margination of platelets by the layer of red blood cells that occupies the central portion of the blood stream. In addition, the lateral forces are increased with increasing shear rate. This increase results in more frequent collision between the platelets and the vessel wall. Higher hematocrits increase this effect (7, 8). Also, rupture of atherosclerotic plaque exposes surfaces that are capable of initiating thrombosis (1).

Arterial thrombi are predominantly composed of platelets, a scanty amount of fibrin, and a few red blood cells, hence the term “white thrombi.” Because of the high platelet composition of these thrombi, antiplatelet agents, rather than anticoagulants, have been used in the treatment and prevention of arterial thrombosis. Thrombus formation remains limited to the injured site, because plasma-dependent and vessel wall–dependent inhibitory pathways limit its propagation (1).

Venous thrombi are mainly composed of red blood cells in a fibrin mesh, hence the term “red thrombi.” Warfarin has been the empiric agent used in the treatment of venous thrombosis and thromboembolism (9).
ANTITHROMBOTIC STRATEGIES

Strategies for antithrombotic therapies aim at securing full antithrombotic benefits while minimizing the risks of undue bleeding. The evolving strides in the development of antithrombotic agents target the interruption of the thrombotic process by modulating specific molecular interactions that lead to thrombus formation. Antithrombotic strategies for preventing and treating arterial thrombosis include 1) the interruption of platelet reactivity, 2) the direct inactivation of thrombin, and 3) the inhibition of thrombin production. In venous thrombosis, the antithrombotic strategy is to inhibit fibrin formation and to promote the destruction of fibrinogen (1).

** Interruption of platelet reactivity**

When platelets come into contact with an abnormal blood vessel or are exposed to extravascular tissue after injury to a blood vessel, they undergo a variety of changes collectively known as platelet activation. Activation stimuli include tissue components such as collagen, shear force, epinephrine, and local mediators released by platelets themselves, such as ADP, serotonin, and thromboxane A₂ (10).

Regardless of the activation stimulus, platelet aggregation ultimately is mediated by the integrin GPIIb/IIIa. This platelet receptor complex can bind several different glycoprotein ligands, including fibrinogen and vWF. The dimeric structure of fibrinogen allows interaction with 2 platelets simultaneously, leading to platelet aggregation. Binding with a certain ligand differs depending on the agonist that induces aggregation. Fibrinogen is the predominant ligand for GPIIb/IIIa binding when platelet aggregation is triggered by agonists such as thrombin, ADP, or collagen, whereas vWF is the predominant ligand when aggregation is induced by shear force alone. Shear-induced platelet aggregation, which may be an important factor in vivo at the site of vascular stenosis, is not affected by aspirin. Antibodies and small molecules that selectively inhibit the interaction of vWF, but not other ligands, with GPIIb/IIIa have recently been developed (10). A novel murine monoclonal antibody to human vWF, GUR76-23, inhibited high shear-induced platelet aggregation and blocked adhesion of ADP plus epinephrine-stimulated platelet to vWF, indicating that it interferes with the interaction with the GPIIb/IIIa complex (11).

** Antiplatelet drugs**

Platelet activation offers many potential targets for inhibitory drug action. Traditionally, attention has focused on inhibiting the synthesis or action of platelet-derived thromboxane A₂, as exemplified by aspirin. More recently, appreciation that platelet activation can proceed via different pathways has increased the interest in interventions directed towards cAMP and GPIIb/IIIa complex as being likely to interfere with the final step in the process (10) (Figure 4).

** Platelet cAMP elevators (dipyridamole)**

Elevation of intracellular cAMP levels by agents that activate adenylate cyclase (i.e., PGE₁, PGI₂, PGD₂) or that inhibit the cyclic phosphodiesterases results in inhibition of platelet responses. Dipyridamole (Persantine), a weak phosphodiesterase inhibitor, appears not to inhibit aggregation responses to collagen, epinephrine, and ADP at usual doses but has a synergistic effect with aspirin in preventing platelet aggregation in thromboembolic disorders. Other weak phosphodiesterase inhibitors, such as caffeine, theophylline, and aminophylline, inhibit ADP-induced platelet aggregation in vitro but may not have clinical significance (12). Within the platelet, cAMP is either
degraded to inactive 5'AMP by the enzyme cAMP phosphodiesterase or becomes bound to inactive protein kinase which, in turn, becomes activated. The activated protein kinase acts together with ATP to phosphorylate substrate proteins to inhibit platelet activation. Agents that retard degradation of cAMP by inhibiting phosphodiesterase cause increased intracellular cAMP, which inhibits platelet aggregation (7, 13) (see Figure 4). Dipyridamole is now indicated in patients with prosthetic heart valves and as an alternative to aspirin for the secondary prevention of acute myocardial infarction, for the prevention of stroke in patients with transient ischemic attacks, and for the maintenance of patency of coronary bypass grafts. It has coronary vasodilator activity as well but has been mostly disappointing in clinical trials (14).

**PGG/H synthase inhibitors (aspirin and aspirin-like drugs)**

Thromboxane A₂, a product of arachidonic acid metabolism, mediates vasoconstriction as well as platelet aggregation and release. Arachidonic acid is released intracellularly by diverse stimuli from phospholipids of both endothelial and platelet cell membranes by the action of phospholipases (10, 15) (see Figure 1). The PGG/H synthase controls the enzymatic cyclooxygenation of free intracellular arachidonate in platelets and endothelial cells. This synthase system contains 2 activities: a bis-oxygenase (cyclooxygenase; COX) that catalyzes PGG₂ formation from arachidonate, and a hydroperoxidase that catalyzes the reduction of PGG₂, resulting in PGH₂ synthesis. PGG₂ and PGH₂ are cyclic endoperoxides. PGH₂ is converted in the platelet to thromboxane A₂. The enzymatic activity of COX is blocked by aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). After aspirin ingestion, a serine residue of COX becomes acetylated by the acetyl portion of the aspirin molecule. The effectiveness of aspirin appears to be dependent on its ability to block the formation of thromboxane A₂ irreversibly by blocking the COX activity of the PGG/H synthase system. Other NSAIDs inhibit platelet function and aggregation. They differ, however, from aspirin in that their effects are reversible within several hours, necessitating multiple daily doses to maintain functionally important inhibition of thromboxane A₂. Ibuprofen is an NSAID. In addition to its reversible inhibition of COX, ibupofen also competes with free arachidonate for COX binding (10, 16).

Two isoforms of COX exist. Cyclooxygenase-1 is constitutively expressed, and COX-2 is an inducible isoform. Cyclooxygenase-1 has clear physiological functions. Its activation leads to the production of prostacyclin, which when released by the endothelium is antithrombogenic, and when released by the gastric mucosa is cytoprotective. Cyclooxygenase-1 in platelets leads to thromboxane A₂ production and causes platelet aggregation, whereas COX-2 is a distinct isoform encoded by a different gene than COX-1. Cyclooxygenase-2 is induced by inflammatory stimuli (17). Aspirin inhibits COX-2 at higher concentrations than those required to inhibit COX-1. This may account, in part, for the different dose requirements of analgesic and anti-inflammatory versus antiplatelet effects of the drug. The anti-inflammatory action of corticosteroids and NSAIDs is caused by the inhibition of COX-2 (18). The unwanted side effects, such as irritation of the stomach lining and toxic effects on the kidney, are caused by the inhibition of COX-1. Individual NSAIDs show different selectivity against COX-1 and COX-2 isoforms. Nonsteroidal anti-inflammatory drugs that are selective towards COX-2, such as meloxicam, may have an improved side-effects profile over current NSAIDs (17).

Meloxicam, which has a selectivity towards COX-2 of up to 100-fold over COX-1, is used for rheumatoid arthritis and osteoarthritis. Celoxib, which is used as an analgesic following tooth extraction, has inhibitory selectivity against COX-2 (15).
GPIIb/IIIa inhibitors

Platelet recruitment involves the activation of ambient platelets by agonists derived from activated platelets. Glycoprotein IIb/IIIa receptor is expressed following agonist stimulation. This receptor binds with multiple adhesive ligand molecules, including fibrinogen, vWF (in conditions of high shear as might exist in stenotic arteries), fibronectin, vitronectin, and thrombospondin. Fibrinogen generally is the functional ligand because it is present in much greater concentration in the plasma. Platelet recruitment is inhibited by anti-GPIIb/IIIa agents, such as monoclonal antibodies, naturally occurring peptides containing arginine-glycine-aspartic acid or dodecapeptide sequences, and by synthetic competitive analogues (1).

The monoclonal antibody 7E3 (abciximab or ReoPro), which inhibits the GPIIb/IIIa receptor, has undergone extensive clinical trials and received approval for clinical use. It has been shown to prevent thrombus formation after vascular injury and to be effective in reducing early reocclusion following coronary interventional procedures (13, 19). Abciximab is a popular drug at BUMC and ranks in the top in annual pharmaceutical expenditures (The Baylor Drug Newsletter, June 1998; 10[6]). Striking inhibition of platelet hemostatic functions and substantial bleeding at intervention sites can occur, but complications were low in the Epilogue Stent Trial. Thrombocytopenia has been observed but is a rare occurrence (20).

Naturally occurring peptides containing arginine-glycine-aspartic acid sequences have been isolated from snake venom and leeches. The peptides are potent inhibitors of the binding of fibrinogen to GPIIb/IIIa receptors, and they abolish platelet aggregation. This group of peptides includes trigramin, bitistatin, kistrin, apallggin, and echistatin (1). A protein from the African saw-scaled viper was researched for its inhibitory effect on thrombus formation. Its chemical sequence was only 3 amino acids, which made it easy to replicate and study. This protein inhibits platelet aggregation by blocking GPIIb/IIIa. A synthesized protein, echistatin (marketed as Agrastat), received new drug approval in April 1998 for clinical use. Clinical studies involving 7300 patients with non–Q-wave myocardial infarction revealed prevention of myocardial infarction occurrence for 6 months when echistatin was combined with heparin therapy (21, 22).

Synthetic GPIIb/IIIa-antagonist peptides have been synthesized and characterized in vitro and in vivo as competitive inhibitors of platelet GPIIb/IIIa binding with adhesive proteins. One such inhibitor, eptifibatide (Integrelin), is a cyclic peptide with a lysine-glycine-aspartic acid sequence rather than an arginine-glycine-aspartic acid sequence. The substitution of lysine for arginine makes this agent specific for the GPIIb/IIIa receptor. The PURSUIT trial confirmed that in patients with unstable angina or non–Q-wave myocardial infarction, eptifibatide injection reduces the combined incidence of death or myocardial infarction, regardless of patient management strategy. This product became available in June 1998 and may prove to be as effective as abciximab in maintaining coronary artery patency (13, 20).

Nonpeptide antagonists that mimic the charge and geometric characteristics of the arginine-glycine-aspartic acid sequence have been developed. These agents have the potential to be orally administered and, thus, effective for chronic antiplatelet therapy. One such agent, tirofiban, is currently in Phase III trials (18).

Anti–ADP-induced GPIIb/IIIa agents (ticlopidine and clopidogrel)

Ticlopidine, a thienopyridine derivative, has a proposed mechanism of action that interferes
selectively with ADP-induced transformation of GPIIb/IIIa complex expression in activated platelets. It also inhibits platelet aggregation induced by thrombin, collagen, arachidonic acid, and platelet-activating factor. The efficacy of ticlopidine in the prevention of stroke has been established by some clinical trials. Ticlopidine, in a dose of 250 mg, twice daily, reduced the incidence of a combined endpoint of stroke, myocardial infarction, or vascular death by roughly 30%. Adverse effects of ticlopidine include gastrointestinal side effects; neutropenia (1% in the first 3 months of treatment), which is reversible with discontinuation of the drug; elevation in serum cholesterol; and thrombocytopenia (10).

Clopidogrel is another ADP antagonist from the thienopyridine group. By binding the ADP receptor, clopidogrel inhibits the binding of fibrinogen to its platelet receptor, the GPIIb/IIIa integrin. It does not directly modify the GPIIb/IIIa complex, suggesting that clopidogrel acts indirectly to reduce fibrinogen binding. In healthy volunteers, it inhibited ADP and thrombin-induced platelet aggregation. The drug selectively reduced the number of functional ADP receptors mediating the inhibition of stimulated adenylate cyclase. Clopidogrel-induced platelet inhibition persists several days after withdrawal of the drug and diminishes in proportion to platelet renewal. In comparison with ticlopidine, clopidogrel is more potent, and neutropenia has not been demonstrated. Clopidogrel is significantly more active than aspirin. Compared with aspirin, clopidogrel has less severe gastrointestinal bleeding but more severe rash incidence (23).

### Thrombin receptor antagonists

Each platelet has about 1000 thrombin receptors. Thrombin receptors also are present on endothelium and vascular smooth muscle cells. A single molecule of thrombin activates multiple receptors. In vitro, platelet thrombin receptor activity is inhibited by monoclonal antibodies and synthetic peptides that target specific receptor sites. Novel synthetic thrombin receptor inhibitor peptides possess a hirudin-like binding sequence that inhibits thrombin receptor function in vivo. Thrombin receptor inhibitor peptides offer an improved benefit by specifically interrupting thrombin-dependent platelet recruitment at the site of vascular injury, while sparing the production of thrombin-dependent fibrin for the formation of hemostatic plugs at sites of tissue damage. Thrombin receptor inhibitor peptides may block other thrombin receptor-dependent responses at the site of vascular injury that lead to occlusive lesion formation, including the mitogenic stimulation of vascular smooth-muscle cell proliferation, leukocyte chemotaxis, and cell adhesion receptor expression (1).

### Direct antithrombin agents

Thrombin is the most potent platelet activator and plays a central role in regulating local thrombus formation at the site of vascular injury. Active thrombin does not generally circulate freely in plasma because of rapid inactivation by ATIII, which also inactivates factor Xa and factor IXa but not factor VIIa. The rate of inhibition is accelerated significantly when ATIII is complexed with heparin. Thrombin inactivation by ATIII also is greatly facilitated by heparin-like glycosaminoglycan (heparan sulfate) that is abundant on the luminal surface of endothelial cells. alpha1-Antitrypsin and alpha2-macroglobulin also inhibit thrombin when the antithrombin pathway is overwhelmed. Thus, both thrombin generation and action are limited locally to sites of vascular injury (1).

Several classes of direct antithrombin agents have been developed. They include naturally occurring antithrombin peptides, synthetic competitive peptides, and irreversible antithrombin
peptides. Because these molecules directly inhibit thrombin activity without depending on ATIII, they inactivate thrombus-bound thrombin as well as soluble thrombin. Accordingly, these direct antithrombin agents interrupt thrombin-mediated thrombotic processes that are resistant to aspirin and heparin. The use of these agents prevents reocclusion of arteries after thrombolytic reperfusion (1) (Table).

**PGG/H synthase inhibitors (aspirin and aspirin-like drugs)**

<table>
<thead>
<tr>
<th>Antithrombotic agents</th>
<th>Ligand</th>
<th>Action</th>
<th>Mode of Action</th>
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<tbody>
<tr>
<td>Hirudin, desirudin</td>
<td>Thrombin</td>
<td>Inactive thrombin</td>
<td>Binds to thrombin</td>
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<tr>
<td>Argatroban</td>
<td>Thrombin</td>
<td>Inhibits free and bound thrombin</td>
<td>Competitive inhibitor of thrombin</td>
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<tr>
<td>D-Phe-Pro-Arg-chloromethyl ketone</td>
<td>Thrombin</td>
<td>Irreversibly inactivates thrombin</td>
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<tr>
<td>Low-molecular weight heparins</td>
<td>Thrombin</td>
<td>Inhibits thrombin production</td>
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</tr>
<tr>
<td>Activated protein C</td>
<td>Thrombin</td>
<td>Inhibits thrombin production</td>
<td>Inactivates surface bound Xa and VIII</td>
</tr>
<tr>
<td>Tick anticoagulant peptide, leech-derived recombinant antistatin</td>
<td>Thrombin</td>
<td>Inhibits thrombin production</td>
<td>Selectively inhibits bound Xa</td>
</tr>
<tr>
<td>Tissue factor-pathway inhibitor</td>
<td>Thrombin</td>
<td>Inhibits thrombin generation</td>
<td>Binds to Xa-TF-VIIa complex, causing inhibition of the complex</td>
</tr>
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**Natural antithrombin polypeptides**

Hirudin, a polypeptide produced by the salivary glands of the medicinal leech *Hirudo medicinalis*, has extremely high, specific, and reversible affinity for thrombin. Recombinant hirudin (desirudin) currently is undergoing clinical trials in deep venous thrombosis and acute coronary syndrome. Desirudin attaches directly to thrombin, has no circulating inhibitor, and can inactivate both clot-bound and circulating thrombin. It has shown greater effects than heparins on the platelet-rich thrombi in arteries and a particular efficacy on venous-type thrombi (24).

**Synthetic antithrombin peptides**

Synthetic peptides based on arginine, benzamidine, and hirudin exhibit antithrombin activities that vary in potency and antithrombotic potential. The tripeptide D-Phe-Pro-Arg inhibits thrombin-induced platelet aggregation and cleavage of fibrinogen in vitro and in vivo (1). Argatroban is a synthetic competitive inhibitor of thrombin derived from arginine. It inhibits free and bound thrombin in a reversible action that does not cause thrombocytopenia (1, 25).

**Irreversible antithrombin agents**

The synthetic antithrombin D-Phe-Pro-Arg-chloromethyl ketone (PPACK) is unique among the ATIII-independent, direct antithrombins because it potentially and irreversibly inactivates thrombin, both soluble and thrombus bound. Systemic infusions of this agent into primates
interrupt platelet-rich, aspirin- and heparin-resistant thrombi on Dacron vascular grafts, vascular stents, and hemodialyzers. Transient 1-hour intravenous infusions of D-Phe-Pro-Arg-chloromethyl ketone produce lasting interruption of platelet deposition at sites of surgical carotid endarterectomy by irreversibly inactivating thrombin generated by, and bound to, a forming thrombus. Moreover, delaying its infusion until surgical hemostasis becomes established obviates the bleeding complications caused by earlier initiation of therapy without compromising the antithrombotic benefits. D-Phe-Pro-Arg-chloromethyl ketone, but not competitive antithrombins such as hirudin, interrupts subsequent thrombus formation after topical application at a site of established thrombus. Long-term toxicity associated with the administration of this agent systemically or locally has not been adequately investigated in humans (1, 25).

Thrombin production inhibitors

Platelet recruitment at the site of vascular injury depends on the local production of thrombin through prothrombinase complex binding to constituents within the forming thrombus. Vascular damage generates thrombin by initiating both TF-dependent and intrinsic activation of factor X, which (together with cofactor activated factor V, a phospholipid surface and ionic calcium) forms the prothrombinase complex that cleaves prothrombin (see Figure 3). Platelets accumulating at the site of injury also actively promote the requisite proteolytic complexes that amplify prothrombin’s catalytic conversion to thrombin. Both thrombin and factor Xa amplify their own rates of formation by catalyzing the activation of factors VII, IX, V, and VIII to factors VIIa, IXa, Va, and VIIIa, respectively. Thrombin also activates the expression of cellular binding sites on platelets for assembly of the vitamin K–dependent complexes. Factor VII is auto activated by limited proteolytic cleavage, when complexed with the cofactor cell-bound TF. Tissue factor is abundant on extravascular cells and may be expressed by monocytes/macrophages. Tissue factor is an important trigger for initiating coagulation in ruptured arterial atheromatous lesions because of its abundant presence in these intimal plaques and its exposure to blood flowing at the disrupted vascular intima. Low-molecular-weight heparins inhibit thrombin production through their ability to inactivate factor Xa and fluid phase thrombin (1).

Activated protein C

Thrombin activates the natural antithrombotic zymogen, protein C, by cleaving the amino-terminal dodecapeptide, when bound to thrombomodulin on the vascular endothelial membrane surface. Activated protein C (APC) inhibits thrombin formation by inactivating surface-bound factors Va and VIIIa through proteolytic cleavages. Protein S, the cofactor for APC, forms a complex with APC in the presence of calcium on membrane surfaces. Protein S enhances the efficiency of protein C inactivation of membrane-bound factors Va and VIIIa. Activated protein C inhibits vascular thrombus formation by inhibiting factor Va- and VIIIa-dependent autocatalysis, while permitting sufficient thrombin production to achieve hemostasis. It offers a more favorable antithrombotic to antihemostatic profile (1, 25).

Natural and recombinant forms of APC have been developed and studied experimentally. Findings suggest that APC might be effective as an adjuvant antithrombotic agent during and after thrombolytic therapy (1, 25).

In contrast to findings with antiplatelet agents and direct antithrombins, the administration of APC at full antithrombotic doses was not associated with detectable impairment of platelet hemostatic function; the bleeding times remained normal throughout APC infusions. These findings with APC
have led to the development of soluble thrombomodulin as a potential means for generating endogenous APC (1).

Peptide inhibitors of factor Xa

Two naturally occurring peptide inhibitors of factor Xa have been developed: tick anticoagulant peptide and leech-derived recombinant antistatin. Tick anticoagulant peptide is available as a recombinant product of yeast. Both recombinant tick anticoagulant peptide and leech-derived recombinant antistatin are potent and selective inhibitors of factor Xa (see Table). Their administration in primates effectively interrupted platelet-rich arterial thrombotic processes that are resistant to aspirin and heparin while sparing platelet hemostatic function. Anti-thrombotic benefits and hemostatic safety of factor Xa inhibitors may be explained by their inhibition of bound factor Xa in the intrinsic pathway-dependent amplification loop for thrombin production, thus permitting the formation of small but hemostatically important amounts of thrombin to be generated through the TF pathway (1, 25) (see Figure 3).

Tissue factor–pathway inhibitors

One of the potential mechanisms for reocclusion following successful thrombolysis is the reexposure of TF in the subendo-thelium and within the lipid- and collagen-rich atherosclerotic plaque. Tissue factor–pathway inhibitor forms a complex with factor Xa that binds to the TF–factor VIIa complex, thus inhibiting thrombin generation (see Table and Figure 3). Experimentally, TF-pathway inhibitor infusion prevented reocclusion after tissue plasminogen activator (tPA)-induced reperfusion. These findings suggest that the TF pathway may be important in the process of reocclusion after successful thrombolysis (1, 25).

Vascular wall thrombogenicity impairment: dietary n-3 fatty acids

Epidemiologic, biochemical, experimental, and clinical studies indicate that dietary n-3 fatty acids benefit individuals who are at risk of developing arteriosclerotic disease, including symptomatic patients undergoing interventional procedures. The reduction in thrombotic vascular events has been attributed to the metabolic effects of substituting n-3 fatty acids for arachidonic acid and the resulting generation of eicosanoid products capable of modifying platelet and vascular functions. Dietary n-3 fatty acids also reduce experimental vascular proliferative lesion formation, whereas fish consumption is associated epidemiologically with decreased mortality rates from coronary artery disease (1). Dietary n-3 fatty acids are presumed to attenuate the thrombogenic properties of damaged vessels by interfering with the cellular membrane functions that mediate thrombogenicity.

Vascular injury initiates thrombin production by inducing TF/factor VIIa–dependent activation of factor X to active factor X(Xa) on phospholipid surfaces in the presence of factor Va and ionic calcium to form a prothrombinase complex that cleaves prothrombin to form thrombin (see Figure 3). Thrombin generation is greatly augmented through the activation of factor X to active factor X (Xa) on phospholipid surfaces by proteolytic complexes composed of TF/factor VIIa and thrombin-activated factor VIII (VIIIa) in concert with factor IXa. Membrane phospholipids of platelets and vascular wall cells incorporate dietary n-3 fatty acids that affect their physicochemical properties, particularly with respect to the complex membrane changes required to enhance conversion of coagulant zymogens to their respective protease complexes. Dietary n-3 fatty acids may prevent vascular thrombosis at sites of vascular injury by impeding the exposed subendothelial matrix from initiating and sustaining the membrane-dependent generation of thrombin. Defective thrombin
production may also be responsible for the interruption of vascular proliferative lesion formation by dietary n-3 fatty acids (1).

VENOUS THROMBOSIS

Venous thrombi are composed mainly of red cells in a fibrin meshwork. Their production is mediated by a combination of hypercoagulability, reduced fibrinolytic activity, stasis, and local vessel damage. Interruption of venous thrombosis is achieved by inhibiting the production of or promoting the destruction of fibrinogen (1).

Classically, heparin and coumarins have been commonly used agents in the treatment of venous thrombosis. Current anti-thrombotic strategies target inhibition of fibrin formation by using LMW heparins and heparinoids (1). Although heparin is effective in the prevention and treatment of venous thrombosis, its usefulness is limited by its pharmacokinetic properties, its biophysical mechanism of action, its antihemostatic effects, its susceptibility to inhibition by platelet-dependent mechanisms, and the heparin resistance occasionally seen in immune heparin-induced thrombocytopenia. Heparin-induced thrombocytopenia may present with life-threatening thrombosis, a phenomenon called “white clot syndrome.” Immune heparin-induced thrombocytopenia (type I) is different from the nonimmune heparin-induced thrombocytopenia (type II). The latter is characterized by a rapid transient decrease in platelet count right after the injection of heparin in normal humans. This effect most likely represents platelet aggregation and sequestration and is not associated with platelet activation or intravascular thrombosis, as seen in the immune type. Platelet count returns to normal once heparin is discontinued and may normalize even if it is not discontinued (26).

Heparin itself has little anticoagulant effect; rather, it functions as a catalytic cofactor for ATIII. Under the catalytic action of heparin, ATIII forms inactive binary (ATIII-enzyme) or ternary (heparin-enzyme-ATIII) complexes with at least 4 coagulation enzymes: thrombin, Xa, IXa, and XIa, in order of decreasing rate. The heparin-antithrombin complex also blocks serine proteases involved in the coagulation cascade besides thrombin, such as factors II, VII, X, and protein C (25, 27).

Heparin is not a homogenous substance. It is a family of glycosaminoglycans of various molecular weights. A specific pentasaccharide in the heparin molecule is the crucial structural element for the high-affinity binding of heparin to ATIII, and thus for heparin anticoagulant activity. Only about one third of the molecules in a solution of pharmaceutical-grade heparin contain this pentasaccharide, and these account for at least 80% of heparin biological activity of neutralizing thrombin. The balance of molecules without the pentasaccharide have very low affinity for ATIII and contribute little to heparin’s ability to inhibit the action of thrombin or other activated clotting factors, although such inert heparin molecules may contribute to the hemorrhagic side effects of heparin by interfering with platelet function (27).

A heparin cofactor, heparin cofactor II, has been identified. Unlike ATIII, heparin cofactor II inhibits only the action of thrombin and not the action of other coagulation enzymes (27). Because fibrin binds thrombin and protects it from inactivation by heparin-ATIII, much higher concentrations of heparin are needed to inhibit thrombin bound to fibrin than is required to inactivate the free enzyme. Thrombin also binds to subendothelial matrix proteins, where it again is
protected from inhibition by heparin. These observations not only explain why heparin is less effective than the ATIII-independent inhibitors of thrombin and factor Xa at preventing thrombosis in experimental animals, but they also suggest that ATIII-independent inhibitors may be more effective than heparin in both arterial and venous thrombosis (1).

Platelets limit the anticoagulant effect of heparin in 2 ways. First, factor Xa generated on the platelet surface is protected from inhibition by heparin-ATIII, a shortcoming that can be overcome by direct factor Xa inhibitors. Second, platelets release heparin-neutralizing proteins, including platelet factor 4. Heparin also exhibits poorly defined, platelet-dependent antihemostatic properties, increases vascular permeability, and promotes experimental microvascular bleeding. These non-anticoagulant, antihemostatic properties are not shared by LMW heparins and heparinoids, which permit them to be given to patients in higher anticoagulant and anti-thrombotic doses than heparin (1, 25). Low-molecular-weight heparins produce less bleeding than heparin. They are effective and safe agents for the prevention and treatment of venous thrombosis (1).

**Fibrin formation inhibition**

If fibrin production is inhibited, venous thrombi formation can be prevented. Inhibition of fibrin production may be achieved by inhibiting thrombin activity and its production, as discussed above. This may also be achieved by inhibiting soluble thrombin using LMW heparins or by destroying the fibrinogen substrate. It is noteworthy that arterial thrombotic processes, mediated by bound thrombin, are not interrupted by the use of LMW heparins or fibrinogen destruction (1).

Low-molecular-weight heparins are fragments of standard, commercial-grade heparin. Their size is about one third the size of the heparin molecule. Like standard heparin, LMW heparins produce their major anticoagulant effect by binding to ATIII through the pentasaccharide sequence. Binding of the penta-saccharide to ATIII produces a conformational change in the ATIII molecule that enhances its ability to inactivate the coagulation enzymes thrombin and factor Xa (1). Standard heparin and LMW heparins amplify the inactivation of thrombin by ATIII by acting as a template that binds ATIII and thrombin through the pentasaccharide sequence to form a ternary complex. In contrast, inactivation of factor Xa by binary heparin ATIII does not require the binding of the heparin molecule to the clotting enzyme factor Xa (Figure 5). This inactivation can be achieved by LMW heparin fragments rather than standard heparin, provided the fragments contain the high-affinity pentasaccharide. Standard heparin has a ratio of anti–factor Xa to antithrombin activity of about 1:1, whereas the LMW heparins have anti–factor Xa to antithrombin ratios of 4:1 and 2:1, depending on their molecular sizes (1, 28).

Evidence suggests that heparin has no direct effect on clot lysis, because it cannot access factor Xa and thrombin when immobilized in thrombus. However, it strongly inhibits the recurrent formation of thrombotic deposits during thrombotic treatment and after the thrombi have been dissolved by plasmin (27).

Heparinoids have been used as anticoagulants. The 2 heparinoids, dermatan sulfate and lomoparin, have different mechanisms of anticoagulant activity than standard heparin and LMW heparin. Dermatan sulfate, a heparin-like sulfated polysaccharide, promotes the activity of heparin cofactor II, a secondary inhibitor of thrombin. Unlike ATIII, heparin cofactor II inhibits thrombin but has no anti–factor Xa activity. Lomoparin contains large amounts of heparin sulfate as well as dermatan sulfate. It therefore enhances both ATIII and heparin cofactor II and has both antithrombin and anti–factor Xa activity (1, 27).
**Fibrinogen destruction**

Impairing fibrin formation by depleting fibrinogen concentration in vivo is another strategy for reducing venous thrombosis. This may be achieved by using a number of snake venom enzymes, including ancrod, batroxobin, and crotalase. Ancrod, which is extracted from the Russell pit viper, has been used clinically. These defibrinogenating enzymes cleave fibrinopeptide A, but not fibrinopeptide B, from fibrinogen to produce fibrin that is very sensitive to endogenous fibrinolysis. Factor XIII is not activated, and the clot remains unstable. The fibrin formed by the action of these snake venoms on fibrinogen is deposited in the microcirculation where it is rapidly lysed by the physiologic vascular fibrinolytic system (1). Within minutes of ancrod administration there is a significant reduction in plasma fibrinogen levels. Additional trials are needed to better define the role of ancrod in the management of stroke, deep vein thrombosis, myocardial infarction, and peripheral arterial thrombosis (27). However, ancrod has been used as a heparin substitute in cardiopulmonary bypass and in individuals with heparin-induced thrombocytopenia (1, 31, 32).

**Coumarin agents**

Coumarin agents have a proven role in the treatment of venous thrombosis and the prevention of venous thromboembolism. Over the past 20 years, important advances have been made in the clinical use of oral anticoagulants and in understanding their mechanisms of action. In addition, the development of the standardized International Normalized Ratio method of reporting prothrombin time has helped to ensure a uniform and proper intensity of therapeutic ranges that decrease the rate of bleeding without reducing efficacy. The oral anticoagulants are 4-hydroxycoumarin compounds that exert their anticoagulant effect by inhibiting the hepatic synthesis of 4 vitamin K–dependent coagulation proteins: factors II (prothrombin), VII (proconvertin), IX (Christmas factor), and X (Stuart-Power factor). The coumarin compounds in common clinical use are warfarin (Coumadin), acenocoumarol, and phenprocoumon. These oral anticoagulants induce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its vitamin K epoxide (28, 30).

Vitamin K is a cofactor. It has a quinone structure with a phytol side chain on the basis of which derivatives are designated as vitamin K₁ (from dietary vegetable sources) and vitamin K₂ (from intestinal bacterial synthesis). The hepatic synthesis of functional forms of factors II, VII, IX, and X depends on vitamin K, the cofactor for a carboxylase enzyme that adds γ-carboxyl groups to glutamic acid residues contained on the nascent coagulation proteins II, VII, IX, and X (4) (Figure 6). Adjacent dicarboxylic residues form a negative charge density that serves as a tight calcium-binding site. This action allows the trace vitamin K–dependent proteins to form calcium bridges to phospholipid surfaces and to achieve sufficient local concentration for activation and interaction with their cofactors and substrates (3). The carboxylation of the nascent endogenous precursor proteins requires the enzyme carboxylase, vitamin K hydroquinone (KH₂), and O₂ to fix CO₂ to the glutamyl residues on these proteins and to convert them to their functional form, the C-γ-carboxyglutamyl residues. A microsomal vitamin K epoxidase activity converts vitamin KH₂ to vitamin K 2,3-epoxide during the carboxylation reaction. The hepatic cell microsomes recycle this metabolite (the 2,3-epoxide) to hydroquinone (vitamin KH₂), the active form. The microsomal activities involved in the metabolic conversions of the liver vitamin K pool are epoxide reductase, which converts the 2,3-epoxide to vitamin K quinone, and vitamin K quinone reductase, which converts the vitamin K quinone to vitamin KH₂, the active form (30) (Figure 6).

The current theory of the action mechanism of warfarin is that the inhibition of vitamin K epoxide
reduction to vitamin K quinone by inhibiting the epoxide reductase prevents efficient recycling of
the vitamin and its subsequent conversion to its enzymatically active form (KH₂), thereby limiting
the action of carboxylase and consequently carboxylation. The quinone reductase that converts
vitamin K quinone into vitamin KH₂ is less sensitive to warfarin inhibition. This explains the
ability of administered vitamin K to counteract the hemorrhagic condition resulting from a massive
dose of warfarin. The 4-hydroxycoumarin anticoagulant effects involve not only interference in the
reduction of vitamin K epoxide to vitamin K quinone, but also the reduction of vitamin K quinone
to vitamin K hydroquinone (28, 30). Hemorrhagic complications of 4-hydroxycoumarin will be
less responsive to treatment with vitamin K because of its interference in the reduction of the
vitamin K quinone (the therapeutic form) to the hydroquinone (30).

SUMMARY

Throughout the 1990s, an enormous volume of basic science and clinical research has aimed at
achieving an optimal balance between preventing thrombosis and maintaining protective
hemostasis. We have endeavored to review the traditional anti-thrombotic agents as well as the
new strategies for procuring more effective and safer agents that inhibit specific sites in the
sequences of platelet aggregation and the coagulation proteins. A few agents (e.g., GUR76-23,
abciximab, echistatin, and eptifibatide) block platelet GPIIb/IIIa. Inhibitors of specific platelet
agonist–receptor interactions (i.e., ADP, thromboxane A₂, thrombin, and collagen) include
ticlopidine and clopidogrel. Inhibitors of arachidonic acid metabolism include omega-3 fatty acids,
aspirin, and NSAIDs. Agents that inhibit thrombin and its production include hirudin, argatroban,
D-Phe-Pro-Arg-chloromethyl ketone, APC, tick anticoagulant peptide, anti-statin, and TF-pathway
inhibitor.

Many areas of research are being investigated, including intracellular adhesion molecules and their
role in the pathogenesis of stroke, additional areas of platelet inactivation, and molecules from
exotic species in the animal kingdom. For example, the human parasite *Necator americanus*
(hookworm) possesses in its saliva an anticoagulant that is 1000 times more potent than heparin.

Upcoming research in the new millennium will further our knowledge of hemostasis and
antithrombotic agents. It will allow more extensive exploration of the previously mentioned
parasites (i.e., leeches, ticks, hookworms) and other zoologic species in the hope that newer agents
will bring us closer to achieving the perfect, sought-after balance between circumventing
thrombosis and maintaining hemostasis.

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