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Haemostasis

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Abstract When the continuity of the vascular endothelium is disrupted, platelets and fibrin seal off the defect. Haemostatic processes are classified as primary (mainly involving platelets) and secondary (mainly related to fibrin formation or blood coagulation). When the blood clot is no longer required for haemostasis, the fibrinolytic system will dissolve it. The pivotal ligand for initial platelet recruitment to injured vessel wall components is von Willebrand factor (vWF), a multimeric protein present in the subendothelium and in plasma, where it is conformationally activated by shear forces. Adhering activated platelets recruit additional platelets, which are in turn activated and form a platelet aggregate. Coagulation is initiated by a reaction, activating factors IX and X. Once critical amounts of factor Xa are generated, thrombin generation is initiated and soluble fibrinogen is converted into insoluble fibrin. Excessive thrombin generation is prevented via inhibition by antithrombin and also via downregulation of its further generation by activation of the protein C pathway. Activation of the fibrinolytic system results from conversion of the proenzyme plasminogen into the active serine proteinase plasmin by tissue-type or urokinase-type plasminogen activators. Plasmin digests the fibrin component of a blood clot. Inhibition of the fibrinolytic system occurs at the level of the plasminogen activator (by plasminogen activator inhibitors) or at the level of plasmin (by α_2-antiplasmin). Together, these physiological processes act to maintain normal functioning blood vessels and a non-thrombotic state.

Keywords Haemostasis · Thrombosis · Bleeding · Platelets · Coagulation · Fibrinolysis

1 Introduction

Integrity of the vascular wall is a prerequisite for normal functioning blood vessels and for maintenance of a non-thrombotic state. When the continuity of the vascular endothelium is disrupted, platelets and fibrin seal off the defect, and the fibrinolytic system dissolves the blood clot. The endothelial cells, which form a monolayer lining the inner surface of blood vessels, synthesise and release activators and inhibitors of platelet aggregation, blood coagulation and fibrinolysis and thus play an active role in the regulation of these systems by providing both procoagulant and anticoagulant substances.

Vessel wall injury exposes subendothelial matrix and collagen fibres to flowing blood; circulating platelets adhere to these structures and initiate arrest of blood flow. Both subendothelial and circulating vWF play an important role in platelet adhesion to sites of injury, in particular in the arterial circulation, where shear forces conformationally activate vWF. Adhering activated platelets recruit additional platelets from the flowing blood, which are in turn activated

via secondary amplification loops resulting in the formation of a platelet aggregate. Activation of the coagulation cascade on the platelet surface results in the formation of a fibrin network that provides a matrix for cell migration, thus supporting wound healing.

In the current model of blood coagulation, the extrinsic PTase reaction initiates coagulation (Broze 1995a, b). Once critical amounts of factor Xa (required for the initiation of thrombin generation) are formed, the extrinsic PTase reaction is efficiently turned off by the tissue factor pathway inhibitor (TFPI), and further formation of thrombin is maintained via positive feedback mechanisms involving thrombin-induced activation of factors V, VIII and XI. Thrombin converts fibrinogen to fibrin. Excess thrombin is efficiently inhibited by its physiological inhibitor antithrombin and downregulates its own generation via stimulation of the protein C pathway.

The fibrinolytic system generates a serine proteinase, plasmin, that degrades fibrin into soluble fibrin degradation products, and thus plays an important role in the dissolution of blood clots and in the maintenance of a patent vascular system.

2
Platelets in Haemostasis

Platelet recruitment to injured vessel wall components depends on several platelet receptors. Strong adhesion to fibrillar collagens I and III, localised in the deeper layers of the vasculature, is determined by specific collagen receptors such as glycoprotein (GP)VI and GP $\alpha_2\beta_1$ integrin (see Sect. 2.3). Platelets adhere to subendothelial vWF. This is a multimeric protein, synthesised by endothelial cells (Jaffe et al. 1974) and stored in specialised inclusion particles, the Weibel–Palade bodies. vWF is released in the circulation and deposited in the subendothelium. Although platelets have several integrin receptors that mediate adhesion to extracellular matrix-associated fibronectin and laminin (Bastida et al. 1987; Hindriks et al. 1992), vascular wall-associated vWF appears to be the pivotal ligand for initial platelet recruitment.

2.1
Von Willebrand Factor

The central role of vWF in haemostasis is supported by several observations. First, vWF is associated with collagen VI in the subendothelium (Rand et al. 1993). Upon de-endothelialisation, subendothelial vWF becomes a potent vascular ligand triggering platelet rolling and tethering. Second, circulating vWF contributes to haemostasis in a dual manner: It carries factor VIII and binds to vascular collagens exposed to the blood stream (Bolhuis et al. 1981). Third, it

participates in platelet–endothelial cell interactions and is thus at the interface between haemostasis and inflammation (Theilmeier et al. 2002).

2.1.1 Structure of vWF

The mature vWF subunit (~250 kDa) consists of four types of repeating functional domains arranged in the following sequence: D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (Shelton-Inloes et al. 1986). The binding site for factor VIII is located within the D′ domain, and that for platelet GPIbα within the A1 domain. A platelet integrin $\alpha_{IIb}\beta_3$ binding sequence Arg-Gly-Asp (RGD) is located in the C1 domain, and the main collagen binding site is located in the A3 domain (Romijn et al. 2003).

The A1 domain is structurally shaped by a disulphide bridge between Cys^{509} and Cys^{695} (Sugimoto et al. 1991). X-ray diffraction studies of the A1 domain revealed a globular shape, comprising a central core constituted of six hydrophobic β-strands, surrounded by six amphipathic α-helices (Celikel et al. 1998; Jenkins et al. 1998). Analysis of naturally occurring loss-of-function mutations, together with mutagenesis and GPIbα peptide docking studies (Cruz et al. 2000; Matsushita et al. 2000; Bonnefoy et al. 2003), has identified a central front groove on the A1 domain next to strand β3, as part of the binding site for GPIbα. Recently, the crystal structure of a gain-of-function A1 domain mutant in complex with the amino-terminal domain of GPIbα (also containing a gain-of-function mutation) confirmed that the frontal part of A1 constitutes the contact area for GPIbα. Furthermore, two distinct areas of tight interaction were revealed, the first and most extensive contact site located near the top of A1, the second involving residues near the bottom face of A1 (Huizinga et al. 2002). In a shear stress field, vWF A1 domains undergo a conformational change, triggering binding to GPIb (Ruggeri 1993; Siedlecki et al. 1996).

The A3 domain (aa 920–1,111) contains the major binding site for fibrillar collagens I and III. Unlike the I domain of integrin chains α_1, α_2, α_{10} and α_{11}, the A3 domain lacks a functional metal ion dependent adhesion site (MIDAS) motif (Pietu et al. 1987). Binding to collagen occurs via residues located in the strand β_3 and the loop $\alpha_3\beta_4$ in the lower half of the front face of A3 (Romijn et al. 2003).

2.1.2 Function of vWF

In small arterioles, in stenosed arteries and at atherosclerotic plaques in partially occluded arteries, platelet adhesion occurs, controlled by elevated fluid shear stress. GPIb/IX/V on flowing platelets interacts with immobilised vWF, initiating platelet tethering to the damaged area (Savage et al. 1992). During translocation, the platelet is progressively activated and adheres by forming

tight bonds between platelet–membrane-activated integrins and vessel wall components, such as collagen and vWF. Subsequently, circulating platelets recognise adhesive molecules (mainly vWF and fibrinogen) on already adhered platelets and initiate platelet aggregation. At elevated shear forces, platelet recruitment and thrombus growth are mainly dependent on platelet binding to vWF, although fibrinogen binding to platelet integrin $\alpha_{IIb}\beta_3$ is also required for thrombus consolidation (Savage et al. 1992; Ni et al. 2000). In the absence of flow, soluble vWF A1 domain sequences are not available for interaction with GPIb, but when exposed to wall shear rates exceeding 600 s^{-1} (Wu et al. 2000), soluble vWF acquires affinity for platelet GPIb and subendothelial vWF is activated. Thus, both subendothelial and collagen-bound vWF participate in platelet recruitment (Sixma et al. 1991).

2.1.3 vWF-GPIb/IX/V Interactions in Arterial Thrombogenesis

Epidemiological studies uncovered a link between elevated plasma vWF levels and the incidence of heart disease caused by arterial thrombosis (Folsom et al. 1997). The plasma of patients with acute myocardial infarction exhibit elevated plasma vWF concentrations and support enhanced shear-induced platelet activation (SIPA), suggestive of a causative role for vWF in acute coronary thrombosis (Goto et al. 1999). Moreover, upregulated vWF antigen contributing to platelet recruitment has been found in atherosclerotic plaques, after balloon angioplasty (Bosmans et al. 1997) or collar placement (De Meyer et al. 1999) and in hyperplastic intima of autogenous arterial grafts (Qin et al. 2001).

2.2 The GPIb Complex as a Platelet Receptor for vWF

The GPIb/IX/V receptor is assembled from four gene products in a hetero-oligomeric complex in the platelet membrane.

2.2.1 GPIb/IX/V Organisation

The GPIb unit is composed of covalently linked GPIbα (~145 kDa) and GPIbβ (~22 kDa) subunits. GPIb is non-covalently associated to GPIX (~17 kDa) and GPV (~82 kDa). GPIb/IX/V seems to be specifically expressed by megakaryocytes and platelets, although treatment of cultured endothelial cells with cytokines has been reported to induce GPIbα messenger RNA (mRNA) expression (Rajagopalan et al. 1992). GPIb/IX/V is expressed on the platelet surface at about 25,000 copies per platelet, each complex assembling with an apparent molecular ratio of 2:2:2:1 (GPIbα:GPIbβ:GPIX:GPV). GPV occupies

the central position, although it does not seem to be needed for GPIb membrane stabilisation and function. GPIbβ and GPIX are required for the correct processing and membrane surface expression of intact GPIb/IX/V. GPIbα is composed of 610 residues, oriented in the platelet membrane with its amino terminus in the extracellular space. It has a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail. The GPIbα subunit contains the binding site for vWF located within a globular amino-terminal domain (~residues 1–290), characterised by leucine-rich-repeats (Shen et al. 2000; Huizinga et al. 2002).

2.2.2 GPIb Mediated Platelet Activation

The cytoplasmic domain of GPIbα (96 residues) binds to filamin-1 and to the adaptor protein 14.3.3ζ (Williamson et al. 2002). These interactions anchor the membrane complex to the cytoskeleton, contributing to the control of dynamic interactions between sheared platelets and vWF. This domain transduces signals, resulting in activation of the integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa) (Yap et al. 2000). A binding site for 14.3.3ζ also exists on the GPIbβ chain, and binding is controlled via phosphorylation of Ser^{166} by a protein kinase A. Finally, binding sites for calmodulin have been described both on GPIbβ and GPV (Andrews et al. 2001). The mechanism by which vWF binding to GPIb/IX/V mediates $\alpha_{IIb}\beta_3$ activation is poorly understood. It involves protein tyrosine phosphorylation (Syk and Src), activation of protein kinase C and phosphoinositol 3 (PI3) kinase, elevation of the intracellular calcium concentration and synthesis of thromboxane A_2 (Wu et al. 2003). Activation is dependent on co-associated transmembrane proteins, such as the FcR γ-chain and FcγRIIA, containing an immunoreceptor tyrosine-based activation motif (ITAM). Recent studies suggest that the p85 subunit of PI3 kinase mediates GPIb-related activation signals and activates Src independently of the enzymatic activity of the PI 3-kinase (Wu et al. 2003).

2.3 Platelet Collagen Receptors

Collagens are structural proteins found in many tissues including the vascular wall. Containing collagen-like domains, the collagen superfamily consists of some 20 members and some 10 additional proteins. The most abundant collagens in the vascular extracellular matrix are type I and III. These are organised in fibrils, providing extracellular strength to the vascular system. Other collagens, present in smaller amounts in the vessel wall, are fibrillar collagen V, the network-forming collagens type IV and VIII, the beaded filament-forming microfibrillar collagen VI, and fibril-associated collagens, with interrupted triple helices, type XII and VIV. Collagen type IV is a major component of the base-

ment membrane underlying the endothelium, and together with types I and III, it represents the most reactive collagen with regard to platelet activation (Madri et al. 1980; Palotie et al. 1983).

2.3.1 Integrin $\alpha_2\beta_1$ Structure

Integrin $\alpha_2\beta_1$ (GPIaIIa, VLA-2 or CD49b/CD29) is expressed on endothelial cells, fibroblasts, lymphocytes and platelets. On platelets, $\alpha_2\beta_1$ mainly serves as a collagen receptor, whereas on endothelial and epithelial cells it reacts both with collagen and laminin (Elices and Hemler 1989). On platelets, $\alpha_2\beta_1$ has a low density, approximately 1,000–2,900 copies per platelet. It is an integrin, composed of two non-covalently linked transmembrane polypeptides α and β, with the overall shape of a globular head standing on two long legs, ending in a pair of single-pass transmembrane helices and short cytoplasmic tails (Humphries 2000). The α_2-subunit is 1,181 amino acids long (~165 kDa). The short C-terminal α_2 cytoplasmic tail contains a highly conserved GFFKR motif, important for integrin activation (Wang et al. 2003) and binding of several intracellular proteins such as F-actin and calreticulin (Rojiani et al. 1991).

Common to several integrin α-chains, the α_2-subunit contains a 200-amino-acid inserted domain (I-domain) between the second and the third repeat, probably presented on the upper surface of the β-propeller. This I-domain is homologous to the vWF A-domains and recapitulates many of the ligand binding properties of the parent integrin. Unique to the α_2 I-domain is an additional short α-helix, called a C-helix, at the top of the domain in close proximity to the MIDAS. Both mutagenesis and crystallography studies showed that the MIDAS-motif is required for ligand binding (Emsley et al. 2000). The β_1-subunit has a molecular weight of 130 or 110 kDa under reducing or non-reducing conditions, respectively. Like the α_2-subunit, the β-chain is a type I transmembrane protein with a large extracellular domain, a single passing transmembrane region and a short cytoplasmic tail. The extracellular part further contains four cysteine-rich epidermal growth factor (EGF)-like repeated segments that all have a high number of internal disulphide bridges. This domain has endogenous disulphide isomerase activity that might be responsible for regulating conformational changes in the integrin (Lahav et al. 2003).

2.3.2 Recognition Site in Collagen for Integrin $\alpha_2\beta_1$

The sequence GFOGER was identified as the ligand for $\alpha_2\beta_1$ in the CB3 peptide of collagen type I (Knight et al. 1998), whereas two weaker recognition sites, GLOGER and GASGER, were found (Xu et al. 2000). Sequence alignment of the α-chains of collagen type I and III showed that GAOGER and GLSGER occupy the same position. Another GAOGER motif was found in collagen type III

at the same position as the GFOGER in collagen type I. An additional $\alpha_2\beta_1$-binding sequence, GMOGER, was identified in the same position relative to GFOGER and GAOGER in collagen type I and type III respectively (Morton et al. 1989; Knight et al. 2000). The spatial distribution of these $\alpha_2\beta_1$-recognition sites is strongly preserved between the fibrillar collagens, suggesting that their organisation might have a role in platelet binding and signalling. The GFOGER peptide induced spreading of platelets through activation of Src and Syk family kinases, leading to tyrosine phosphorylation of PLCγ2 (Inoue et al. 2003). This pathway is very similar to the one utilised by GPVI (see below).

2.3.3 GPVI on Platelets

GPVI is a 63-kDa type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. In contrast to integrin $\alpha_2\beta_1$, GPVI is restricted to platelets and megakaryocytes (Jandrot-Perrus et al. 2000). It consists of 319 amino acids with two extracellular Ig-like domains formed by disulphide bonds, followed by a highly glycosylated stem of approximately 60 amino acids. The transmembrane region contains an arginine residue, critical for interaction with the FcR γ-chain (Zheng et al. 2001). The 51-amino-acid cytoplasmic domain contains binding sites for calmodulin (Andrews et al. 2002) and Src homology SH3 binding proteins (Suzuki-Inoue et al. 2002). The GPVI-Fc complex probably operates as dimers on the platelet surface, since pairs of GPO motifs separated by three or four intervening triplets interact best with the receptor.

Recently, the putative primary collagen binding site was localised to the apical area of GPVI, where the first Ig-like domain meets the interdomain linker, with lysine 59 as a crucial residue (Smethurst et al. 2004). GPVI does not require a specific recognition site, as it strongly binds to the collagen-related-peptide (CRP), which consists of a triple helical polymer of ten GPO triplets. In contrast, triple helical GPP polymers are very poor ligands. Platelet adhesion starts in the presence of one GPO triplet and gradually increases up to four GPO triplets. Only a slight additional increase is observed with CRP (ten GPO triplets). In the three-dimensional structure of collagen fibres, single GPO triplets of one strand may neighbour those of adjacent strands and thus constitute the required GPVI recognition motif (Farndale et al. 2003).

GPVI is non-covalently associated with the signal-transducing FcR γ-chain, also belonging to the Ig superfamily (Tsuji et al. 1997).This association is required both for surface expression and for the functional activity of GPVI. The cytoplasmic tail of GPVI contains a proline-rich domain that binds to the SH3 domains of the tyrosine kinases Fyn and Lyn (Quek et al. 2000). Cross-linking of GPVI by ligand binding may bring the SH3-associated kinases to the FcR γ-subunit, enabling phosphorylation of the ITAM of the FcR γ-subunit. This leads to binding and activation of tyrosine kinase, Syk, which further signals through a cascade of tyrosine phosphorylations in which the adaptor

molecules LAT (linker for activation of T cells) and SLP-76 play an important role. The result is an activation of several effector molecules such as PLCγ2 and PI3 kinase, finally leading to activation of protein kinase C and Ca^{2+} mobilisation from internal stores (Nieswandt and Watson 2003).

2.4 Secondary Platelet Recruitment and Aggregation

Bound adhering platelets are activated via transducing signals delivered to collagen and GPIb receptors by bound collagens and vWF, respectively. The vWF-mediated platelet activation is a consequence of shear stress-induced Ca^{2+} influx and is aspirin-insensitive (Kroll et al. 1996). Activated adhering platelets undergo morphological modifications associated with platelet spreading and the secretion of their granular contents. Thus, the released thromboxane A_2, serotonin, vWF and fibrinogen will further activate neighbouring platelets, finally resulting in glycoprotein $\alpha_{IIb}\beta_3$ receptor inside-out activation. This membrane receptor thus acquires the capacity to react with fibrinogen and to support platelet aggregation. Fibrinogen binding to $\alpha_{IIb}\beta_3$ is mediated primarily via the fibrinogen γ-carboxyterminal dodecapeptide as well as by its Arg-Gly-Asp (RGD) sequences (Steiner et al. 1989). Whereas initial platelet–vWF–collagen interactions are co-ordinated primarily via $\alpha_2\beta_1$ and GPIb, these interactions are consolidated via $\alpha_{IIb}\beta_3$, creating stable bonds between platelets (Savage et al. 1996).

Platelet degranulation releases nucleotides, such as adenosine diphosphate (ADP) and ATP, strongly amplifying platelet activation during the secondary recruitment phase of flowing platelets. The rapid secretion of nucleotides is capable of triggering even Ca^{2+}-dependent platelet activation steps involved in the permanent activation of the first layer of adhering platelets. Purines and pyrimidines act by interacting with distinct cell-surface receptors. Purinergic receptors were first recognised by Burnstock et al. (1978). They were divided into two classes: At P1 purinoceptors, adenosine is the principal natural ligand, while P2 purinoceptors recognise both purine and pyrimidine nucleotides, namely ATP, ADP, uridine triphosphate (UTP) and UDP (Abbracchio and Burnstock 1994).

The extensive and heterogeneous group of P2 receptors is subdivided into P2X ligand-gated cation channels and G protein-coupled P2Y receptors (Fredholm et al. 1997). The two main types of purinoceptors for extracellular nucleotides operate on different scales of time and distance. P2X receptors act within milliseconds whereas P2Y receptors trigger second-messenger cascades (Communi et al. 2000) that amplify and prolong the duration of the signal over hundreds of milliseconds or even seconds. Platelets have two P2Y receptors whose combined action is required for full activation and aggregation in response to ADP (Gachet 2001). One of these, $P2Y_1$, is coupled to the heterotrimeric guanosine triphosphate (GTP)-binding protein G_q and to

phospholipase C-β activation; it induces mobilisation of cytoplasmic Ca^{2+} and mediates shape change followed by an initial wave of rapidly reversible aggregation. The other receptor, $P2Y_{12}$ (Cattaneo et al. 1997), is negatively coupled to adenylyl cyclase through G_i; it mediates progressive and sustained platelet aggregation in the absence of shape change and plays an important role in the potentiation of secretion induced by several agonists via its interaction with released ADP. This process is independent of the formation of large aggregates and of thromboxane A_2 synthesis. $P2Y_{12}$-mediated activation of the PI3 kinase pathway contributes to stabilise thrombin-induced platelet aggregates (Trumel et al. 1999), although thrombin and thrombin-related peptides can cause platelet aggregation independently of G_i signalling (Kim et al. 2002). Comparison of the relative potency of $P2Y_1$ and $P2Y_{12}$ during experimental thrombosis in gene-deficient mouse models has demonstrated the central role of $P2Y_{12}$, the receptor inactivated by thienopyridines (see Sects. 2.6 and 2.5.2). Thrombosis studies in gene-deficient mice and in a mouse model overexpressing the ion channel $P2X_1$ have demonstrated that platelet activation also depends on contributions by degranulated ATP during shear stress-controlled events and during collagen-induced platelet aggregation (Hechler et al. 2003; Oury et al. 2003).

2.5 Lessons from Disease: Loss and Gain of Function

2.5.1 Bernard–Soulier Syndrome and Platelet-Type Von Willebrand Disease

Mutations affecting GPIb/IX/V integrity are associated with a prolonged bleeding time. In Bernard–Soulier syndrome, mutations in the GPIbα, GPIbβ or GPIX gene may affect the transport of the protein chains to the cellular membrane, leading to deficient vWF binding. Alternatively, non-functional mutations in *GPIb*α may allow normal transport, while resulting in defective platelet aggregation and reduced platelet adherence to subendothelium, especially at high shear stress (Hayashi and Suzuki 2000). In platelet-type von Willebrand disease (VWD), the bleeding tendency is due to gain-of-function mutations Gly233Val or Met239Val, in the GPIbα subunit. The effects of these mutations resemble type 2B VWD (see below). vWF spontaneously binds to the platelets, leading to depletion of large vWF multimers from the circulation and to moderate thrombocytopaenia and bleeding (Tait et al. 2001).

2.5.2 Von Willebrand Disease

VWD is an inherited bleeding disorder classified in three main groups according to biosynthesis defects and protein dysfunction. In type 1 VWD, vWF displays a partial quantitative deficiency with normal multimerisation. Patients with type 2 VWD manifest qualitative deficiencies categorised into four

variants: 2A, 2B, 2M and 2N. Type 2A VWD shows an absence of large vWF multimers due to defective vWF multimerisation in the Golgi or to increased proteolytic degradation in the plasma (Lyons et al. 1992). vWF from type 2B patients has an increased affinity for platelet GPIbα and shows spontaneous binding of multimers to platelets in vivo. This paradoxically results in a haemostatic defect due to large vWF multimer clearance from the circulation and intermittent thrombocytopaenia. vWF type 2M mutations cause defective binding to platelets, without dysfunctional multimerisation. Most 2M mutations are located in the A1 domain of vWF, compatible with defective binding to GPIbα. vWF type 2N mutations affect factor VIII binding, resulting in a reduced factor VIII stabilisation. This bleeding tendency resembles mild haemophilia A. Finally, type 3 VWD, the most severe subtype, is characterised by the absence of plasma, tissue or cellular vWF. Type 3 VWD is caused by frameshift, deletion and nonsense mutations (Sadler 1998).

2.5.3
Collagen Receptor Deficiency and Bleeding

Evidence for the importance of integrin $\alpha_2\beta_1$ in platelet function was obtained in a patient with mild bleeding problems related to strongly reduced expression of integrin $\alpha_2\beta_1$ (Nieuwenhuis et al. 1985). Platelet aggregation in response to various types of collagen and adhesion to collagen under static and flow conditions was markedly reduced, and the few platelets that adhered failed to spread. In this and in a second female patient, symptoms disappeared after menopause, accompanied by normalisation of the $\alpha_2\beta_1$ expression, suggesting that the gene defect was located in the promoter region of $\alpha_2\beta_1$ chains, the bleeding defect thus rather reflecting defective hormone regulation of gene expression. In a 66-year-old man with a myeloproliferative disorder and prolonged bleeding time but no bleeding history, a deficient collagen-induced aggregation and aberrant adhesion to collagen were found, due to an acquired deficiency in integrin $\alpha_2\beta_1$ (Handa et al. 1995). The first patient with a GPVI deficiency (Sugiyama et al. 1987) suffered from autoimmune thrombocytopaenia caused by antibodies against a 65-kDa protein (i.e. GPVI) that was present in healthy individuals but absent in the patient. His platelets failed to respond to collagen. A few additional patients were described with low GPVI expression levels, suffering from mild bleeding problems and with platelets responding poorly to collagen (Moroi et al. 1989) or to CRP (Kehrel et al. 1998). The molecular basis for these GPVI deficiencies is, however, poorly defined.

2.6
Inhibition of Platelet Deposition on the Vessel Wall

Pharmacological inhibition of platelet deposition onto damaged vessel wall structures is potentially antithrombotic. However, adhering platelets also re-

lease vasoactive substances and growth factors, predominantly the platelet-derived growth factor (PDGF), promoting smooth muscle cell activation and migration (Ferns et al. 1991). Vessel wall injury thus not only predisposes to thrombosis but also initiates neointima formation, resulting in vessel wall thickening and eventually in stenosis, a problem encountered in about one-third of patients undergoing a percutaneous transluminal coronary angioplasty (PTCA) (Glazier et al. 1989). Neointima formation is impaired in thrombocytopaenic animals, in agreement with the progression of restenosis, under control by platelet-derived vasoactive substances (Friedman et al. 1977). Therefore, such receptor–ligand interactions involved in platelet adhesion to the vessel wall may represent interesting targets. These include collagen, vWF and fibronectin (Melis et al. 2004).

At present, during acute coronary interventions, $\alpha_{IIb}\beta_3$ antagonists have become the standard treatment to block platelet aggregation. Yet $\alpha_{IIb}\beta_3$ antagonists have a poor effect on the deposition of (single) platelets and therefore have a poor outcome in the prevention of restenosis (Nguyen and Harrington 2003). In contrast, the potent inhibition by $\alpha_{IIb}\beta_3$ antagonists causes a bleeding risk, which narrows their therapeutic window and requires careful patient monitoring. Furthermore, poor bioavailability and immune-mediated thrombocytopaenia, in about 1% of patients treated, precludes the chronic use of these antagonists.

Anti-adhesive anti-platelet drugs with antithrombotic potential, reducing neointima formation, have been studied in animal models. The murine anti-human GPIbα monoclonal antibody 6B4 (Cauwenberghs et al. 2001) prevented arterial thrombosis in a baboon model of femoral artery stenosis, without prolonging the bleeding time (Wu et al. 2002). Even in combination with a neutralising anti-human $\alpha_{IIb}\beta_3$ antibody, a strong antithrombotic effect was achieved without bleeding time prolongation. Likewise, the mouse anti-vWF monoclonal antibody AJvW-2 is a potent inhibitor of GPIbα–vWF interactions. In vitro and ex vivo, AJvW-2 inhibits SIPA, as well as high shear stress-induced platelet adhesion and aggregation onto surface coated collagen (Kageyama et al. 1997). It also inhibits the enhanced SIPA in platelet-rich plasma of patients suffering from acute coronary syndromes (Eto et al. 1999). In several animal models, AJvW-2 prevents both arterial and venous thrombosis; it exerts a protective effect during neointima formation after balloon injury in the guinea-pig (Kageyama et al. 2000) due to inhibition of platelet deposition on the vessel wall. Its antithrombotic effect is not accompanied by a bleeding time prolongation, in contrast to that of the $\alpha_{IIb}\beta_3$ antagonist lamifiban, studied in parallel, or the widely used anti-$\alpha_{IIb}\beta_3$ antibody abciximab.

Drugs such as aspirin (inactivating cyclooxygenase, thus eliminating thromboxane A_2 production by thromboxane A_2 synthase in platelets) and thienopyridines (inactivating $P2Y_{12}$ via reactive metabolites that couple to a critical thiol of the receptor) inhibit specific amplification pathways of platelet activation and are efficient in the primary and secondary prevention of thrombosis, at

the expense, however, of a well-defined bleeding risk. Blood platelet activation relies on the synergistic interplay of several activation pathways, and it is clear that selected combinations of inhibitors of separate pathways offer the potential of inhibiting thrombosis to a variable degree, with variable effects on the haemostatic balance. Anti-adhesive inhibition of GPIbα–vWF interactions and of collagen receptor–collagen interactions may have the potential to control thrombosis by inhibiting primarily arterial thrombosis, while maintaining an acceptable bleeding risk.

3
Coagulation System

Haemostatic processes are traditionally divided in two parts: Primary haemostasis mainly involves platelets (see Sect. 2), and secondary haemostasis mainly relates to fibrin formation or blood coagulation following an extrinsic or intrinsic pathway (MacFarlane 1964). This model, although still valuable for laboratory diagnosis of haemostatic abnormalities, has recently been revised based on (1) the discovery of TFPI (Rapaport 1989; Broze et al. 1990), (2) the activation of factor XI by thrombin (Gailani and Broze 1991; Naito and Fujikawa 1991), (3) the finding that primary and secondary haemostatic processes strongly interact and (4) the notion that tissue factor may be blood-borne (Giesen et al. 1999).

In the current model of coagulation, the extrinsic tenase reaction initiates coagulation by activating factors IX and X. Platelets play a crucial role in the exposure of tissue factor and deliver the first trace amounts of activated factor V. Once critical amounts of factor Xa, required for the initiation of thrombin generation, are formed, the extrinsic tenase reaction is efficiently turned off by TFPI, and further formation of thrombin is maintained via positive feedback mechanisms involving thrombin-induced activation of the plasma factors V, VIII and XI. Excess thrombin is efficiently inhibited by its physiological inhibitor antithrombin and downregulates its own further generation via stimulation of the protein C pathway.

3.1
Structure of the Main Procoagulant and Anticoagulant Proteins

Procoagulant and anticoagulant proteins are composed of multiple domains, which have a high degree of structural and functional homology (Table 1; Colman et al. 1994; Bloom et al. 1994).

3.1.1
Signal Peptide

Both procoagulant and anticoagulant proteins in plasma are initially synthesised with a signal peptide. This short (usually very hydrophobic) peptide,

Table 1 Overview of the main procoagulant and anticoagulant proteins with some of their properties

	Function or main substrate of the active form	M_r (kDa)	Chain composition	Plasma conc. (μg/ml)	$t_{1/2}$ (h)	Domains				
						Gla	EGF	Kringle	Catalytic	Other
Zymogens										
Prothrombin	Fibrinogen, factor XIII	72	Sc, 581 AA	100	72	10 AA	None	2	Ser protease	
Factor VII	Factor X, factor IX	50	Sc, 406 AA	0.5	5	10 AA	2	None	Ser protease	
Factor X	Prothrombin	59	Tc, 254 AA, 139 AA	8	32	11 AA	2	None	Ser protease	
Factor IX	Factor X	56	Sc, 415 AA	5	24	12 AA	2	None	Ser protease	
Factor XI	Factor IX	160	Tc, 607 AA each	5	72	None	None	None	Ser protease	
Factor XII	Factor XI	80	Sc, 596 AA	30	60	None	2	1	Ser protease	
Protein C	Factor Va, factor VIIIa	62	Tc, 262 AA, 155 AA	3–5	7	9 AA	2	None	Ser protease	
Cofactors										
Tissue factor	Extrinsic tenase cofactor	45	Sc, 263 AA	Cell-bound	–	None	None	None	–	2 Barrel-like structures Transmembrane module Cytoplasmic tail
Factor V	Prothrombinase cofactor	330	Sc, 2196 AA	7–10	12	None	None	None	–	A1, A2, B, A3, C1, C2

Table 1 (continued)

	Function or main substrate of the active form	M_r (kDa)	Chain composition	Plasma conc. (μg/ml)	$t_{1/2}$ (h)	Domains				
						Gla	EGF	Kringle	Catalytic	Other
Factor VIII	Intrinsic tenase cofactor	280	Tc, 1313 AA, 684 AA	0.2	12	None	None	None	–	A1, A2, B, A3, C1, C2
Protein S	Cofactor for activated protein C	75	Sc, 635 AA	20	42	11 AA	4	None	–	Sex hormone-binding globulin-like module
Thrombo-modulin	Cofactor for protein C activation	60	Sc, 557 AA	Cell-bound	–	None	6	None	–	Lectin-like module Hydrophobic region Transmembrane module Cytoplasmic tail
Inhibitors										
Antithrombin	Inhibitor of thrombin and factor Xa	58	Sc, 432 AA	125	48	None	None	None	–	
Tissue factor pathway inhibitor	Inhibitor of extrinsic tenase and factor Xa	42	Sc, 276 AA	0.1	–	–	–	–	–	Kunitz domains 1, 2 and 3

Sc, single-chain; Tc, two-chain; AA, number of amino acids

required for translocation of the growing polypeptide chain into the endoplasmatic reticulum, is cleaved off prior to secretion.

3.1.2 Propeptide/γ-Carboxyglutamic Acid-Rich Domain

All vitamin K-dependent proteins (prothrombin, factors VII, IX and X, protein C and protein S), contain a γ-carboxylation recognition site located on the propeptide domain between the signal peptide and the γ-carboxyglutamic acid-rich domain (Gla domain). This site directs γ-carboxylation of the γ-carboxyglutamic acid residues located in the adjacent, approximately 40-residue-long Gla domain. After carboxylation of the Gla domain, which is crucial for the Ca^{2+}-mediated binding of vitamin K-dependent proteins to negatively charged membranes, the propeptide is cleaved off.

3.1.3 Epidermal Growth Factor Domain

Several procoagulant and anticoagulant proteins contain two or more EGF-like domains. These domains consist of about 43 to 50 amino acid residues, and their structure is determined by three characteristic disulphide bonds. The function of EGF-like domains in many coagulation proteins, although not fully understood, appears to be in the formation of protein complexes. The EGF-like domains in factor VII are important for the binding to tissue factor. The second EGF-like domain of factor IX contains a binding site for activated factor VIII. The second EGF-like domain of protein C is involved in the binding of protein S. The binding sites on thrombomodulin for protein C and thrombin are located on the fourth and fifth EGF-like domains respectively.

3.1.4 Kringle Domain

Kringle domains consist of about 100 amino acids, and their structure is determined by three disulphide bonds. These domains are involved in interactions with other proteins. Only two procoagulant proteins, prothrombin and factor XII, contain kringle domains. The second kringle of prothrombin probably contains the main binding site for activated factor V.

3.1.5 Catalytic Domain

The catalytic domain of all procoagulant enzymes contains an active site and an internal core that is similar to that of trypsin. Conversion of an inactive proenzyme to an active enzyme depends on limited proteolysis and, for some

proteins, on cleavage of so-called activation peptides. The active site of all clotting enzymes (as with all serine proteases) contains a catalytic triad consisting of serine, aspartic acid and histidine.

3.1.6 Pseudosubstrates

The natural inhibitors of coagulation, antithrombin and TFPI, are pseudosubstrates with high affinity for their specific target enzymes. Antithrombin is a single-chain globular molecule which depends on heparin to obtain its optimal inhibitory conformation required for docking and locking the catalytic centre of its target enzymes, thrombin and factor Xa. It forms 1:1 stoichiometric complexes which are rapidly cleared from the circulation. TFPI is a single-chain molecule with three Kunitz domains, which contain about 58 residues and three characteristic disulphide bonds. They act as pseudosubstrates for their target serine proteases. The first Kunitz domain of TFPI inhibits the factor VIIa/tissue factor complex, whereas the second inhibits factor Xa; the function of the third Kunitz domain is unknown.

3.2 Procoagulant Mechanisms

3.2.1 Initiation of Coagulation

Tissue factor is the vascular trigger required to initiate coagulation (Rapaport and Rao 1995). In healthy blood vessels, tissue factor is mainly located in the extracellular matrix beneath and between endothelial cells and therefore appears to form a protective lining around blood vessels, capable of activating blood coagulation after vascular injury (Drake et al. 1989).

Tissue factor binds to factor VIIa and accelerates the activation of factor IX and factor X by factor VIIa (Rapaport and Rao 1995). The physiological importance of tissue factor has been confirmed by the finding that disruption of the tissue factor gene in mice is associated with impaired vascular development and lethal embryonic bleeding (Carmeliet et al. 1996; Bugge et al. 1996). Association of tissue factor with phospholipids is required for significant procoagulant activity (Nemerson 1995). Relipidation experiments with recombinant tissue factor have shown that both phosphatidylcholine and phosphatidylethanolamine support the procoagulant properties of tissue factor, whereas phosphatidylserine is inactive.

At high tissue factor concentrations, factor X is mainly activated by the factor VIIa–tissue factor complex, whereas at low concentrations, factor IXa-/factor VIIIa-dependent activation becomes more pronounced (Osterud and Rapaport 1977; Marlar et al. 1982). Factor X activation by the extrinsic tenase reaction is responsible for the initiation phase. Activated factor X activates

prothrombin on a phospholipid surface upon association with its cofactor, activated factor V, secreted from the α-granules of activated platelets (Gould et al. 2004). The cleavage of prothrombin is sequential. In a first stage, meizothrombin is generated (Krishnaswamy et al. 1986); this active enzyme remains attached to the phospholipid surface. Subsequent removal of fragments including the Gla domain of prothrombin results in soluble thrombin that diffuses away from the catalytic surface.

3.2.2 Blood-Borne Tissue Factor

Endothelial cells themselves have little or no tissue factor activity, but it can be strongly induced in vitro by endotoxin, thrombin, fibrin and several cytokines, as well as by shear stress and hypoxia (Contrino et al. 1997; Lin et al. 1997; Rapaport 1989; Nemerson 1995). Both monocytes and natural killer cells have also been found to upregulate tissue factor expression in endothelial cells (Napoleone et al. 1997). However, it is doubtful whether this phenomenon occurs in pathological conditions in vivo. Tissue factor is highly concentrated in the areas surrounding the cholesterol clefts of diseased coronary vessels (Fuster et al. 1997; Nemerson 1995), but whether it initiates thrombus formation after plaque rupture is doubtful. Recent work indicates that platelets adhering to a ruptured plaque effectively prevent contact between the plaque tissue factor and the blood (Hathcock and Nemerson 2004). In addition, it was shown that when native human blood is allowed to flow over a glass coverslip at high shear, platelets adhere to the coverslip and biologically active tissue factor containing microparticles adheres to the platelet layer (Giesen et al. 1999). Until recently, tissue factor was believed to be located essentially extravascularly; now, however, the surprising concept of blood-borne tissue factor has emerged. Monocytes and possibly polymorphonuclear leucocytes are the source of these tissue factor-positive microparticles, which are transferred to the adhering platelets (Rauch et al. 2000; see Fig. 1).

During platelet activation the α-granule membranes, containing P-selectin (CD62P) (Johnston et al. 1989), fuse with the plasma membrane which becomes decorated with P-selectin. Surface P-selectin then interacts with CD15 (a leucocyte membrane-bound carbohydrate known as sialyl Lewis X) or with P-selectin glycoprotein ligand 1 (Sako et al. 1993), also on leucocytes.This interaction results in the formation of conjugates between activated platelets and leucocytes or leucocyte microparticles. Under normal conditions, most cell surface tissue factor is encrypted, which means that it binds factor VIIa but is not capable of initiating coagulation. Encrypted tissue factor allows circulating tissue factor-positive monocytes to be present in the circulation in the absence of generalised coagulation (Maynard et al. 1975). However, when the phospholipids in the monocyte plasma membrane are scrambled by cal-

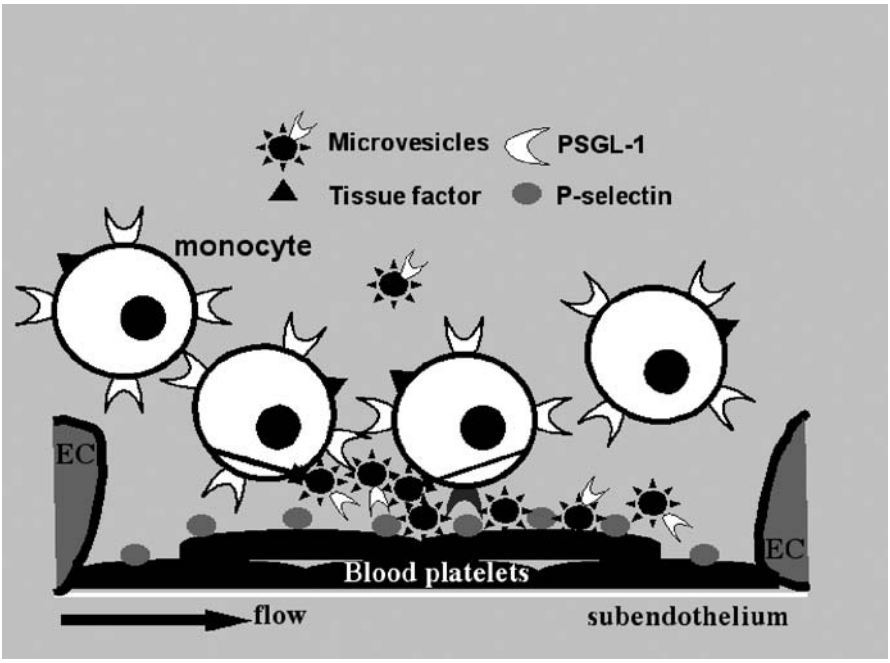

Fig. 1 Blood-borne tissue factor. Platelets rapidly adhere to injured vessels and expose P-selectin. Surface P-selectin then interacts with PSGL-1 (P-selectin glycoprotein ligand-1) on monocytes and monocyte-derived microvesicles, delivering blood-borne tissue factor

cium ionophore, allowing binding of clotting factors as described above, tissue factor becomes de-encrypted and coagulation ensues (Bach and Rifkin 1990). The transfer of tissue factor-positive microparticles to the surface of a spread platelet that has bound clotting factors therefore allows thrombin generation.

The co-localisation of platelets, blood-borne tissue factor and fibrin in blood flowing over an ex vivo surface has recently been visualised in real time (Balasubramanian et al. 2002). Furie et al. (2001) used intravital confocal microscopy of the microcirculation of living mice to study thrombosis induced by laser injury. Co-localisation of platelets, leucocytes and fibrin was observed. Their preliminary experiments have shown that thrombus formation is significantly reduced in mice either deficient in P-selectin or in P-selectin glycoprotein ligand 1.

If platelet–leucocyte interaction via P-selectin is the cellular basis for intravascular thrombus formation, then inhibition of P-selectin function seems an attractive therapeutic strategy that is currently being actively pursued. Both anti-P-selectin antibodies (Palabrica et al. 1992; Downing et al. 1997) and recombinant soluble P-selectin glycoprotein ligand 1 (Khor et al. 2000) are being evaluated. In primate models, pretreatment with a blocking monoclonal antibody to P-selectin accelerated pharmacological thrombolysis of

arterial thrombosis (Toombs et al. 1995) and reduced stasis-induced venous thrombosis (Downing et al. 1997).

3.2.3 Propagation of Coagulation

Both meizothrombin and thrombin are responsible for the propagation phase of coagulation. Meizothrombin, by lateral diffusion on the phospholipid surface, effectively activates factor V and factor XI (Tans et al. 1994; von dem Borne et al. 1997). Thrombin causes further platelet activation and factor XI activation on the platelet surface (Walsh 2001), and dissociates factor VIII from von Willebrand factor and activates it (Vlot 1998). Activated factor VIII binds to the phospholipid surface through its C_2 domain. Factor VIIIa is the cofactor for factor IXa and is required for the propagation phase induced by the intrinsic tenase reaction. Recent studies show that factor Xa generation via the intrinsic tenase reaction occurs after that of the extrinsic tenase reaction, as it requires thrombin-dependent activation of factor VIII (Butenas et al. 1997).

The propagation phase, involving the intrinsic tenase reaction, consists of a new burst of factor X activation which leads, on the one hand, to the formation of a factor Xa-TFPI complex shutting down the extrinsic tenase reaction (Broze et al. 1990, Broze 1995a, b) and, on the other hand, to an explosive generation of thrombin (see Fig. 2). The propagation phase of coagulation results in a high local concentration of thrombin that converts fibrinogen into a fibrin network that is stabilised through covalent cross-linking by thrombin-activated factor XIII.

Endothelial cells may promote the propagation phase of coagulation in several ways. They synthesise and bind factor V, and its expression on the endothelial cell surface is enhanced by mechanical injury (Annamalai et al. 1986). Endothelial cells also contain factor VIII (Kadhom et al. 1988); although its cellular localisation is not clearly identified, it is conceivable that factor VIII is stored in the Weibel–Palade bodies associated with vWF, since both are concomitantly released upon infusion of DDAVP (1-deamino-8-D-arginine vasopressin). Thrombin-activated endothelial cells release vWF that plays a role in platelet adhesion, whereas concomitant release of factor VIII may cause an increased concentration at the site of thrombus formation.

3.2.4 Blood Coagulation as a Surface-Catalysed Process

With the exception of fibrinogen and prothrombin, the coagulation factors are trace proteins (see also Table 1). For efficient interactions they need to be concentrated on a cell surface. The main physiological catalytic surface is a layer of phospholipid containing negatively charged phospholipids such as phosphatidylserine. Phosphatidylserine normally is sequestered in the inner

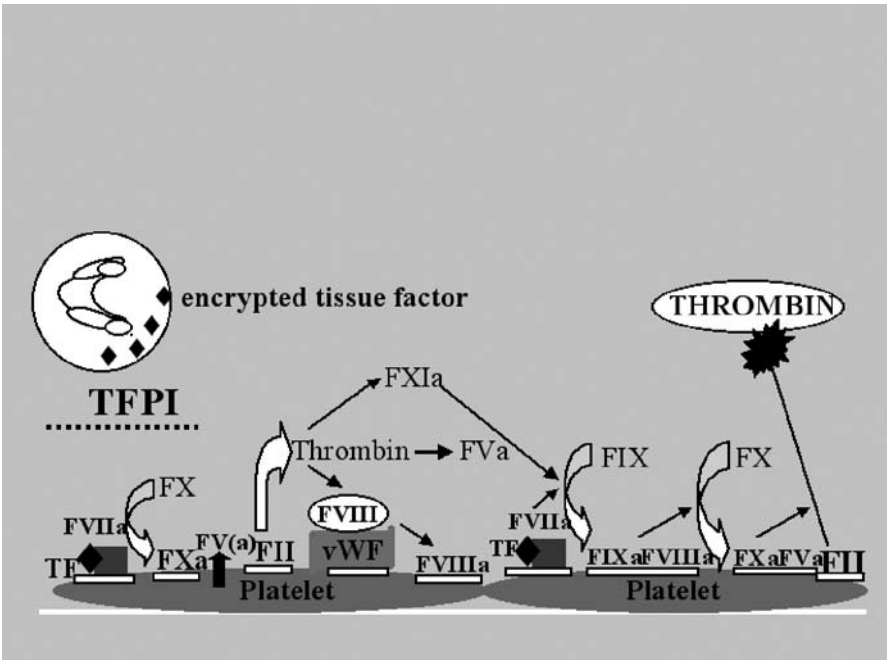

Fig. 2 Blood coagulation as a surface-catalysed process. For efficient interactions, procoagulant factors need to be concentrated on a cell surface enriched in phosphatidyl serine. FVIIa binds to blood-borne tissue factor on activated platelets and initiates coagulation by generating limited amounts of FXa. This enzyme together with FVa, released from platelets, generates the first traces of thrombin, which amplifies its own generation by activating FV, FVIII and FIX. As soon as a critical concentration of FXa is formed, TFPI (tissue factor pathway inhibitor) inhibits the extrinsic tenase reaction. The end result of the process is an explosive generation of thrombin

leaflet of a cellular phospholipid bilayer. Upon activation of cells, in particular of platelets, phospholipid scrambling occurs (Sims and Wiedmer 2001). Cell surface-exposed phosphatidylserine following scrambling serves as a receptor for the vitamin K-dependent coagulation factors (prothrombin, factors VII, IX and X), for factor V and for factor VIII (Heemskerk et al. 2002). Glutamic acid (glu) residues at the NH_2-terminal ends of vitamin K-dependent coagulation factors are carboxylated to ɣ-carboxyglutamic acid (gla) residues (Stenflo et al. 1974). This "gla-domain" anchors these proenzymes to the negatively charged phospholipid membrane in a Ca^{2+}-dependent manner. Factors V and VIII are protein cofactors that facilitate the interaction of the vitamin K-dependent (pro)enzymes. Their sequence contains six sequential domains arranged in the order A_1-A_2-B-A_3-C_1-C_2. They bind to phospholipid through the C_2 domain by the burial of hydrophobic residues within the phospholipid bilayer; these hydrophobic residues are surrounded by positively charged residues that

interact with the negatively charged phospholipid head groups (Pratt et al. 1999). Activated factor V is secreted from the α-granules of activated platelets and binds with high affinity to the phospholipid surface (Gould et al. 2004). Factor VIII is concentrated on activated platelets via its carrier protein, vWF (Kawasaki et al. 1999). The latter, subjected to shear stress, binds to activated platelets through their membrane GPIb/IX/V and GPIIb/IIIa complexes (Ruggeri 1997). Finally, the proenzyme factor XI also binds to the platelet GPIb/IX/V complex, where it is activated by meizothrombin or thrombin (Tans et al. 1994; von dem Borne 1997; Walsh 2001).

Thus, primary and secondary haemostasis interact strongly. Upon adhesion to and spreading on collagen, activated platelets assemble on their surface a number of proenzymes and protein cofactors that interact efficiently through lateral diffusion on the phospholipid surface, resulting in thrombin generation and further platelet activation.

3.3 Anticoagulant Mechanisms

The formation of thrombin and the deposition of fibrin on the surface of quiescent endothelial cells is impaired by several pathways.

The extrinsic tenase activity is inhibited by the Kunitz-type inhibitor, TFPI (Broze et al. 1990; Broze 1995a, b; Rapaport 1989) and by the serine protease inhibitor, antithrombin (Rapaport and Rao 1995; van 't Veer and Mann 1997). Both inhibitors neutralise factor VIIa only when it is bound to tissue factor. The mature full-length TFPI is a 43-kDa protein with an acidic NH_2-terminal region followed by three tandem Kunitz-type protease inhibitory domains and a basic COOH-terminal region. TFPI inhibits the extrinsic tenase reaction via a two-step mechanism. In the first step, factor Xa is inhibited by binding to an arginine residue in the reactive centre of the second Kunitz domain. In the second step, the TFPI/factor Xa complex forms a quaternary complex with factor VIIa/tissue factor in which factor VIIa binds to a lysine residue in the reactive centre of the first Kunitz domain. TFPI is predominantly located in the endothelial cell extracellular matrix, where it is bound to heparan sulphate or other glycosaminoglycans. The plasma concentration of TFPI (2 nM) is increased several-fold after intravenous injection of heparin. Plasma TFPI has a lower molecular weight (34–41 kDa) than its endothelium-bound form and appears to be truncated at the COOH-terminal end. It circulates bound to lipoproteins and has substantially lower factor Xa inhibitory activity than the full-length form. The physiological importance of TFPI has been extensively studied in animal models. Infusion of high concentrations of TFPI prevents thrombosis and intravascular coagulation following tissue factor or endotoxin infusion in rabbits (Broze 1995). Neutralisation of TFPI by polyclonal antibodies promotes tissue factor-induced intravascular coagulation (Broze 1995a; Rapaport and Rao 1995). Targeted TFPI gene disruption has recently been

shown to cause intrauterine lethality in mice due to yolk sac haemorrhages or fatal bleeding, compatible with a consumptive coagulopathy (Huang et al. 1997).

Antithrombin, in the presence of heparin, rapidly inhibits the extrinsic tenase reaction (Broze 1995a; Huang et al. 1997; van 't Veer and Mann 1997). In solution, this inhibitory pathway is probably equivalent to the TFPI-dependent inhibition of factor VIIa/tissue factor, whereas on cell surfaces TFPI-dependent inhibition is much faster (Broze 1995a; van 't Veer and Mann 1997). Antithrombin is the major thrombin-inactivating protein (Beresford and Owen 1990). This serpin also inactivates factors Xa, IXa, XIa and kallikrein. Antithrombin only displays its full inhibitory activity in the presence of heparin or other sulphated glycosaminoglycans which are synthesised and expressed by endothelial cells. Some cell surface heparan sulphate proteoglycans may be involved in thrombin–antithrombin interactions (Mertens et al. 1992). Heparan sulphate proteoglycans are also a major constituent of the extracellular matrix, which explains why the thrombogenicity of balloon-injured vessels can be abolished by treatment with antithrombin, whereas heparin is ineffective (Frebelius et al. 1994).

Another endothelial cell-dependent anticoagulant pathway involves the integral membrane glycoprotein thrombomodulin (Esmon 1995). Its physiological importance is well established and supported by gene disruption studies (Rosenberg 1997). Thrombomodulin consists of a lectin-like NH_2-terminal domain, followed by six EGF-like domains, a serine–threonine-rich domain, a transmembrane domain and a short cytoplasmic tail. The fifth and sixth EGF-like domains are essential for thrombin binding, while the calcium-dependent binding of protein C requires the linker region between the third and fourth EGF-like domain. Thrombomodulin has both direct and indirect anticoagulant properties. The direct anticoagulant action of thrombomodulin involves binding, neutralisation and degradation of thrombin (Esmon 1995). Thrombomodulin accounts for about half of the thrombin-binding sites on endothelial cells. Agents such as endotoxin, interleukin-1 and tumour necrosis factor, which stimulate tissue factor activity, downregulate thrombomodulin activity by suppressing its transcription. Thrombomodulin-bound thrombin cannot cleave fibrinogen and cannot activate factor V, factor XIII or platelets and is rapidly endocytosed and degraded (Esmon 1993, 1995).

The indirect anticoagulant action of thrombomodulin involves the generation of activated protein C (Esmon 1989). The zymogen protein C, a vitamin K-dependent protein, is activated by thrombin, and this activation is accelerated up to 20,000-fold by thrombomodulin. Activated protein C has anticoagulant properties by inhibiting factors Va and VIIIa. This reaction is moderately catalysed at the endothelial cell surface by protein S, another vitamin K-dependent cofactor that is synthesised and expressed by endothelial cells in the liver (Dahlback 1991). Protein S binds to the endothelial cell membrane and to protein C, forming a cell surface-bound complex. Due to the

exposure of negatively charged phospholipids, activated platelets may provide the appropriate surface for the inactivation of factors Va and VIIIa. However, activated protein C is also active on endothelial cells, probably involving the recently described endothelial cell protein C receptor (Fukudome and Esmon 1994). Protein S not only functions as a cofactor in the protein C pathway, but also directly inhibits the prothrombinase and tenase reactions on phospholipid vesicles, platelets and human endothelial cells or matrices (Heeb et al. 1993, 1994; Koppelman et al. 1995; van Wijnen et al. 1996).

Other potential anticoagulant proteins include annexin V, protease nexin 1 (PN-1) and protease nexin 2 (PN-2), their roles as endothelial anticoagulants are, however, not firmly established. Annexins are a family of non-glycosylated proteins that bind calcium and phospholipids. Annexin V is localised in the endothelium of venous and arterial blood vessels (van Heerde et al. 1995); it preferentially binds to phosphatidylserine, thereby preventing the assembly of activated coagulation factors on phospholipid surfaces. Anticoagulant properties of annexin V have been reported on phospholipid vesicles, platelets and endothelial cells. PN-1 is a serpin that inhibits thrombin, plasmin, urokinase, activated protein C, kallikrein, factor Xa and trypsin (Bombeli et al. 1997). It is localised on the surface of vascular endothelial cells, fibroblasts and platelets. PN-1 bound to cell surfaces or endothelial cell matrix retains its inhibitory properties for thrombin but not for urokinase or plasmin. Inhibition of thrombin and factor Xa by PN-1 is accelerated by heparin, whereas that of plasmin is not. PN-2 is the secreted form of the transmembrane amyloid β-protein precursor. It is abundantly present in the α-granules of platelets but also in monocytes and endothelial cells (van Nostrand et al. 1992). PN-2 is a much more potent inhibitor of factors IXa and XIa than of thrombin and may be involved in the regulation of the intrinsic tenase reaction on endothelium (Schmaier et al. 1993).

4
Fibrinolysis

4.1
Regulation of Physiological Fibrinolysis

The fibrinolytic system (Fig. 3) comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme plasmin that degrades fibrin by two immunologically distinct physiological plasminogen activators: tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activator. Inhibition of the fibrinolytic system may occur either at the level of the plasminogen activators, by specific plasminogen activator inhibitors (PAI-1 and PAI-2), or at the level of plasmin, mainly by α_2-antiplasmin (Collen and Lijnen 1991). The main biochemical properties of these components are summarised in Table 2. The

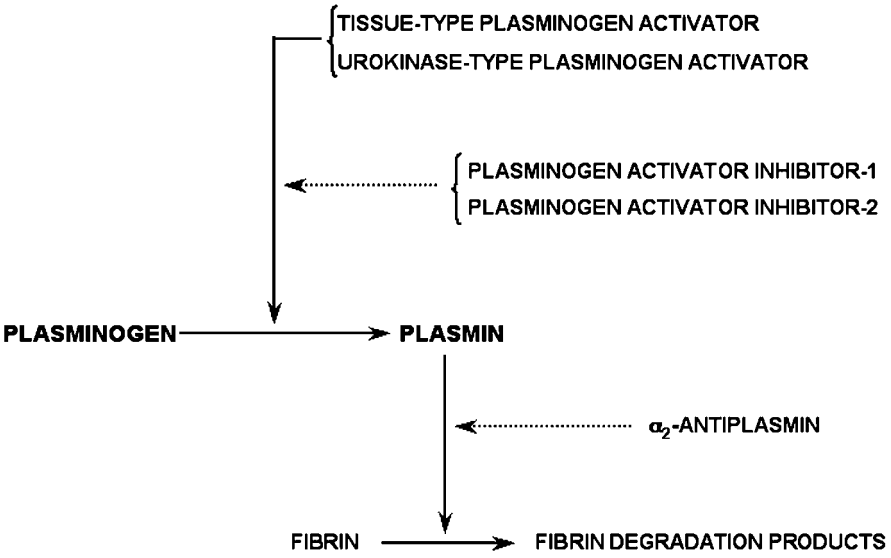

Fig. 3 Schematic representation of the fibrinolytic system

Table 2 Biochemical properties of the main components of the fibrinolytic system

	M_r (kDa)	Chain composition	Carbohydrate content (%)	Amino acids	Catalytic triad or reactive site	Plasma concentration (mg/l)
Plasminogen	92	1	2	791	His603, Asp646, Ser741	200
Plasmin	85	2	2	±715	His603, Asp646, Ser741	–
t-PA	68	1/2	7	527	His322, Asp371, Ser478	0.005
u-PA	54	1/2	7	411	His204, Asp255, Ser356	0.008
α_2-Antiplasmin	67	1	13	464	Arg364-Met365	70
PAI-1	52	1	ND	379	Arg346-Met347	0.05
PAI-2	47	1	ND	393	Arg358-Thr359	<0.005

ND, not determined

fibrinolytic system thus is regulated by controlled activation and inhibition, but also by increased or decreased synthesis and/or secretion of t-PA and of PAI-1, primarily from the vessel wall (Lijnen et al. 2000a).

Impaired fibrinolysis—due to a defective synthesis and/or release of plasminogen activators, a deficiency or functional defect in plasminogen, or in-

creased levels of inhibitors of plasminogen activators or plasmin—is associated with thrombosis. In turn, excessive fibrinolysis resulting from increased levels of t-PA or from α_2-antiplasmin or PAI-1 deficiency may result in bleeding complications.

4.1.1 Plasminogen Activation by t-PA

In the absence of fibrin, t-PA is a poor enzyme, but the presence of fibrin strikingly enhances the activation rate of plasminogen. Fibrin indeed provides a surface to which t-PA and plasminogen adsorb in a sequential and ordered way, yielding a cyclic ternary complex (Hoylaerts et al. 1982). Plasminogen binding to fibrin involves the lysine-binding sites in its kringle structures, whereas binding of t-PA to fibrin is mediated via its finger and kringle 2 domains. Formation of this complex results in an enhanced affinity of t-PA for plasminogen, yielding up to three orders of magnitude higher efficiencies for plasminogen activation. Increased binding of both enzyme and substrate to degrading fibrin is mediated in part by COOH-terminal lysine residues generated by plasmin cleavage. Their interaction with lysine-binding sites on t-PA and plasminogen may allow an improved alignment and allosteric changes enhancing the rate of plasminogen activation (Thorsen 1992).

Consequently, proteins that remove COOH-terminal lysine residues from the fibrin surface, such as the thrombin activatable fibrinolysis inhibitor (TAFI), may have an antifibrinolytic action (Nesheim et al. 1997). TAFI is a 60-kDa single-chain protein, identical to plasma procarboxypeptidase B, that occurs at a concentration of 75 nM and is activated by thrombin, trypsin or plasmin.

4.1.2 Plasminogen Activation by u-PA

u-PA is secreted as a single-chain molecule (scu-PA) that is converted to a two-chain moiety (tcu-PA) by plasmin. In contrast to tcu-PA, scu-PA displays very low activity towards low molecular weight chromogenic substrates, but it appears to have some intrinsic plasminogen-activating potential, which represents 0.5%, or less of the catalytic efficiency of tcu-PA (Lijnen et al. 1990). Other investigators, however, have claimed that scu-PA has no measurable intrinsic amidolytic or plasminogen activator activities. In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; in the presence of a fibrin clot, scu-PA, but not tcu-PA, induces fibrin-specific clot lysis (Gurewich et al. 1984). The fibrin specificity of scu-PA does not require its conversion to tcu-PA, nor its binding to fibrin, but is mediated by enhanced binding of plasminogen to partially digested fibrin (Fleury et al. 1993).

4.1.3
Inhibition of Plasmin by α_2-Antiplasmin

α_2-Antiplasmin forms an inactive 1:1 stoichiometric complex with plasmin. The inhibition involves two consecutive reactions: a fast, second-order reaction producing a reversible inactive complex, followed by a slower first-order transition resulting in an irreversible complex. The second-order rate constant of the inhibition is very high ($2–4 \times 10^7$ $M^{-1}s^{-1}$), but this high inhibition rate is dependent on the presence of a free lysine-binding site and active site in the plasmin molecule and on the availability of a plasminogen-binding site and reactive site peptide bond in the inhibitor. The half-life of plasmin molecules on the fibrin surface, which have both their lysine-binding sites and active site occupied, is estimated to be two to three orders of magnitude longer than that of free plasmin (Wiman and Collen 1978).

4.1.4
Inhibition of Plasminogen Activators by PAI-1

PAI-1 reacts very rapidly with t-PA and with tcu-PA, with second-order inhibition rate constants of the order of 10^7 $M^{-1}s^{-1}$, and it does not react with scu-PA (Kruithof 1988). Like other serpins, PAI-1 inhibits its target proteinases by formation of a 1:1 stoichiometric reversible complex, followed by covalent binding between the hydroxyl group of the active site serine residue of the proteinase and the carboxyl group of the P1 residue at the reactive centre ("bait region") of the serpin.

PAI-1 occurs as an active inhibitory form that spontaneously converts to a latent form, due to insertion of part of the reactive centre loop in the major β-sheet of PAI-1, which is thereby not accessible to the target enzyme (Mottonen et al. 1992). Another molecular form of intact PAI-1 has been isolated that does not form stable complexes with t-PA but is cleaved at the P1-P′1 peptide bond ("substrate PAI-1") (Declerck et al. 1992).

4.2
Pharmacology of Plasminogen Activators

Following intravenous administration of wild-type recombinant t-PA (rt-PA) to man, it is cleared from the circulation with an initial half-life of 4–8 min. Clearance is the result of interaction with several receptor systems. Liver endothelial cells have a mannose receptor which recognises the high mannose-type carbohydrate side-chain at Asn^{117} in the kringle 1 domain, whereas liver parenchymal cells contain a calcium-dependent receptor which interacts mainly with the growth factor domain of t-PA (Otter et al. 1992; Kuiper et al. 1996). In addition, the low-density lipoprotein receptor-related protein (LRP), expressed in

high copy number on hepatocytes, binds free t-PA and complexes with PAI-1 (Orth et al. 1992; Bu et al. 1992).

The recommended dose of rt-PA [alteplase, Activase (Genentech, South San Francisco), Actilyse (Boehringer Ingelheim, Ingelheim)] for the treatment of acute myocardial infarction was 100 mg administered as 60 mg in the first hour (of which 6–10 mg was given as a bolus over the first 1–2 min), 20 mg over the second hour and 20 mg over the third hour. Later it was proposed to give the same total dose of 100 mg but "front loaded", starting with a bolus of 15 mg followed by 50 mg in the next 30 min and the remaining 35 mg in the following hour (Neuhaus et al. 1989). In the GUSTO trial, a dose of 15 mg intravenous bolus of alteplase followed by 0.75 mg/kg over 30 min (not to exceed 50 mg) and then 0.50 mg/kg over 60 min (not to exceed 35 mg) was utilised (GUSTO Investigators 1993). In the COBALT (1997) trial, double bolus administration of rt-PA (50 mg given 30 min apart) was evaluated in patients with myocardial infarction. Whichever regimen is used, it is important to co-administer intravenous heparin during and after alteplase treatment. For catheter-directed local thrombolysis with alteplase in patients with recent peripheral arterial occlusion, a dose of 0.05–0.10 mg/kg per hour over an 8-h period is used.

During thrombolytic therapy, there is a vast excess of t-PA over PAI-1 in the circulation, but critical lysis occurs at the surface of an arterial thrombus, where the local PAI-1 concentration can be very high. Therefore, mutants with resistance to PAI-1 may be useful to reduce re-occlusion. In addition, mutants with a prolonged half-life allow efficient thrombolysis by bolus administration at a reduced dose (Collen and Lijnen 2003).

The main mechanism of removal of u-PA from the blood is by hepatic clearance. Scu-PA is taken up in the liver via a recognition site on parenchymal cells and is subsequently degraded in the lysosomes (Kuiper et al. 1992). Following intravenous infusion of recombinant scu-PA (saruplase) in patients with acute myocardial infarction, a biphasic disappearance was observed with an initial half-life in plasma of 8 min (Van de Werf et al. 1986).

With a preparation containing 160,000 IU/mg of saruplase, the dose used successfully in patients with acute myocardial infarction was 20 mg given as a bolus and 60 mg over the next 60 min, immediately followed by an intravenous heparin infusion (20 IU/kg per hour) for 72 h (PRIMI Study 1989). In the LIMITS Study in patients with acute myocardial infarction, the same dose regimen of saruplase was used, but with a prethrombolytic heparin bolus of 5,000 IU and an i.v. heparin infusion for 5 days starting 30 min after completion of thrombolysis (Tebbe et al. 1995). A recombinant glycosylated form of scu-PA (A-74187) has been evaluated in patients with acute myocardial infarction, using 60 or 80 mg monotherapy or 60 mg primed with a preceding bolus of 250,000 IU of recombinant tcu-PA, always combined with aspirin and i.v. heparin (Weaver et al. 1994).

4.3 Role of Fibrinolysis in Arterial Restenosis

Vascular interventions for the treatment of atherothrombosis induce restenosis of the vessel within 3–6 months in 30%–50% of treated patients. Arterial stenosis may result from remodelling of the vessel wall (such as occurs predominantly after balloon angioplasty) or from accumulation of cells and extracellular matrix in the intimal layer (such as occurs predominantly after intraluminal stent application).

Proteinases from the plasminogen/plasmin system participate in the proliferation and migration of smooth muscle cells (SMC), and in matrix remodelling during arterial wound healing. To assess their role in SMC migration and neointima formation, a perivascular electric injury model in the mouse has been extensively used (Carmeliet et al. 1997a). In this model, surgically exposed femoral arteries are injured perivascularly via delivery of an electric current, which destroys all medial SMC, denudes the injured segment of intact endothelium and transiently induces platelet-rich mural thrombosis. A vascular wound-healing response results that is characterised by degradation of the mural thrombus, transient infiltration of the vessel wall by inflammatory cells and progressive removal of the necrotic debris. Topographic analysis reveals repopulation of the media and accumulation in the neointima of SMC, originating from the uninjured borders and progressing into the necrotic centre. Within 3 weeks after injury, a neointima is formed that contains up to 12 layers of smooth muscle α-actin-immunoreactive cells. Evans blue staining in injured arteries reveals progressive re-endothelialisation from the uninjured borders.

This electric injury model has been applied to wild-type mice and to mice with deficiency of the main components of the plasminogen/plasmin system. At 1 week after vascular injury in wild-type mice, t-PA activity in arterial sections or extracts was not significantly altered, whereas u-PA activity levels were two- to threefold higher than control at 2 days after injury of the femoral artery. Prolonged fibrin overlay with femoral or carotid artery sections from t-PA-deficient mice revealed that the fibrinolytic activity in injured versus control segments was markedly enhanced. This activity was reduced by approximately 50% upon inhibition of u-PA (Lijnen et al. 1998).

In plasminogen-deficient mice, wound healing was significantly impaired with delayed removal of necrotic debris, reduced leucocyte infiltration and SMC accumulation, and decreased neointima formation. SMC accumulated at the uninjured borders but failed to migrate into the necrotic centre (Carmeliet et al. 1997b). Neointima formation and neointimal cell accumulation were also reduced in u-PA-deficient and in combined t-PA-and u-PA-deficient arteries but not in t-PA-deficient arteries. Similar to the plasminogen-deficient arteries, SMC accumulated at the uninjured borders but failed to migrate into the necrotic centre in u-PA-deficient and in the double-deficient arter-

ies (Carmeliet et al. 1997c). Proliferation of SMC and re-endothelialisation were not affected by a deficiency in plasminogen, u-PA or t-PA. Thus, u-PA and plasminogen play a significant role in vascular wound healing and arterial neointima formation after injury, most likely by promoting cellular migration. In this model, binding of u-PA to its cellular receptor u-PAR is not required to provide sufficient pericellular u-PA-mediated plasmin proteolysis to allow cellular migration into a vascular wound (Carmeliet et al. 1998).

Deficiency of PAI-1, the main inhibitor of both u-PA and t-PA, in contrast, improved vascular wound healing in this model. SMC migrated more rapidly from the uninjured borders into the necrotic centre of the arterial wound than in wild-type SMC. When PAI-1-deficient mice were intravenously injected with replication-defective adenovirus expressing human PAI-1, plasma PAI-1 antigen levels increased in a dose-dependent fashion and luminal stenosis was significantly suppressed. By impairing cellular migration, PAI-1 thus plays an inhibitory role in vascular wound healing and arterial neointima formation after electric injury (Carmeliet et al. 1997d). In contrast, in murine models of vascular injury induced by ferric chloride, rose bengal or copper, a positive overall correlation was observed between PAI-1 levels and neointima formation (Konstantinides et al. 2001; Eitzman et al. 2001; Ploplis et al. 2001). These discrepancies may be explained to some extent by subtle differences in the genetic background of the mice strains or by differences in the experimental models. PAI-1 binds with high affinity to its cofactor vitronectin (VN), which stabilises its activity and mediates binding to fibrin clots. PAI-1 and VN play a role in the thrombotic response to ferric chloride-induced carotid artery injury in mice by preventing premature thrombus dissolution and embolisation. The effect of PAI-1 and VN on restenosis after vascular injury may depend on which phase of the wound healing response and what part of the vasculature are analysed; a critical feature may be the presence or absence of thrombus/fibrin (Konstantinides et al. 2002). Thus, PAI-1 may inhibit neointima formation in the absence of fibrin, but enhance it in the presence of fibrin. Indeed, the mechanical and electric injury models are usually associated with only transient thrombosis, in contrast to the prominent thrombotic reaction in the injury models induced with ferric chloride, rose bengal or copper.

In wild-type mice and in mice deficient in α_2-antiplasmin—the main physiological plasmin inhibitor—the neointimal and medial areas at 1–3 weeks after electric injury of the femoral artery were similar, resulting in comparable intima/media ratios. Nuclear cell counts in cross-sectional areas of the intima of the injured region were also comparable. Fibrin deposition was not significantly different in arteries of both genotypes at 1 day after injury, and no mural thrombosis was detected at 1 week after injury. Thus, α_2-antiplasmin does not seem to play a major role in SMC migration and neointima formation after vascular injury in mice (Lijnen et al. 2000b).

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