THROMBOGENESIS

# Role of Platelet Activation and Fibrin Formation in Thrombogenesis

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Further progress in the search for more effective but safe antithrombotic agents is coupled to an improved understanding of the factors involved in arterial and venous thrombogenesis. Although arterial thrombosis is initiated by formation of a layer of platelets on modified endothelium or subendothelial constituents and subsequent recruitment of passing-by platelets, this phenomenon is not sufficient to lead to a full thrombus. Further growth of such a platelet mass depends, to a large extent, on the presence of free thrombin. Thrombin is mainly generated by activation of factor XI on the platelet contact with collagen. In addition, thrombin leads to formation of fibrin, which maintains the stability of the arterial platelet thrombus and is the main component of the venous thrombus. The search for agents that inhibit platelet activation and thrombin formation is, therefore, a logical endeavor.

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## Nonactivation of Platelets in the Normal Circulation

Platelets are versatile fragments of membrane-enclosed cytoplasm that travel as single elements in the bloodstream. Although sticky, they do not adhere to the normal intact endothelial surface of vessel walls. Even after removal of endothelium, the exposed basement membrane is hardly reactive to platelets. Deeper located structures such as fibrillar collagen (particularly types I and III), elastin and microfibrils, when exposed, become covered with platelets, but soon gain the property of nonreactiveness to further platelet deposition. When these platelets are progressively detached from the denuded subendothelium, the surface still remains nonthrombogenic as is the neointima that forms after deendothelialization. However, if this neointima is damaged a second time, microthrombi rapidly form, particularly when injured smooth muscle cells are exposed (1,2).

It is difficult to explain why platelets do not adhere to normal endothelium. One explanation is that both platelets and endothelium have a negative electrical charge and, therefore, would be mutually repulsive (3). A second hypothesis states that the platelet repulsion is an active process, involving continuous synthesis by cells of prostacyclin (4). However, this hypothesis does not explain why endothelium treated with aspirin does not induce platelet adhesion (5).

Moreover, repeated stimulation of endothelial cells in vivo (6) and in tissue culture results in rapid refractoriness of prostacyclin release. A third hypothesis suggests the continuous synthesis by endothelium of a chemorepellent lipoxygenase metabolite coded LOX (7).

Obviously, there is no satisfactory answer to the simple question: Why do platelets remain apparently indifferent to the vessel wall in the arterial circulation with its high pressure system, to the intimate contact with the vessel structures in the microcirculation and to the slow flow conditions in veins?

## Platelet Adhesion as the Initial Step in Thrombus Formation

One of the first events in the thrombotic process is the *adherence* of platelets to modified or injured endothelial cells (Fig. 1). This is a complex process that involves structures of the subendothelium, physical factors, plasma proteins and platelet receptors.

**Subendothelium.** Among the constituents of normal subendothelium, the various types of collagen and its ubiquitous companion fibronectin are the most active toward platelets. Platelet adhesion occurs more readily to collagen structures that lie deep within vascular tissues (such as in an ulcerated atherosclerotic plaque) than to those that are found immediately beneath the endothelium (8,9).

**Physical factors.** An increase in shear rate will cause, up to a certain value, more pronounced platelet adhesion. Furthermore, adherence is linearly related to the platelet
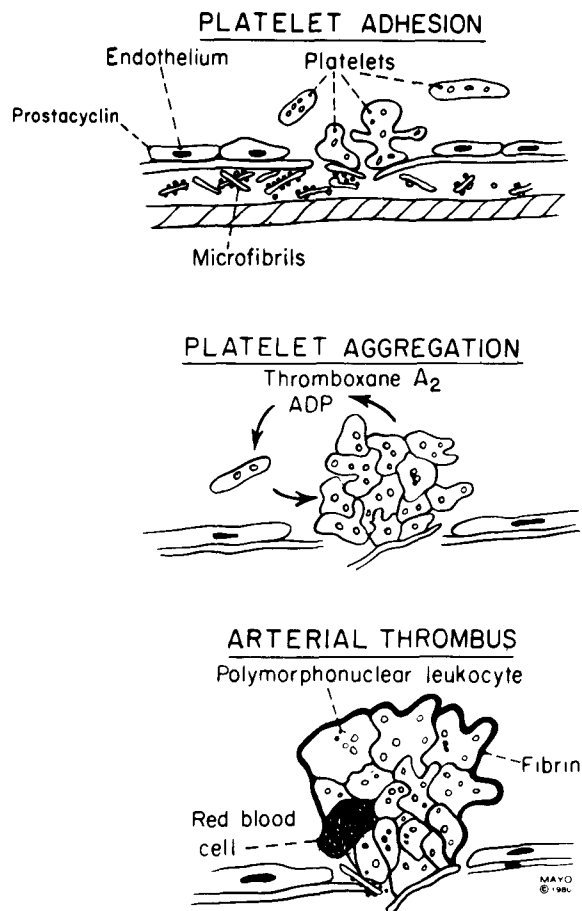
## PLATELET ADHESION



## PLATELET AGGREGATION

## ARTERIAL THROMBUS

**Figure 1.** Phases of arterial thrombus formation. Scheme of microscopic events. ADP = adenosine diphosphate. (Reprinted with permission from Fuster V, Chesebro JH [8].)

in platelets is also involved in the factor VIII/von Willebrand-mediated process; raising the cyclic AMP level impedes adhesion.

*On artificial surfaces, the process of platelet adhesion is less well understood.* A film of adsorbed plasma proteins forms immediately after exposure of the surface to blood, and apparently different surfaces become coated with different proteins that, by means of their surface charge, react differently with platelets (13). Among the adsorbed plasma proteins that have been studied are fibrinogen, which tends to increase adherence, and albumin, which tends to have the opposite effect.

## Shape Change and Platelet Aggregation

Attachment of platelets to surfaces (adhesion) precedes attachment of platelets to each other (aggregation) (Fig. 1). These two phenomena are separate, although it is difficult to study them independently.

**Platelet membrane receptors.** There are several specific receptors on the platelet membrane. On formation of an activator (see below) and receptor complex, a signal is transferred to the interior of the cell (Fig. 4). Most agonists seem to act by means of a common pathway, namely, the hydrolysis of polyphosphatidyl-inositol into diacylglycerol and inositol triphosphate, the latter being responsible for calcium mobilization. Such cytoplasmic increase in calcium is responsible for 1) the contraction of the platelet, 2) the secretion of its constituents (ADP and serotonin), and 3) the activation of the membrane phospholipases and the

number, is increased in a constant flow system as compared with a pulsatile one, is dependent on viscosity and is strikingly enhanced by the presence of red blood cells. The latter effect is partly due to the fact that in the flowing blood, red cells occupy a central position and push the platelets toward the periphery and, thus, increase platelet-vessel wall contact. Blood turbulence at the branching points or in the vicinity of a vessel injury may also release adenosine diphosphate (ADP) from red cells, ADP stimulates the adherence of platelets.

**Plasma proteins.** A large multimeric protein termed factor VIII/von Willebrand factor binds to the subendothelium and gives rise to platelet adherence.

**Platelet receptors.** On their surface, platelets have *glycoproteins*, and one of them (glycoprotein Ib) plays a part in the adhesive process to subendothelium (Fig. 2 and 3) (10,11). The deficiency of this protein, as in the bleeding disorder Bernard-Soulier syndrome, or antibodies against this glycoprotein, prevents the adhesion of platelets to damaged vascular endothelium (12). The balance between free calcium ions and cyclic adenosine monophosphate (AMP)

**Figure 2.** Binding of adhesive macromolecules (von Willebrand factor [vWF] and fibrinogen) to platelet membrane glycoproteins. Both macromolecules are enlarged compared with platelets. Fibrinogen binds to the glycoprotein IIb-glycoprotein IIIa-Ca$^{++}$ complex, which is formed in presence of adenosine diphosphate (ADP) derived from exogenous sources or secreted from platelet storage granules. von Willebrand factor can either bind to glycoprotein Ib in absence of ADP or to the glycoprotein IIb-glycoprotein IIIa-Ca$^{++}$ complex in the presence of ADP. (Reprinted with permission from Hawiger J, Kloczewiak, Timmons S. Platelet-receptor mechanisms for adhesive macromolecules. In: Oates JA, Hawiger J, Ross R, eds. Interaction of Platelets With the Vessel Wall. Bethesda: American Physiological Society, 1985:1–19.
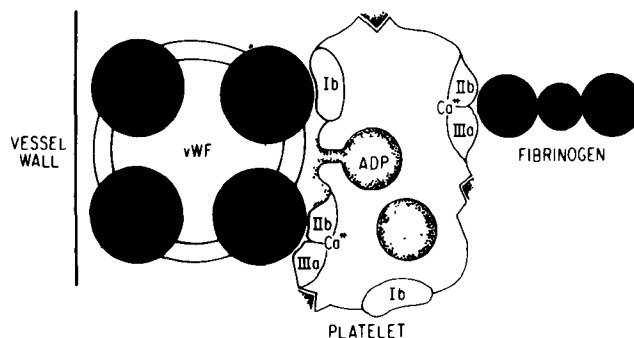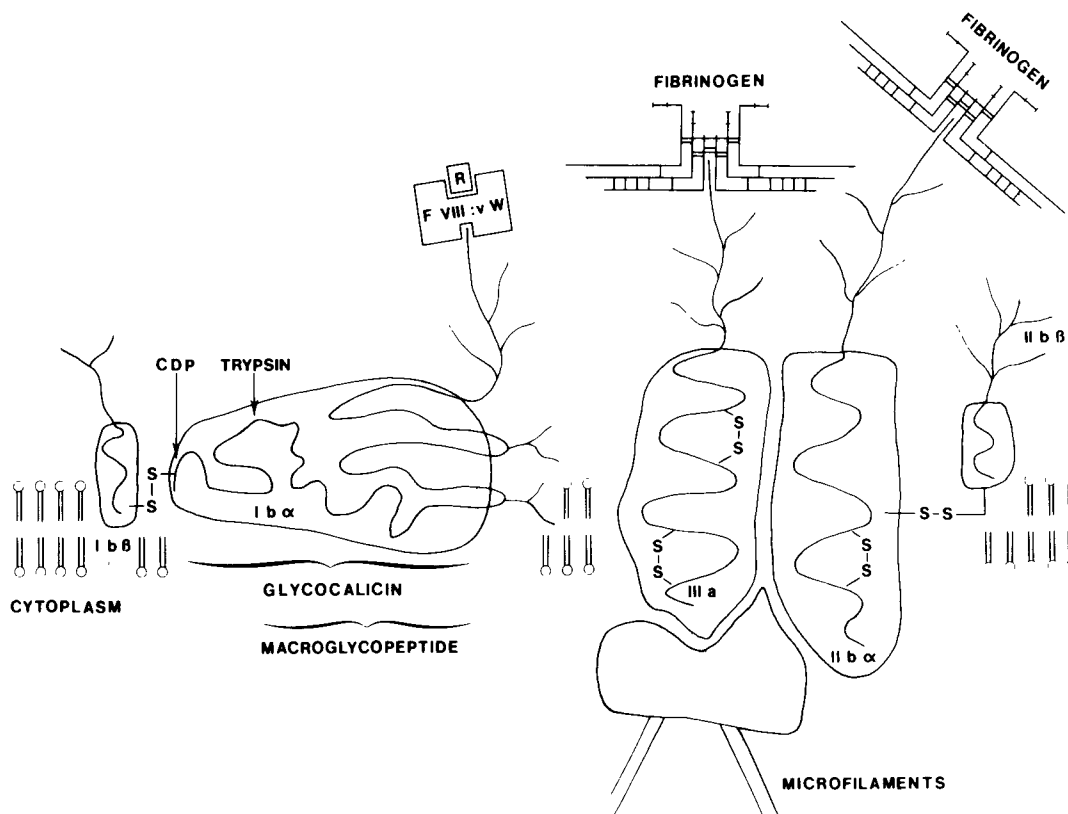
Figure 3. Orientation of glycoproteins Ibα, Ibβ, IIbα, IIbβ and IIIa on platelet plasma membrane. A calcium-dependent protease (CDP) hydrolyzes glycoprotein Ibα, producing glycocalicin, and trypsin produces macroglycopeptide as indicated. Ristocetin bridges the interaction of factor (F) VIII: von Willebrand (vW) factor with glycoprotein Ib (absent in Bernard-Soulier syndrome). Glycoproteins IIb and IIIa (defective in thrombasthenia) interact with fibrinogen after platelet activation. S = disulfide bond. (Reprinted with permission from Verstraete M, Vermylen J [20].)

arachidonate pathway with synthesis and secretion of thromboxane A$_2$ (Fig. 4).

**Platelet contraction.** Specifically, after activation, platelets are transformed from discs into spiny spheres: there is a centripetal movement of the circumferential band of microtubules forcing the granules towards the center of the cell. As mentioned, such a contraction, as in most if not all cells, is due to a cytoplasmic increase in calcium, an activation of myosin light chain kinase through calmodulin and interaction of phosphorylated myosin with actin.

**Platelet aggregation.** During the process of platelet activation, ADP is released and is a potent inducer of platelet aggregation, provided divalent calcium and intact fibrinogen are present. As a first step, ADP binds to a specific receptor, independent of calcium ions, and induces the shape change of the platelets. This reaction leads to exposure of fibrinogen binding sites on the platelets and depends on divalent cat-

ions. Fibrinogen then binds to its receptors on adjacent platelets, thereby forming bridges between these cells that are the key factors in establishing platelet aggregation.

**Synthesis of thromboxane A$_2$.** Part of the platelet calcium made available during activation seems to activate platelet membrane phospholipases, which catalyze the liberation of arachidonic acid. This polyunsaturated fatty acid is then converted into cyclic endoperoxides by means of the cyclooxygenase enzyme; the latter is transformed into thromboxane A$_2$ by means of the thromboxane synthetase enzyme. Similar to the release of ADP, the synthesis and release of thromboxane A$_2$ activates the surface membrane of neighboring platelets, resulting in an increase in cytoplasmic calcium and, therefore, in further release of ADP and "stickiness" or aggregation of platelets.

**Extrinsic activation of platelet activation.** In pathologic situations such as when an atherosclerotic plaque ruptures, there are extrinsic activators that may be much more potent in independently triggering calcium release and platelet aggregation than the physiologic low concentration of ADP and thromboxane A$_2$ (14–16). These are the already mentioned exposed collagen from the vessel wall and thrombin resulting from the activation of the intrinsic and extrinsic coagulation systems, large amounts of ADP released from erythrocytes (hemorrhage with lysis) and presumably platelet-activating factor released from the neighboring cells (for example, endothelial cells). These powerful extrinsic ag-

**Figure 4.** Membrane receptors of the arachidonic acid-dependent and independent pathways, leading to mobilization of $Ca^{++}$ from storage sites and its cyclic nucleotide-dependent inhibition. AMP = adenosine monophosphate; ATP = adenosine triphosphate; $PGG_2$ and $PGH_2$ = prostacyclin $G_2$ and $H_2$, respectively; $\uparrow$, $\uparrow\uparrow$ and $\uparrow\uparrow\uparrow$ = increased availability of calcium. (Reprinted with permission from Verstraete M, Vermylen J [20].)
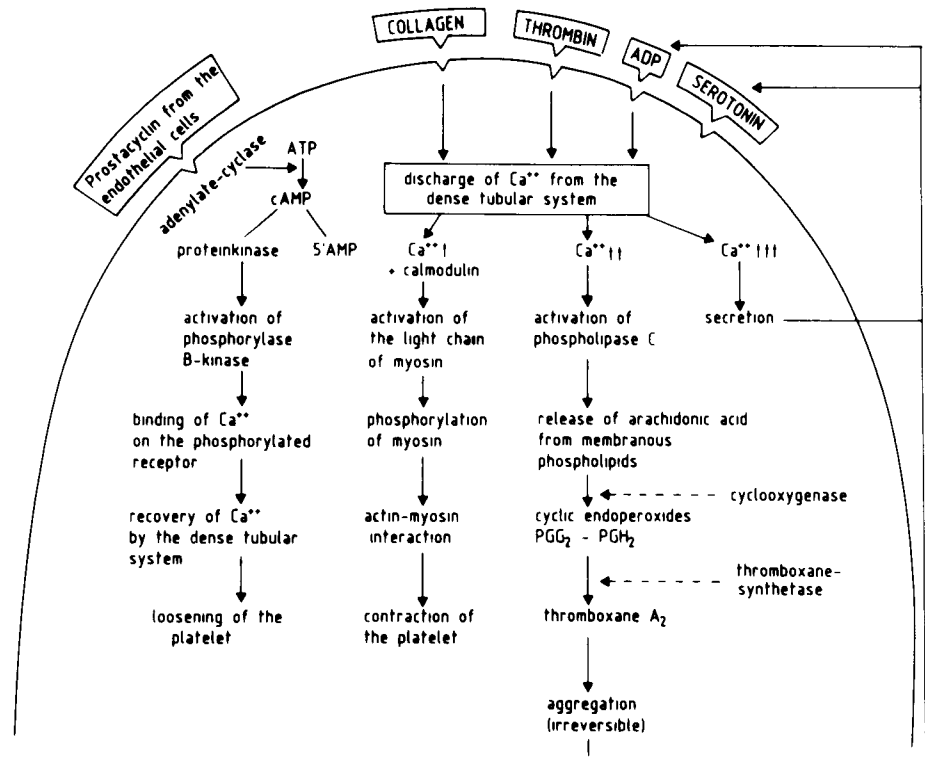
gregating factors may, in part, explain why platelet inhibitor drugs may be inefficient in preventing some arterial thrombotic phenomena (16).

## Secretion of Platelet Granules

Platelets contain three types of secretory granules, which are listed in Table 1. Besides being extruded from the platelet at different sites, the quantitative response of the various granules to stimuli differs. Alpha granule release is induced by lower concentrations of stimuli than are required for secretion of dense body constituents, which could imply a difference in the energy requirement for release from the two kinds of granules.

*Thrombin* causes the alpha granules to fuse with each

**Table 1.** Content of Platelet Granules

Dense granules
  5-Hydroxytryptamine (serotonin), ADP, ATP, calcium
Alpha granules
  Proteins not present in plasma: platelet factor IV, beta-thromboglobulin, platelet derived growth factor
  Proteins present in plasma: fibrinogen, fibronectin, von Willebrand factor, albumin, factor V, thrombospondin
Lysosomes
  Acid hydrolases
  Cathepsin D, E
Peroxysomes
  Catalase

other and with elements of the surface-connected canalicular system. Platelet factor IV and beta-thromboglobulin are tetramers of basic peptides present in alpha granules, which, after activation of platelets, are released in the bloodstream. Normal plasma does not contain measurable levels of these proteins so that their presence in plasma is a marker of platelet activation and secretion (17).

*ADP* is released by platelets in quantities sufficient to induce aggregation. Thus, it is apparent that secretion of dense body contents is an important feedback mechanism for promoting growth of the platelet thrombus.

## Activation of the Clotting Mechanism: Role of Thrombin and Fibrin Formation

During the process of platelet adhesion and aggregation, the clotting mechanism may be activated and thrombin generated; this further promotes platelet aggregation and, most importantly, leads to formation and polymerization of fibrin, which stabilizes the platelet mass and allows the arterial thrombus to resist the high intravascular pressure (Fig. 1).

**Formation of fibrin.** As discussed in more detail in this symposium by Wessler and Gitel (18), the formation of thrombin is the end point of a chain of enzymatic reactions in which a proenzyme is activated to an enzyme, which in turn activates another proenzyme and so on (Fig. 5). These proenzymes (clotting factors) are present in the fluid phase of blood; their level is very low. Their interaction is mark-

## INTRINSIC ACTIVATION

negatively
charged surface
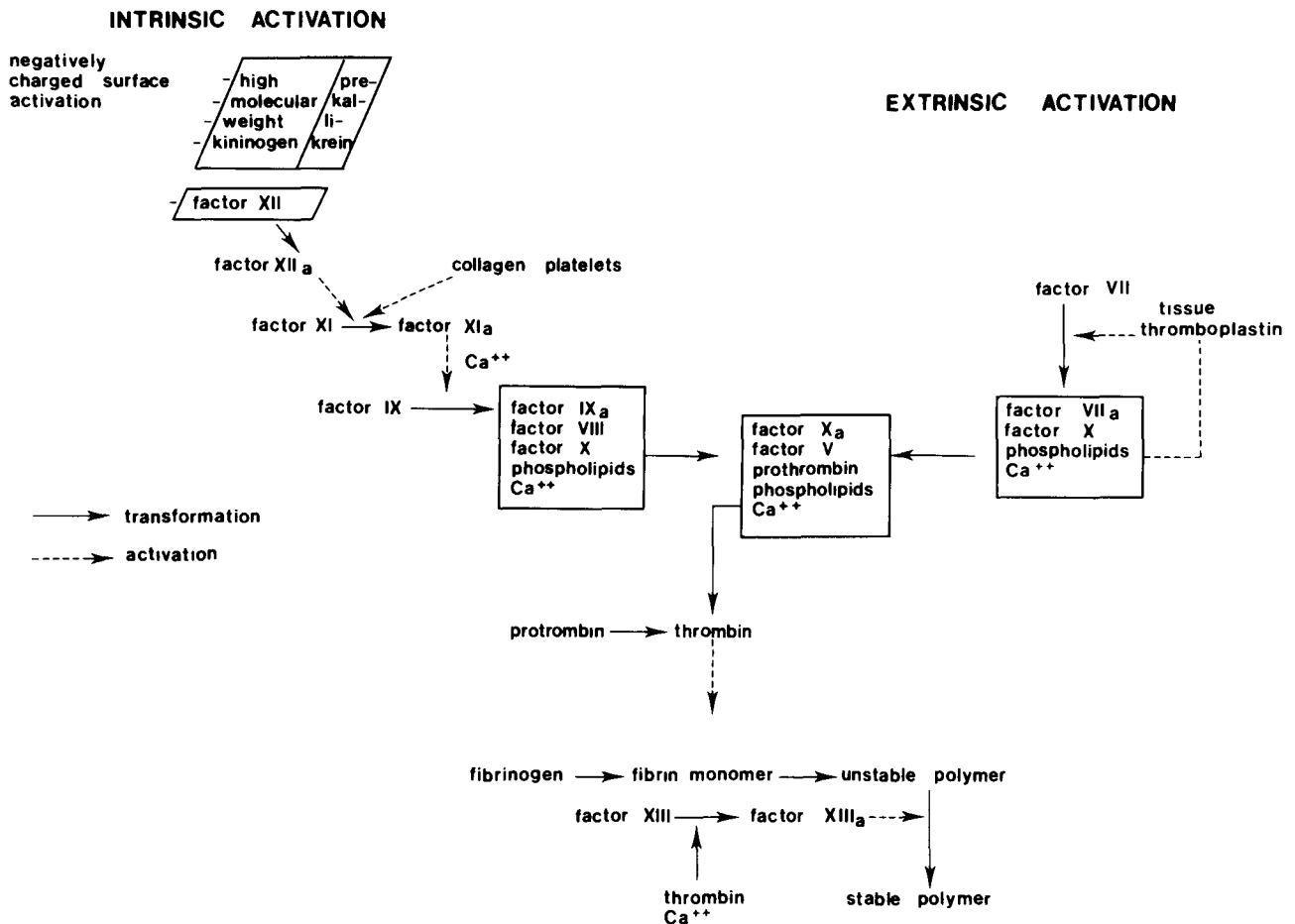activation

### EXTRINSIC ACTIVATION

Figure 5. Mechanisms of clotting factor interactions. Coagulation is initiated by either an intrinsic or extrinsic pathway. In the intrinsic pathway, the phospholipid is furnished by platelets. In the extrinsic system, the phospholipid portion of tissue thromboplastin functions on the activation of factor X. From factor Xa on, both pathways converge on a common path. Omitted from the diagram are inhibitors of the various steps, the augmentation of action of each pathway by activated factors and the interaction between the intrinsic and extrinsic systems. (Reprinted with permission from Verstraete M, Vermylen J [20].)

edly enhanced by their being adsorbed and concentrated on surfaces. Blood coagulation, therefore, can be considered a series of surface-catalyzed events. For the clotting process in vivo, the platelets would be a major supplier of these surfaces. The chain of reaction is viewed as an amplification system, allowing activation of a few molecules at the outset to result in an explosive generation of thrombin (19).

**Intrinsic versus extrinsic pathways of activation.** Traditionally, a division is made between the "intrinsic" and "extrinsic" pathways of activation (Fig. 5) (20). In the intrinsic pathway, all necessary factors are present in the circulating blood with a negatively charged surface (for example, subendothelial collagen) or with a foreign surface

(for example, the glass wall of a test tube). In the extrinsic activation pathway, it is not a plasma component but tissue fluid that initiates the blood coagulation process; after vessel damage this tissue factor, called tissue thromboplastin, mixes with the blood and starts the coagulation process. After activation of factor X, the two pathways merge into one. In addition, both appear to be equally necessary to ensure normal hemostasis.

**Mechanisms of clotting factor interactions (Fig. 6).** More specifically, in the intrinsic pathway, coagulation is initiated by the adsorption of factor XII onto a foreign surface or collagen. Both kallikrein and high molecular weight kininogen are required for the rapid surface activation of factor XII. In addition, high molecular weight kininogen increases the reactivity of factor XIIa in the conversion of factor XI to XIa. Factor XIa converts factor IX to the active protease factor IXa, whereupon factors VIII and IXa form a complex, activating factor X. Both platelet phospholipid, made available by aggregated platelets, and calcium ($Ca^{2+}$) are essential for maximal activation of factor X. In the presence of factor V, calcium and platelet phospholipid, factor Xa subsequently converts prothrombin (factor II) to thrombin. The extrinsic pathway is triggered by the release of the tissue thromboplastin, a protein-phospholipid mixture
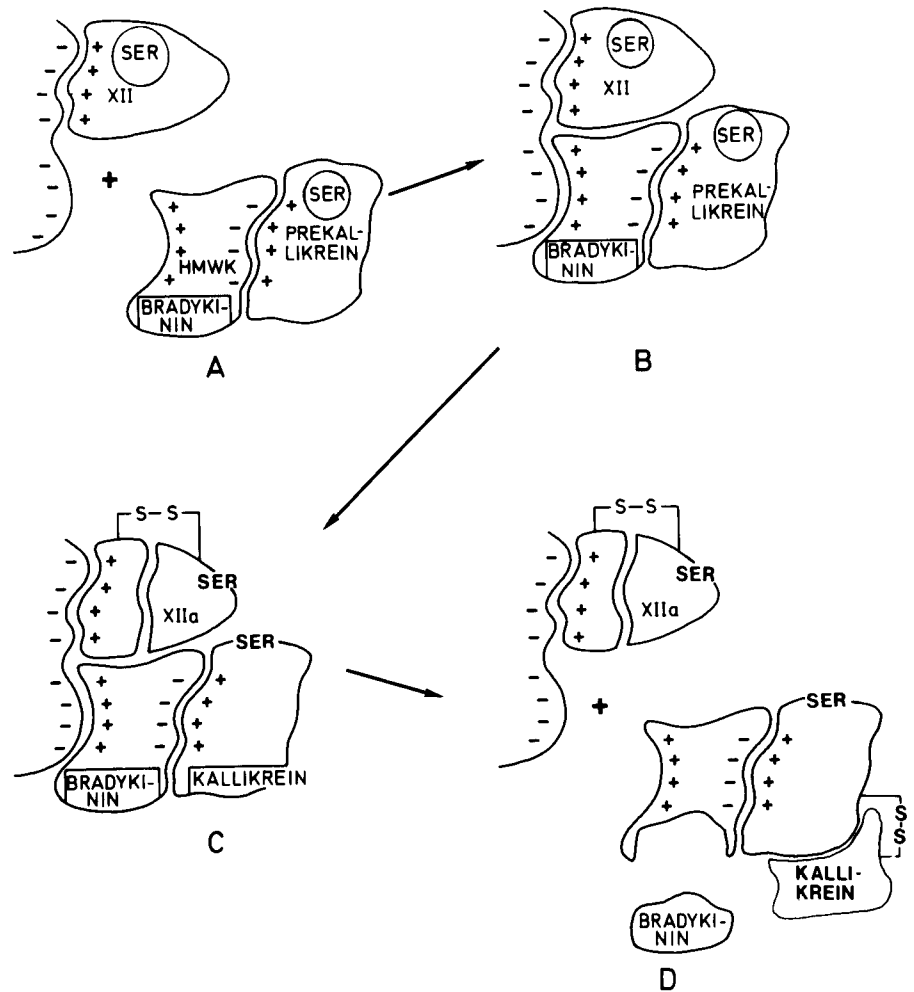
**Figure 6.** In the intrinsic pathway of coagulation, contact activation refers to a series of reactions following adsorption of factor XII and XI as well as prekallikrein and high molecular weight kininogen (HMWK) to highly negatively charged surfaces. The contact activation does not require calcium and results in surface-mediated conformational changes of the molecules. **A to D** = sequence of events. S = disulfide bond; SER = serotonin. (Reprinted with permission from Verstraete M, Vermylen J [20].)

that activates factor VII to VIIa. Together they serve as cofactors for the activation of both factor IX and factor X. Once factor Xa is formed, thrombin production proceeds as described. Thrombin cleaves fibrinogen to fibrin, activates factor XIII, which stabilizes fibrin and also, as previously mentioned, induces platelet aggregation.

## Endogenous Inhibitors of Thrombus Formation: The Role of Prostacyclin, Protein C, Fibrinolysis and Antithrombin III

During platelet activation and fibrin formation there are endogenous mechanisms that tend to limit thrombus for-

**Figure 7.** Protein C can be activated by thrombin, and this reaction is greatly accelerated by a protein that is present on the endothelial cell surface (this cofactor has been termed thrombomodulin). Protein C inhibits activated factors Va and VIIIa and initiates fibrinolysis. The inhibition of factors Va and VIIIa is also enhanced by another vitamin K-dependent moiety, protein S. (Reprinted with permission from Wessler S, et al. Warfarin: from bedside to bench. N Engl J Med 1984;311:645–52.)
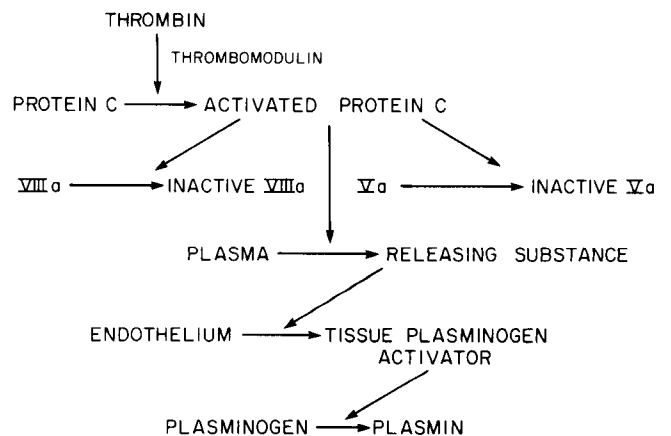
mation. The four mechanisms that appear to be most important are the generation of prostacyclin, the activation of protein C, fibrinolysis and the presence of antithrombin III.

**Generation of prostacyclin.** Prostacyclin, a compound discovered by Moncada et al. (22), seems to be the main prostaglandin metabolite in vascular tissue. It is most highly concentrated on the intimal surface, particularly the endothelium, and progressively decreases in activity toward the adventitial surface. Prostacyclin is a potent systemic vasodilator and, most importantly, is the most potent inhibitor of platelet aggregation yet discovered. The platelet-inhibitory action of prostacyclin is related to an activation of the platelet membrane adenylate cyclase enzyme, which leads to an increase in platelet cyclic adenosine 5'-monophosphate and a decrease in platelet-free calcium and, therefore, in platelet susceptibility to activation. Similar to the synthesis of the prostanoid thromboxane $A_2$ by the platelet (as previously discussed), the vessel wall synthesizes prostacyclin from its own precursors. That is, arachidonic acid is converted into cyclic endoperoxides by means of cyclooxygenase enzyme, and such endoperoxides are subsequently converted into prostacyclin by means of prostacyclin synthetase enzyme. It has been shown that prostacyclin can be produced by the cells of the vessel wall in response to stimulation by endothelial injury or thrombin; most importantly, the platelets adhering to sites of vascular damage not only release thromboxane $A_2$, which promotes the concomitant aggregation of platelets and release of endoperoxides, which potentiate prostacyclin synthesis by the arterial wall. Thus, the process of platelet aggregation and thrombosis may tend to be limited or prevented.

In this context, Greenland Eskimos, who have a bleeding tendency but in whom thrombosis or atherosclerosis does not develop, seem to have little platelet thromboxane $A_2$ and a substantial amount of a prostacyclin-type substance (prostaglandin $I_3$), all of these substances presumably related to diet (23,24). Indeed, it has been suggested that an imbalance between platelet proaggregating and disaggregating activity of both prostaglandin systems (thromboxane $A_2$ and prostacyclin) may be an important factor leading to thrombosis and vascular disease (25). Thus, it has been postulated that certain of the so-called risk factors of atherosclerosis and thrombosis may promote vascular disease by altering this thromboxane $A_2$-prostacyclin equilibrium system (8).

**Activation of protein C (Fig. 7).** Thrombin associated with its endothelial cofactor thrombomodulin has an additional essential role, that of transforming protein C to activated protein C. Activated protein C limits thrombosis (by destroying factors VIIIa and Va) and initiates fibrinolysis (that is, it makes available a substance that releases tissue-derived plasminogen activator, which in turn converts plasminogen to plasmin) (Fig. 7). Plasmin cleaves fibrin into soluble fragments and degrades fibrinogen into fibrin degradation or split products (19). Patients who are congenitally deficient in protein C are prone to thrombosis, an indication of the importance of this protein (6). Also, recurrent thrombosis has been reported among persons with a low fibrinolytic response (27). Apart from protein C, fibrin in itself favors the release of tissue plasminogen activator. Because plasminogen binds to fibrin during fibrin formation and is only active when such binding takes place, fibrinolysis occurs only in the thrombus (28). In this manner, plasmin generation proceeds within the thrombus, where it is protected from rapid inactivation by plasma alpha$_2$-antiplasmin.

**Presence of antithrombin III.** In addition to prostacyclin, protein C and fibrinolysis, which limit thrombosis, intravascular coagulation is regulated by other naturally occurring plasma inhibitors. The most important is antithrombin III (29). In addition, the endothelial cell tends to bind free thrombin by means of a cofactor that favors a thrombin-antithrombin III complex (30). A deficiency in antithrombin III is associated with thrombosis, evidence of the clinical relevance of this control mechanism (31).

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