relate with the severity of disease. Nash et al. [23] have found that adhesion, which is initially weak and shear reversible, is subsequently stabilized by cooperative effects of multiple receptor interactions. Three endothelial proteins have been identified *in vitro* as receptors for infected erythrocytes: thrombospondin, CD36 and ICAM-1 [24]. The precise role of these receptors in the pathogenesis of the disease is not yet clear. Thrombospondin is a complex glycoprotein of high molecular weight (420 kDa) composed of three identical chains linked by disulfide bonds. As a major component of platelet α-granules, it is synthesized by many adherent cell types such as endothelial cells and macrophages. It is a multifunctional protein involved in cell-cell interactions, carrying binding sites for fibrinogen and fibronectin. CD36 is a glycoprotein of 85 kDa expressed on the endothelial cell membrane and also on circulating monocytes macrophages and some tumor cell lines (leukemias and melanomas). This molecule is a receptor for thrombospondin and collagen type I. ICAM-1 (CD54) is a 95-112 kDa glycoprotein belonging to the immunoglobulin superfamily, which is widely distributed on cells of the immune system and on the endothelium. Its specific ligands are the integrins LFA-1 (Leukocyte Function-associated Antigen-1, CD11a/CD18) and Mac-1 (CD11b/CD18). However, ICAM-1 is ubiquitous and binds to the human rhinovirus. Using monoclonal antibodies (MAbs) which recognize different epitopic sites of ICAM-1, the molecular domain-1 has been identified as the binding site for infected erythrocytes and been shown to be distinct from the LFA-1 sites [25], while the domain-2 appears to differ from this binding site but to play an essential role in the structural conformation of the first domain. LFA-1 recognizes a molecular region bordering domains 1 and 2 but on the opposite side to the binding site for infected erythrocytes [25]. Ockenhouse et al. [26] have further demonstrated the importance of domains 1 and 2 in the interaction of ICAM-1 with infected erythrocytes by means of COS cells transfected with a cDNA encoding wild type or mutant ICAM-1. These transfected cells have been tested for the adhesion of malaria infected erythrocytes. Soluble ICAM and synthetic peptides derived from the amino-acid sequence of ICAM bind to infected erythrocytes. These results suggest that such molecules could be employed in the treatment of severe and cerebral malaria.

Among other endothelial cell molecules constituting putative receptors for malaria infected erythrocytes, adhesion molecules induced by cytokines may also mediate the cytoadhesion of parasitized cells to the endothelium. Adhesion is inhibited by antibodies against VCAM-1 and E-selectin (ELAM-1). E-selectin and VCAM-1 are present on the endothelium of post-mortem brain tissue from patients dying of cerebral malaria [26], while during clinical episodes of malaria, increases in the plasma levels of soluble ICAM-1 and E-selectin are positively correlated with plasma concentrations of soluble IL-2. The association of acute-phase proteins with inflammation marked by rises in cytokines such as IL-1ß and TNF, may create conditions which favor the adhesion of parasitized erythrocytes to the endothelium. Blood levels of TNF may be increased in patients with malaria. This cytokine is frequently elevated in cerebral complications [27]. Its systemic effects include effects on the vasculative which may participate in the pathogenesis of the disease. Thus, cytokines activating endothelial cells induce the expression of receptors which can potentiate the adhesion of Plasmodium falciparum infested erythrocytes.

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Blood Coagulation

Definition *Process that leads to clot formation. The blood coagulation system is constituted of a series of biochemical reactions that lead to the formation of fibrin.*

See: →Coagulation factors; →Fibrinolytic, hemostatic and matrix metalloproteinases, role of; →Thrombosis

BSS

Definition *Bernard-Soulier syndrom*

See: →Bleeding disorders

Cadherins

Calcium-dependent intercellular adhesion molecules. Three main types have been described, Epithelial

Cadherin (E-cadherin), Neural cadherin (N-cadherin) and vascular endothelial cadherin (VE-cadherin).

See: →Signal transduction mechanisms in vascular biology

CAI

Definition *Carboxyamido-triazol*

See: →Angiogenesis; →Angiogenesis inhibitors

CAM

Definition *Chorioallantoic membrane*

See: →Angiogenesis; →Angiogenesis inhibitors; →Atherosclerosis

cAMP

Definition *Cyclic adenosine monophosphate*

See: →Fibrinolytic, hemostatic and matrix metalloproteinases, role of; →Nitric oxide; →Platelet stimulus-response coupling; →Vasomotor tone regulation, molecular mechanisms of

CD142

See: →Procoagulant activities

Cell Cycle

Definition *In order to divide, cells pass through different phases that include G1, S (DNA synthesis), G2 and M (mitosis) which together form the cell cycle. The major regulators of the cell cycle are cyclins/cyclin-dependent kinases which are connected to the signalling machinery.*

See: →Signal transduction mechanisms in vascular biology

CETP

Definition *Cholesteryl ester transfer protein*

See: →Lipoproteins

CFU

Definition *Colony-forming unit*

See: →Megakaryocytes

CFU-MK

Definition *Megakaryocytic colony forming unit*

See: →Megakaryocytes

cGMP

Definition *Cyclic guanidine monophosphate*

See: →Cyclic nucleotides; →Nitric oxide; →Platelet stimulus-response coupling; →Vasomotor tone regulation, molecular mechanisms of

Chemokines

Definition *Family of regulatory molecules. Different subtypes have been described based on the occurrence of the cysteine motif. Among CXC chemokines, some contain an ELR motif and have stimulatory properties while others are ELR negative and are inhibitory.*

See: →Angiogenesis; →Angiogenesis inhibitors

CHO

Definition *Chinese hamster ovary*

See: →FGF receptors; →FGF-1, FGF-2; →Vascular integrins; →von Willebrand factor

Chronic Inflammatory Disorders

Definition *Family of chronic diseases characterized by a non-resolved process of inflammation*

For mouse models see: →Fibrinolytic, hemostatic and matrix metalloproteinases, role of

CL

Definition *Corpus luteum*

See: →Hormonal regulation of vascular cell function in angiogenesis

cNOS

Definition *Constitutive nitric oxide synthase*

See: →Nitric oxide

Coagulation Factors

Roman numbers identify the majority of coagulation factors However, several coagulation factors have a synonym that in some cases is associated with a disease state or the first individual identified with the deficiency of the corresponding fac tor. The synonym for each coagulation factor (when applied) will be always given below in parenthesis.

Definition *Serine proteases that constitute the extrinsic and intrinsic blood coagulation pathway.*

See also: →Fibrin/fibrinogen; →Fibrinolytic, hemostatic and matrix metalloproteinases, role of; →Procoagulant activities; →Thrombin; →Thrombosis

Introduction Following vascular injury, the blood clotting process is initiated in order to stop blood from leaking outside the vasculature. Blood clotting involves a multitude of proteins, which act in concert to produce the procoagulant enzyme α-thrombin, which in turn is responsible for the generation of the fibrin plug. However, while generation of the fibrin plug is required for the arrest of excessive bleeding, unregulated clotting will result in the occlusion of the vessels and thrombosis. Thus, the regulation of the delicate balance between the procoagulant and anticoagulant mechanisms is of extreme importance for survival. The maintenance of hemostasis in human blood is a complex event, requiring the controlled interaction of protease, zymogens, cofactors and inhibitors on surfaces presented to the blood by the platelets, blood cells and vessel wall. Procoagulant events must be regulated and are localized to damaged regions of vessels; otherwise, disseminated coagulation throughout the vasculature will result in the occlusion of blood flow to tissues and organs. Blood clotting occurs when platelets adhere and aggregate at sites of injury, leading to the exposure of procoagulant membrane surface which localizes and concentrates the proteins required for normal hemostasis and formation of polymeric fibrin strands which stabilize the initial platelet mass. These events are dependent upon the conversion of prothrombin to α-thrombin, the central enzyme of blood coagulation. The factors, which regulate thrombin generation, are manifold, and diseases of abnormal coagulation are often associated either with defects in the pathway leading to thrombin or in the processes, which regulate thrombin production. Deficient or unrestricted thrombin generation can lead to bleeding (hemorrhage) or vessel occlusion (thrombosis, infarction, etc.), respectively. Thus, an understanding of the origins of hemorrhagic and thrombotic disease must be rooted in an understanding of the factors regulating the thrombin response.

Theories of blood coagulation have recognized that initiation of thrombin generation and clotting of plasma *in vitro* arises from two distinct sources: the contact (or intrinsic) pathway and the tissue factor (or extrinsic) pathway. First Paul Morawitz in 1905 [1] proposed that tissue thromboplastin is required for the initiation of blood clotting and established the basis for the extrinsic pathway of blood coagulation. Davie and Ratnoff [2] later outlined the classical "cascade" models of the intrinsic pathway of blood coagulation. Both pathways are characterized by a series of reactions initiated by the presentation of a procoagulant surface to the blood flow. No procoagulant complex can by assembled in the absence of an adequate membrane surface. Every reaction in the series involves the activation of an inactive plasma protease precursor (zymogens) via limited proteolysis to an activated plasma protease. Each newly activated protease activates other zymogens later in the sequence, producing a string of activation processes, which culminate in thrombin generation and subsequent clot formation. However, the activity of all of the complexes is also limited by the availability of an adequate membrane surface provided by endothelial cells, platelets, and monocytes. The cell surface provides a site for the recruitment of the appropriate proteins and allows for fast and efficient clot formation. Subsequent investigations detailed the architecture of each active enzyme at the procoagulant surface as an assembly, requiring calcium, a proenzyme activated by proteolysis with a cofactor also activated by proteolysis. Thus, timely exposure of the adequate membrane surface is an important step in the regulation of α-thrombin formation and since each reaction in the series requires this surface-dependent assembly for efficient expression of activity, coagulation can be modulated in vivo by regulation of either the surface presented to the blood, as in platelet activation; the protease component, by inhibitors, or the cofactor component.

Structure and Characteristics

Molecular Weight; Domains

Antithrombin III (AT-III) AT-III contains an NH_2-terminal heparin binding domain, a carbohydrate rich domain, and a COOH-terminal serine protease-binding domain (Figure 1). AT-III is a member of the serine protease inhibitor (SERPIN) family which inhibit serine proteases. AT-III (M_r 58,000) circulates in plasma at a concentration of 150-400 μg/ml (2.6 μM-3.4 μM).

AT-III contains a COOH-terminal arginine-serine reactive site that interacts with the serine proteases of the blood coagulation process as well as two positively charged clusters of amino acid residues that bind sulfated polysaccharides (Figure 1).

Factor II (Prothrombin) Prothrombin circulates in plasma at a concentration of 1.4 μM as an inactive zymogen of M_r 72,000 [3]. The NH_2-terminal portion of the molecule contains the γ-carboxyglutamate residues, which are involved in the metal binding properties of the molecule and the two kringle domains (Figure 2). The entire catalytic domain is located at the COOH-terminus of the molecule. Prothrombin is a vitamin-K dependent protein. The light chain includes 46 amino acid residues containing the γ-carboxyglutamate residues, which are required for the metal binding and the proper interaction of the molecule with the membrane surface. Two kringle domains of 79 amino acids each follow this region. The serine protease domain is localized at the COOH-terminal domain, is composed of 259 amino acids and contains the catalytic triad which is responsible for the enzymatic activity of the molecule (Figure 2).

Factor V (Proaccelerin) Human factor V circulates in plasma at a concentration of 20 nM [4], as a large single chain procofactor with a M_r 330,000 [5]. The cDNA and deduced amino acid sequence shows that the molecule

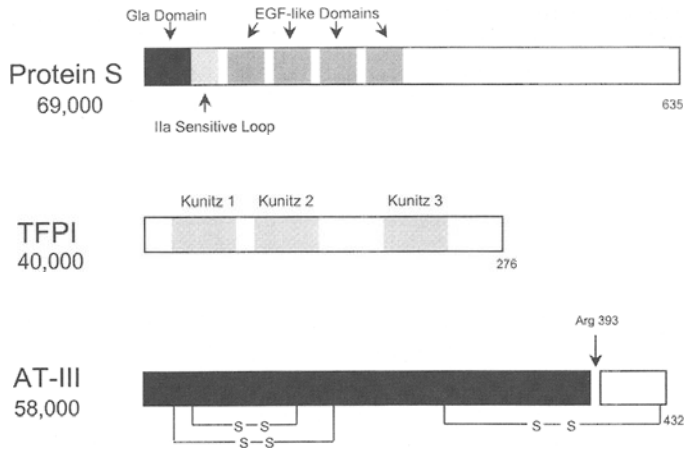

Figure 1. *Schematic representation of the organization of the protein S, TFPI, and ATIII molecules.* The specific domains are indicated. (Reprinted with permission from The Regulation of Clotting Factors by Kalafatis et al. In: Critical Reviews in Eukaryotic Gene Expression, 7(3):241-280 (1987), Ed Begell House Inc).

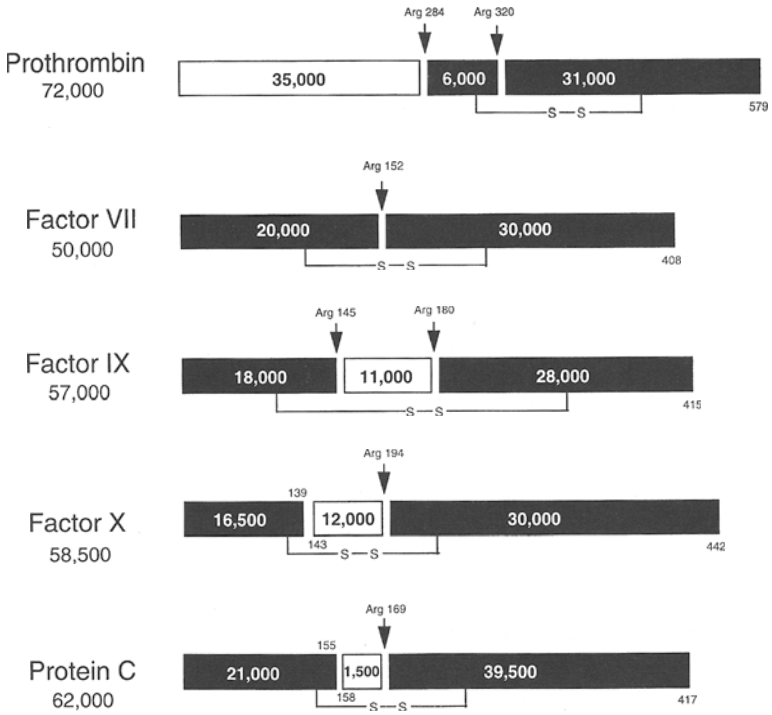

Figure 2. *Schematic representation of the structure of the vitamin K-dependent proteins.* The active molecule is identified in each case by the black shading. The specific activating cleavage sites are also shown. The NH_2-terminal portion of each protein contains the γ-carboxyglutamic acids which are required for the metal binding of the proteins (not shown). (Reprinted with permission from The Regulation of Clotting Factors by Kalafatis et al. In: Critical Reviews in Eukaryotic Gene Expression, 7(3):241-280 (1987), Ed Begell House Inc).

contains triplicate "A" domains which share a high degree of homology with ceruloplasmin, duplicated "C" domains which share homology with discoidin and a connecting B region which functions as an activation peptide (Figure 3).

Factor V is cleaved by α-thrombin to generate the active cofactor, and two heavily glycosylated activation peptides [6] (Figure 3). The factor Va molecule is a heterodimer composed of a heavy chain of M_r 105,000 and a light chain of M_r 74,000. Both subunits are non-covalently associated via a divalent metal ion process (Figure 3). The heavy chain contains the NH_2-terminus of the procofactor (residues 1-709) and is composed of two "A" domains (residues 1-303, and 317-656), connected by an amino acid region containing basic amino acids (residues 304-316). The COOH-terminal portion of the heavy chain (residues 657-709) is rich in acidic amino acids. The light chain of the cofactor contains the COOH-terminus of the factor V molecule (residues 1546-2196) and is composed of one "A" domain (residues 1546-1877) and two "C" domains (residues 1878-2036, and 2037-2196). Factor Va has 18 cysteines residues (Fig 3). Four of these cysteines are present as free -SH while the remaining fourteen are involved in disulfide bridges as follows (Figure 3): three 26 amino acid residue α-loops are present, one in each "A" domain; the "A1" and "A2" domains also contain one

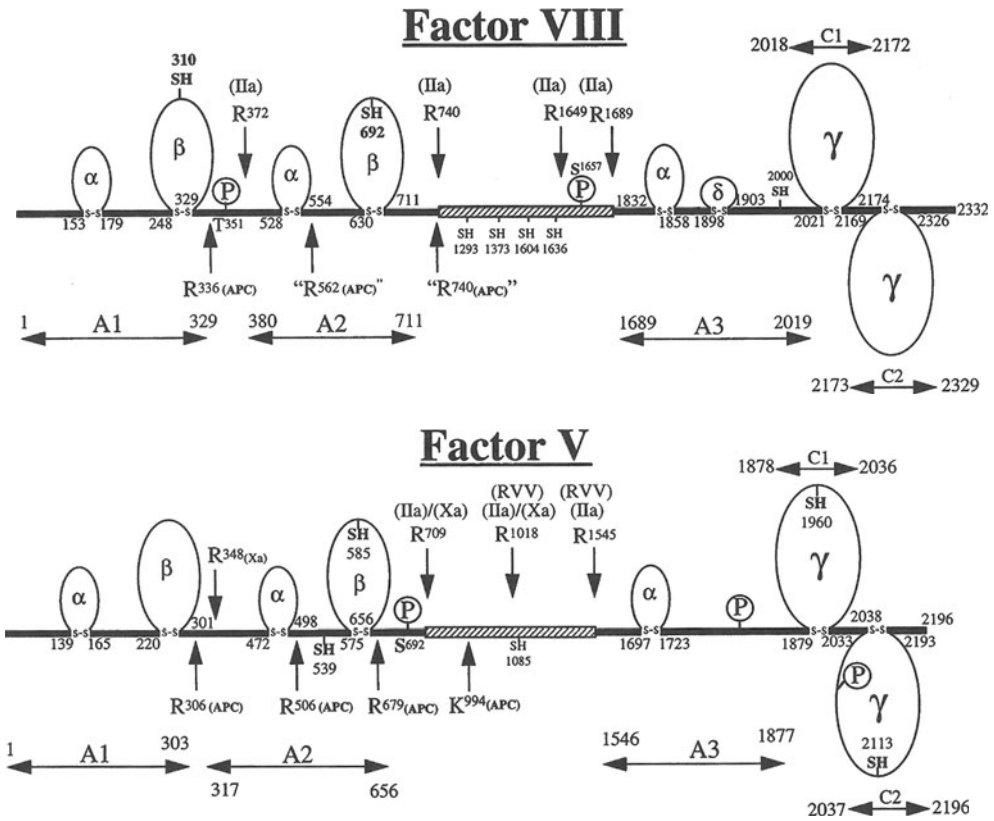

Figure 3. *Structural features of factor VIII and factor V.* The activating cleavage sites of the procofactors are shown on top. The APC-inactivating cleavage sites that are required for factor V/Va inactivation by APC are shown at the bottom. The APC cleavage sites for factor VIII are also shown. The location of the free cysteines (indicated by SH) and of the disulfide bridges in each molecule are also shown. The phosphorylation sites of both procofactors are identified by a "P". (Reprinted with permission from The Regulation of Clotting Factors by Kalafatis et al. In: Critical Reviews in Eukaryotic Gene Expression, 7(3):241-280 (1987), Ed Begell House Inc).

β-loop each of which composed of 82 amino acids; two γ-loops which are composed of 154 and 155 residues represent the "C1" and "C2" domains. The factor V molecule has multiple potential N-linked glycosylation sites in the B region and on the heavy and light chains that are required for secretion. The molecule is also phosphorylated by a membrane associated casein kinase II-like enzyme on the heavy chain (at Ser^{692}) and by a protein kinase C isoform on 2 sites of the light chain (Figure 3). Phosphorylation at Ser^{692} is required for fast and efficient inactivation of the cofactor by APC. Finally, Tyr^{665}, Tyr^{696},Tyr^{698}, Tyr^{1494}, Tyr^{1510}, Tyr^{1515}, and Tyr^{1565} are believed to be sulfated. Inhibition of sulfation by sodium chlorate results in a cofactor molecule 5-fold less active that the native molecule. It has been thus suggested that sulfation of factor V is important for α-thrombin activation of the procofactor. It has been also suggested that sulfation may by important for the interaction of factor Va with factor Xa.

FactorVII Factor VII circulates in plasma predominantly as a single chain inactive zymogen (M_r 50,000) at a concentration of 10 nM [7]. The NH_2-terminal part of the molecule contains the γ-carboxyglutamate residues, which are involved in the metal binding properties of the protein and a β-hydroxyaspartic acid at position 63 (Figure 2).

Factor VII is a vitamin-K dependent protein. The light chain includes 49 amino acid residues containing the γ-carboxyglutamate residues, which are required for the metal binding and the proper interaction of the molecule with the membrane surface (Figure 2). Two epidermal growth factor (EGF) domains of 32 and 37 amino acids respectively follow this region. The serine protease domain is localized at the COOH-terminal domain, is composed of 254 amino acids and contains the catalytic triad, which is responsible for the enzymatic activity of the molecule.

Factor VIII (Antihemophilic Factor) Human factor VIII (M_r 280,000) circulates in plasma at 0.7 nM [8] in non-covalent association with von Willebrand factor (vWf) (M_r~$15x10^6$). Deficiency of factor VIII constitutes one of the most common inherited coagulation disorders (hemophilia A). The cDNA and deduced amino acid sequence of factor VIII shows that the molecule is com-

posed of triplicated "A" domains, duplicated "C" domains and a B region (Figure 3).
Human factor VIII (M_r 285,000) circulates in plasma as a dimer composed of a multitude of heavy chain fragments (M_r 90-200,000) and a light chain (M_r 80,000) [9]. α-Thrombin converts factor VIII to an heterotrimer. Factor VIIIa is a composed of fragments of M_r 50,000 (A1 domain) containing a COOH-terminal portion rich in acidic amino acids (residues 337-372), M_r 40,000 (A2 domain), and M_r 74,000 (A3C1C2) containing the carboxyl-terminal part of the factor VIII molecule (Figure 3). The three subunits are non-covalently associated via divalent metal ions. Factor VIIIa contains 19 cysteines residues (Figure 3). Three of these cysteines are present as free -SH while the remaining sixteen are involved in disulfide bridges. Three γ-loops are present, one in each "A" domain. The "A1" and "A2" domains also contain β-loops. Two γ-loops encompass the "C1" and "C2" domains of the light chain of factor VIIIa. A small δ-loop is also present in the A3 domain of the cofactor (Figure 3).
Factor VIII is subject to several post-translational modifications. The molecule contains several potential N-linked glycosylation sites that are necessary for secretion and is phosphorylated by a platelet membrane casein kinase II at Thr^{351} and Ser^{1657}. Tyr^{346}, Tyr^{718}, Tyr^{719}, Tyr^{723}, Tyr^{1664}, and Tyr^{1680} are sulfated and sulfation at residues 346 and 1664 increases the rate of factor VIII activation by α-thrombin, whereas sulfation at residues 718, 719, and 723 is required for optimum interaction of factor VIIIa with the components of the intrinsic tenase. Finally, sulfation of Tyr^{1680} is necessary for the proper interaction of factor VIII with vWf.

Factor IX (Christmas Factor) Human factor IX circulates in plasma as a single chain zymogen (M_r 55,000) at a concentration of 90 nM [10]. Deficiency of this glycoprotein constitutes an inherited coagulation disorder called hemophilia B. The NH_2-terminal part of the molecule contains the γ-carboxyglutamate residues, which are involved in the metal and membrane binding properties of the protein (Figure 2).
Factor IX is a vitamin-K dependent protein. The light chain includes 50 amino acid residues containing the γ-carboxyglutamate residues which are required for metal ion binding and the proper interaction of the molecule with the membrane surface. This region is followed by two EGF domains of 32 and 37 amino acids respectively. The serine protease domain is localized at the COOH-terminal region, is composed of 235 amino acids and contains the catalytic triad which is responsible for the enzymatic activity of the molecule (Figure 2).

Factor X (Stuart Factor) Human factor X circulates in plasma as an inactive zymogen (M_r 59,000) [11] composed of a heavy chain (M_r 42,000) and a light chain (M_r 16,500) which are covalently associated through a disulfide bond at a concentration of 170 nM. The light chain contains the γ-carboxyglutamate residues, which are required for the metal binding and the proper interaction of the molecule with the membrane surface (Figure 2).
Factor X is a vitamin-K dependent protein. The light chain includes 49 amino acid residues containing the γ-carboxyglutamate residues which are required for metal ion binding and the proper interaction of the molecule with the membrane surface. This region is followed by two EGF domains of 32 and 36 amino acids respectively. The serine protease domain is localized at the COOH-terminal domain, is composed of 254 amino acids and contains the catalytic triad, which is responsible for the enzymatic activity of the molecule (Figure 2).

Factor XI Factor XI circulates in plasma at a concentration of 30 nM [12] in a non-covalent complex with high molecular weight kininogen. Factor XI (M_r 160,000) contains two identical polypeptide chains (M_r 80,000) associated through disulfide bridges.
The mature factor XI molecule is a two-chain homodimer that are linked by disulfide bonds. A monomer is composed of four tandem amino acid sequence repeats, which constitute the heavy chain of factor XIa. The catalytic domain, which is located at the carboxyl terminal portion of the molecule, will constitute the light chain of factor XIa. Following activation the proenzyme will give rise to an enzyme with two active sites per molecule.

Factor XII (Hageman factor) Factor XII has a M_r 85,000 and circulates in plasma at a concentration of 375 nM. Factor XII is single chain protein of 596 amino acids. The protein was found to contain multiple domains, which have considerably homology with the EGF and kringle regions of tissue-type plasminogen activator (tPA) and fibronectin.

Factor XIII (Plasma Transglutaminase) Factor XIII is a heterodimer of M_r 320,000 composed of two subunits: the α subunit (M_r 82,000) and the β subunit (M_r 76,500). Factor XIII ($\alpha_2\beta_2$) circulates in plasma at a concentration of 70 nM. Factor XIII is a dimer composed of two non-identical subunits α and β with the overall structure $\alpha_2\beta_2$. The active site is on the α chain whereas the β chain functions as a carrier protein.

Fibrinogen Fibrinogen is a M_r=340,000 protein which is present in plasma at a concentration of 7 μM [13]. Fibrinogen is composed of three pairs of polypeptides, which are linked via disulfide bridges. The three polypeptides are: Aα (M_r 66,500), Bβ (M_r 52,000) and γ (M_r 46,500).
Fibrinogen is a disulfide linked dimer composed of 3 pairs of disulfide linked non-identical polypeptide chains (Aα, Bβ, and γ). The two subunits are aligned in an antiparallel manner forming a trinodular arrangement of the six chains. The nodes are formed by disulfide bridges between the three chains. The Aα chain contain an NH_2-terminal fibrinopeptide A [amino acid residues 1-16], the factor XIIIa cross-linking sites and 2 phosphorylation sites. The Bβ chain contains the fibrinopeptide B [amino acid residues 1-14] one N-linked carbohydrate moiety and an NH_2-terminal pyroglutamic acid. The γ chain contains all the other N-linked glycosylation sites and a factor XIIIa cross-linking site.

High molecular weight kininogen (Fitzgerald factor) Two forms of kininogens are found in plasma. The high molecular weight kininogen circulates in plasma at 670 nM as a single chain protein with a M_r 120,000 whereas, the low molecular weight kininogen (M_r 66,000) circulates in plasma at a concentration of 2.4 μM.
The kininogens are proteins which are composed of multiple domains (D1–D6) and can be divided into three structural portions: a heavy chain that is common to the high and low molecular weight kininogens, the bradykinin portion, and the light chains that are unique to each of the two forms of kininogens. Domains 1–3 constitute the kininogens' heavy chain and domain 4 is the bradykinin moiety. Domain 5 is a unique domain that represent the light chain of low molecular weight kininogen, whereas domains 5 and 6 are unique to the light chain of the high molecular weight kininogen.

Prekallikrein (Fletcher Factor) Prekallikrein, which is a single chain protein with a M_r 85,000, circulates in plasma at a concentration of approximately 500 nM.
Prekallikrein is composed of a 371 amino acid heavy chain and a 248 amino acids light chain. Both chains are held together by a disulfide bond. The amino acid sequence of prekallikrein is 58% homologous to the amino acid sequence of factor XI. Prekallikrein has four tandem repeats located at the amino terminal portion of the molecule. Because of the intrachain disulfide bond arrangement the molecule has four "apple" domains each composed of 90 or 91 amino acids. These structures have been also observed in factor XI.

Protein C Protein C which is a vitamin K-dependent protein of M_r 62,000 (Figure 2) [14] circulates in plasma at a concentration of 60 nM and is composed of a heavy chain (M_r 41,000) and a light chain (M_r 21,000) which are associated covalently through a disulfide bond. The light chain contains the γ-carboxyglutamate residues, which are required for the metal binding and the proper interaction of the molecule with the membrane surface.
Single chain protein C represents approximately 20% of the circulating plasma zymogen suggesting that the protein is initially generated as a single chain protein which is converted to a two chain form by an as yet unidentified protease. Protein C is a vitamin K-dependent zymogen with γ-carboxyglutamate residues located on the NH_2-terminal portion of the light chain. These residues are required for the proper interaction of the protein with the membrane surface and its physiological function. After these residues there is a stack of hydrophobic residues, two EGF repeats (40 and 36 amino acids respectively) and a protease domain (250 amino acids). A β-hydroxyaspartic acid residue is produced by a post-translational modification in the EGF domain. This residue is required for Ca^{2+}-dependent alteration in the molecule (Figure 2).

Protein S Plasma protein S which is another vitamin K-dependent protein (300 nM, M_r 69,000) exists in two states, free-protein S (~150 nM) and in complex with the C4B-binding protein (C4BP) of complement (150 nM). Protein S is also present at low concentrations in platelets (Fig 1).
Protein S is composed of an NH_2-terminal "Gla"-domain, a thrombin sensitive region, four EGF-like structures and a COOH-terminal portion, containing 60% of the whole molecule (Figure 1). Protein S was initially identified due to the presence of the γ-carboxyglutamic acid residues [15]. Plasma protein S exists in two states, free-protein S and in complex with the C4B binding protein (C4BP) of complement. Protein S is also present at low concentrations in platelets. The anticoagulant properties of protein S are only observed for the free fraction of the protein and are the object of intense investigation.

Thrombomodulin Human thrombomodulin (M_r~ 100,000) is an endothelial cell surface glycoprotein that exhibits similarity to the low-density lipoprotein receptor [16]. Thrombomodulin is composed of 559 amino acids and contains five domains: an NH_2-terminal lectin-like domain (residues 1–224) containing a potential α-thrombin binding site, six EGF-like domains (residues 225-461), a 34 amino acid segment which contains eight hydroxyl-amino acids, several potential glycosylation sites for O-linked carbohydrate in a region rich in threonine and serine residues (residues 462–496), a hydrophobic membrane spanning domain (residues 497-520), and a cytoplasmic tail (residues 521–559).

Tissue Factor (TF) TF (has M_r 29,600) is a membrane-bound glycoprotein, which is exposed at the site of vascular injury and, in association with the serine protease factor VIIa, initiates the blood coagulation cascade [17]. Tissue factor is selectively expressed by a variety of vascular and extravascular cells including monocytes, macrophages and endothelial cells. Human tissue factor is composed of 263 amino acids and contains three domains: a putative extracellular domain which contains residues 1–219 (soluble tissue factor), an extremely hydrophobic (region residues 220–242) which is the transmembrane domain, and a cytoplasmic domain (amino acids 243–263) which contains a potential N-linked glycosylation site and one cysteine covalently associated with a palmityl or stearyl thioester.

Tissue Factor Pathway Inhibitor (TFPI) The normal plasma concentration of TFPI is approximately 2.5 nM [18]. TFPI in human plasma (M_r 34,000) is very heterogeneous. (Figure 1). The full-length TFPI has an acidic amino-terminal region, three tandem repeated serine protease inhibitor domains which are structuraly homologous to the Kunitz domain of the trypsin inhibitor, and a basic COOH-terminal domain.

Gene

Chromosomal Localization; Gene Structure, Expression, and Regulation

Antithrombin III (AT-III) The 15 kb gene encoding AT-III is located on chromosome 1 and has seven exons. The promoter region does not possess a TATA box at -25.

There is an 8-bp segment at the 5' flanking region that exhibits high homology with the J_κ-C_κ enhancer element of the human Igκ chain. This element appears to be critical for the efficient synthesis of AT-III. Transcription of the AT-III gene is initiated at a site located 72-bp upstream of the ATG initiation codon and results in a mRNA of 1.8 kb.

Factor II (Prothrombin) Prothrombin is the product of a 21 kb gene, which is located on chromosome 11. Transcription of the prothrombin gene gives rise to a 2 kb mRNA transcript [19]. Prothrombin is primarily synthesized in the liver. The 5' flanking region of the prothrombin gene contains potential binding sites for several transcription factors including EBP20, Sp1, Ap-1, and CTF/NF-1. Low level expression of prothrombin has also been reported in the brain, diaphragm, stomach, kidney, spleen, intestine, uterus, placenta and adrenal tissues.

Factor V (Proaccelerin) The factor V gene is located on chromosome 1 at q21-25. The factor V gene is 80 kb in length, contains 24 introns and following transcription gives rise to a 6.8 kb mRNA [20,21]. Synthesis of factor V has been demonstrated by bovine aortic endothelial cells, a human hepatocarcinoma cell line, HepG2, and by human and guinea pig megakaryocytes, the precursor cells of circulating platelets. The factor V antigen level in megakaryocytes has been shown to increase following treatment with PMA. This increase in antigen is at the protein level and is not associated with an increase in mRNA levels. Platelet factor V (20% of the circulating factor V) is also localized in the platelet α-granules [4].

Factor VII The factor VII gene is located on chromosome 13q34 [22]. The 12.8 kb gene for factor VII is located 3 kb upstream of the gene for factor X and encodes a 2.4 kb mRNA transcript. The factor VII gene is expressed only in the liver. The 5' flanking region of the factor VII gene contains binding sites for the liver enriched transcription factor HNF-4. This region also contains 3 potential AP-1 binding sites. The upstream regulatory region also contains a putative C/EBP-β/NF-IL6 binding site. This site is not important for expression of the factor VII gene because IL6, IL1-β, and IL6 plus dexamethasone do not affect mRNA levels.

Factor VIII (Antihemophilic Factor) The factor VIII gene is present on the X chromosome at q28 [23]. The factor VIII gene is 187 kb in length, contains 25 introns and gives rise to a 9 kb mRNA transcript [17]. While the introns present in the factor VIII gene are larger than the introns in factor V with the exception of intron 6, all of the intron exon boundaries are at approximately the same amino acid residue position. Hepatocytes are the major source of plasma factor VIII. Expression of factor VIII can also be detected in a variety of other cell types. DNase I footprint analysis revealed 19 protein binding sites in the -1175 to -9 region of the factor VIII promoter. This region contains all of the required elements for maximal promoter activity. Cis acting elements in this region include binding sites for HNF-1, NFκB, C/EBPα, C/EBPβ, and HNF-4.

Factor IX (Christmas Factor) The 34 kb factor IX gene is located on the X chromosome and is transcribed into a mRNA of 2.8 kb [24]. Five cis acting elements have been identified in the proximal promoter region of the factor IX gene. The 5' and 3' cis acting sites are liver specific binding sites which bind the transcription factor C/EBP α. The other cis acting sites in the proximal promoter have been shown to bind the transcription factors NF-1 and HNF-4. The factor IX proximal promoter region also contains a consensus androgen response element sequence.

Factor X (Stuart Factor) The gene encoding factor X is on chromosome 13 [25]. The gene is 27 kb in length and encodes a 1.5 kb mRNA. The gene for Factor X is located near the gene for factor VII. Factor X is expressed primarily in the liver. The sequences present in the 279 bp upstream from the AUG site are sufficient for maximal promoter activity in Hep G2 cells.

Factor XI The factor XI gene is 23 kb in length and is located on chromosome 4 at q35. The factor XI gene encodes a 2.1 kb mRNA transcript [26].

Factor XII (Hageman factor) The gene for factor XII is located on chromosome 5, is 12 kb long and encodes for a 2.4 kb mRNA. Human liver and rat hepatocytes were found to synthesize factor XII. In postmenopausal and pregnant women estrogens enhance the concentration of the protein.

Factor XIII (Plasma Transglutaminase) The 160 kb gene for the α chain of factor XIII is located on chromosome 6 at p24-25 and encodes a 3.8 kb mRNA transcript [27]. The gene for the β chain is 28 kb in length and is located on chromosome 1 at q31-32 and encodes a mRNA transcript of 2.3 kb.

Fibrinogen The three genes which code for the three fibrinogen chains are located within a 50 kb length of DNA on chromosome 4 at q23-q32. The gene for the α-chain is 5.5 kb in length and encodes a 2.2 kb mRNA transcript. The 8 kb β-chain gene encodes a 1.9 kb mRNA, and the 8.5 kb γ-gene gives rise to a 1.6 kb mRNA transcript [28].

High molecular weight kininogen (Fitzgerald factor) The two forms of kininogens circulating in plasma are products of a single gene, which is localized to chromosome 3q26-qter producing an mRNA of 27 kb. The gene consisting of 11 exons produces the mRNA for both high and low molecular weight kininogen by alternative splicing. Both molecules share the coding sequence of the first nine exons. Exon 10 contains the coding sequence for the M_r 56,000 light chain of high molecular weight kininogen whereas exon 11 encodes for the M_r 4,000 light chain of low molecular weight kininogen.

Prekallikrein (Fletcher Factor) The gene for prekallikrein is localized on chromosome 4 and is 22 kb long. The gene is composed of 15 exons and 14 introns. Computer analysis of the 5'-promoter region of the gene

revealed that plasma kallikrein and factor XI genes may be derived from a common ancestor.

Protein C The 11.2 kb protein C gene is located on chromosome 2q13-q14. The protein C gene encodes mRNA transcripts of 1.8 and 1.6 kb [29]. The presence of the two transcripts is believed to be a result of alternative polyadenylation signals present in the transcript. The promoter region contains binding sites for the liver enriched transcription factors HNF-3 and HNF-1.

Protein S The protein S gene is located near the centromere on chromosome 3 [30]. The protein S gene produces mRNA transcripts of 4, 3.1, and 2.6 kb. Protein S is synthesized in the liver and endothelial cells, testicular lining cells, and megakaryocytes.

Thrombomodulin The intronless gene for thrombomodulin is on chromosome 20. Thrombomodulin is constitutively expressed on endothelial cells [31]. Expression of thrombomodulin in contrast to TF is downregulated by exposure of endothelial cells to IL-1, TNF-α, endotoxins or hypoxia, thus contributing to the establishment of a procoagulant environment.

Tissue Factor (TF) The 12 kb TF gene located on chromosome 1 contains 6 exons and is transcribed into a 2.3 kb mRNA [32]. The endothelial cells align the luminal side of the blood vessels forming a barrier between the flowing blood and the underlying cells such as smooth muscle cells and fibroblasts, which constitutively express TF on their membrane. TF is selectively expressed by a variety of vascular and extravascular cell types including monocytes, macrophages and endothelial cells. Induction of TF expression occurs in response to expression of a multitude of transcription factors which are constitutively expressed in differentiated cells or as a result of activation by growth factors, and inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) as well as bacterial lipopolysaccharide (LPS). Vessel wall injury or the induction of TF expression results in the establishment of a procoagulant "milieu". Transcriptional expression of the TF gene is regulated in extravascular and vascular cells by three Sp1 binding sites in the TF promoter (−111 to +14 bp). Transient expression of the TF gene is regulated by a distal enhancer (−227 to −172 bp) which contains two AP-1 binding sites and aκB binding site. This enhancer region mediates LPS and cytokine induced expression of TF in cultured monocytes and endothelial cells. The proximal enhancer (−111 to +14 bp) will induce transcription following exposure of cultured epithelial cells to PMA or serum.

Tissue Factor Pathway Inhibitor (TFPI) Under normal conditions, TFPI is expressed in megakaryocytes and the endothelium. Adherent monocytes and macrophages will express TFPI under pathologic conditions. TFPI is encoded by a gene located on chromosome 2 and contains nine exons and eight introns resulting in a 4.0 kb mRNA. The gene was mapped to the region 2q31-2q32.1. The 5' DNA does not contain a TATAA box and several studies demonstrated multiple transcription initiation sites. The three Kunitz domains are encoded by separate exons.

Processing and Fate

Antithrombin III No proteolysis is necessary for activation.

Factor II (Prothrombin) Factor Xa with its cofactor, factor Va, a phospholipid surface in the presence of Ca^{2+} ions, activates prothrombin after proteolysis at two positions to yield α-thrombin. Cleavage at Arg^{320} generates a two-chain intermediate of M_r 72,000 (meizothrombin) which has esterase activity and is very unstable [33]. Meizothrombin is cleaved at Arg^{284} to generate α-thrombin containing the COOH portion of the prothrombin molecule and fragment 1·2 representing the NH_2-terminal part of prothrombin (Figure 2). Fragment 1·2 is further cleaved by α-thrombin to prothrombin fragment 1 plus prothrombin fragment 2.
α-Thrombin is a two chain enzyme which is composed of an A-chain (M_r 6,000) and a B chain (M_r 31,000) associated covalently through a disulfide bond. Purified α-thrombin undergo autolysis after long term storage. The resulting products are less active than α-thrombin. Similar results were found following limited digestion of α-thrombin by trypsin. β-Thrombin is produced following cleavage of α-thrombin at Arg^{70} or/and Arg^{73} in the B-chain of α-thrombin, whereas β'-thrombin is produced following cleavage of α-thrombin at Arg^{154} in the B chain. Cleavage of α-thrombin at both sites will result in the formation of γ-thrombin.

Factor V (Proaccelerin) Factor V is cleaved by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545} to generate the active cofactor, and two heavily glycosylated activation peptides [34] (Figure 3). The factor Va molecule is a heterodimer composed of a heavy chain of M_r 105,000 and a light chain of M_r 74,000. The two subunits are non-covalently associated via a divalent metal ion process.
The inactivation of human factor Va is membrane-dependent and occurs because of limited proteolysis by APC of the heavy chain at Arg^{506}, Arg^{306}, and Arg^{679} (Figure 3) [35]. The light chain of the cofactor is not cleaved by APC. When factor Va is inactivated by physiologically relevant concentrations of APC in the presence of a membrane surface, the inactivation proceeds in a sequential manner. The first cleavage at Arg^{506} appears necessary for optimum exposure of the cleavage sites at Arg^{306} and Arg^{679}. The membrane dependent cleavage at Arg^{306} is required for complete inactivation. Phosphorylation of the cofactor by a platelet-membrane associated casein kinase II enzyme at the COOH-terminal portion of the heavy chain (at Ser^{692}) increases the rate of cofactor inactivation by APC because of acceleration of cleavage at Arg^{506}. It has been recently shown that upon cleavage at Arg^{306}/Arg^{506}, the A2 domain of the cofactor is dissociated from the rest of the molecule.

The procofactor factor V is also inactivated by APC because of cleavages at Arg^{306}, Arg^{506}, Arg^{679}, and Lys^{994} [35]. Cleavage at Arg^{306} in factor V is the inactivating cleavage site of the procofactor.

Factor VII Factor VII is converted to its active form, factor VIIa, by a variety of blood coagulation enzymes: α-thrombin, factor IXa, factor Xa, factor VIIa and factor XIIa through cleavage of a single peptide bond (Arg^{152}-Ile^{153}) (Figure 2). The resulting factor VIIa (M_r 50,000) is composed of a light chain (M_r 20,000) and a heavy chain (M_r 30,000) covalently associated through a disulfide bond.

Factor VIII (Antihemophilic Factor) α-Thrombin converts factor VIII to a heterotrimer following cleavage at Arg^{372}, Arg^{740}, and Arg^{1648}. Cleavage by α-thrombin at Arg^{1689} releases the cofactor from vWf and generates the active cofactor, factor VIIIa (Figure 3). Factor VIIIa is a composed of fragments of M_r 50,000 (A1 domain) containing a COOH-terminal portion rich in acidic amino acids (residues 337-372), M_r 40,000 (A2 domain), and M_r 74,000 (A3C1C2) containing the carboxyl-terminal part of the factor VIII molecule. The three subunits are noncovalently associated via divalent metal ions.
The majority of factor VIIIa cofactor activity (70-80 %) is lost at physiological pH following dissociation of the A2 domain from the rest of the molecule [36]. The remaining cofactor activity is lost because of cleavage by APC at Arg^{336}. The K_d for the dissociation of the A2 domain of factor VIIIa from the A1/light chain dimer is 270 nM [37]. Thus at physiological protein concentrations (0.7 nM), factor VIIIa inactivation is mostly the result of dissociation of the A2 domain rather than of proteolysis at Arg^{336}. Spontaneous loss in factor VIIIa cofactor activity because of dissociation of the A2 domain explains the difficulty of working with factor VIIIa.
Factor VIII is inactivated following cleavages at Arg^{336} and Arg^{562}. The first cleavage at Arg^{336} in factor VIII appears to be the inactivating cleavage site. Cleavage by APC at Arg^{562} and Arg^{740} in factor VIII have been suggested to be necessary for complete inactivation of the factor VIII latent cofactor activity.

Factor IX (Christmas Factor) During activation, factor IX is converted to an inactive intermediate, factor IXα, and then to the active enzyme, factor IXa, by two sequential cleavages at Arg^{145} and Arg^{180} (Figure 3) resulting in the release of a M_r 11,000 activation peptide. These cleavages are catalyzed by either the extrinsic tenase complex (TF/factor VIIa) or factor XIa. Factor Xa on an acidic membrane surface will also activate factor IX. Proteolysis of factor IX at Arg^{145} will first convert factor IX to the inactive intermediate, factor IXα. Generation of this obligate intermediate enhances the overall rate of production of fully active enzyme. The resulting factor IXa (M_r 45,000), which has coagulant and esterase activities, is composed of a heavy chain (M_r 28,000) and a light chain (M_r 18,000) covalently associated through a disulfide bond.

Factor X (Stuart Factor) Factor X is converted to its active form, factor Xa, following cleavage of the heavy chain at Arg^{194}. This cleavage is catalyzed by either the intrinsic tenase (factor IXa/factor VIIIa) or the extrinsic tenase (factor VIIa/TF) and results in the release of a M_r 12,000 peptide. The resulting factor Xa (M_r 48,000) is composed of a heavy chain (M_r 30,000) and a light chain (M_r 18,000) covalently associated through a disulfide bond (Figure 2).

Factor XI Factor XIIa as well as α-thrombin will cleave factor XI at Arg^{369} to generate an active molecule with two active sites. The resulting active enzyme is a homodimer composed of two subunits, each made of a heavy chain and a light chain.

Factor XII (Hageman factor) Factor XII binding to a negatively charged surface result in the autoactivation of the molecule. Following binding of Zn^{2+}, factor XII undergoes a conformational change that induces an enzymatic activity to the molecule only when bound to a negatively charged surface. Activation of the zymogen by kallikrein, trypsin, or plasmin results in an enzyme with decreased coagulant activity. Two major forms of activated factor XII exist: αXIIa form which is a M_r 80,000 protein composed of two chains associated through disulfide bonds and the βXIIa form (or HFf form) which results from cleavage at Arg^{334}, Arg^{343}, and Arg^{353} and is composed of fragments of M_r 28,000 to M_r 30,000 that has no surface binding capabilities but it's still capable in activating prekallikrein.

Factor XIII (Plasma Transglutaminase) α-Thrombin activates factor XIII following cleavage at Arg37 in the α subunit. Following cleavage of the α chain an active site sulfhydryl is exposed and factor XIIIa will catalyze the formation of amide bonds between glutamine and lysyl side chain residues of the α and γ chains of fibrin through intermediate steps of acylation and deacylation resulting in homopolymers. Fibrin was found to have six lysyl glutamyl cross links per molecule.

Fibrinogen Following cleavage of the Aα chain at Arg^{16} and the Bβ chain at Arg^{14} two fibrinopeptides (A and B) are released from the fibrinogen molecule. As a consequence of these cleavages there is formation of the insoluble fibrin plug. The release of fibrinopeptide A generates a fibrin molecule exposing a polymerization site (amino acid residues 17–20) on the Aα chain. These regions will bind to complimentary regions on the D domain of fibrin to form the protofibrils. Subsequent cleavage by α-thrombin on the Bβ chain and release of fibrinopeptide B exposes additional polymerization sites and promotes lateral growth of the fibrin network. Plasmin dissolves the fibrin clot following several sequential cleavages giving rise to several soluble degradation products.

High molecular weight kininogen (Fitzgerald factor) Cleavage of high molecular weight kininogen by kallikrein results in the liberation of the bradykinin

domain and the rearrangement of the cysteine protease inhibitor domain which is opposite to the prekallikrein binding region. Proteolysis of high molecular weight kininogen allows the molecule to perform its cell antiadhesive activity.

Prekallikrein (Fletcher Factor) Prekallikrein is activated to kallikrein by factor XIIa on a surface in the presence of high molecular weight kininogen or by factor βXIIa in a fluid phase. Activation occurs following cleavage at Arg^{371} and results in the formation of a 371 amino acids heavy chain which is associated to the light chain via a disulfide bond. Thus, activation of the molecule will not result in a change in molecular weight. In the absence of factor XII, prekallikrein will not be activated on an artificial surface.

Protein C Protein C is converted to its active form, activated protein C (APC), by cleavage of the heavy chain at Arg^{169} (Figure 2). This cleavage is catalyzed by the α-thrombin-thrombomodulin complex. It has been also shown that the meizothrombin-thrombomodulin and the factor Xa-thrombomodulin complex can also activate protein C to generate APC.

Protein S No proteolysis is necessary for activation. However, both α-thrombin and factor Xa abolish the anticoagulant activity of protein S by proteolysis at specific amino acid bonds located within the 29 amino acid long α-thrombin sensitive loop. This loop is located between the γ-carboxyglutamate and the first EGF domain and corresponds to exon IV of the protein S gene. α-Thrombin converts protein S into a two-chain inactive molecule following cleavage at Arg^{49} and Arg^{70} whereas factor Xa inactivates protein S following single cleavage at Arg^{60}. The latter cleavage is abolished in the presence of factor Va.

Thrombomodulin No proteolysis is necessary for activation.

Tissue Factor (TF) No proteolysis is necessary for activation.

Tissue Factor Pathway Inhibitor (TFPI) No proteolysis is necessary for activation. However, more than 70% of plasma TFPI circulates in various forms which are been truncated at a variety of positions at the COOH-terminal end.

Biological Activity

Antithrombin III (AT-III) The general mechanism of inhibition of proteases by serpin's is a covalent entrapment of the active site of the enzyme by the serpin. In the absence of sulfated polysaccharides (heparin/heparan sulfate) the coagulation proteases interact slowly with the reactive site bond of AT-III producing a stable complex. The initial binding of the protease/inhibitor complex is facilitated by the presentation of a pseodosubstrate by the inhibitor. In the presence of sulfated polysaccharides the reaction is considerably accelerated because of a polysaccharide induced conformational change of the reactive site of the protease inhibitor. Upon interaction at this site with the enzyme the inhibitor changes its conformation which prevents the protease from performing the cleavage at the presented site resulting in a stable intermediate complex. The enzyme may be released from the complex when it is able to escape the trap upon cleavage of the peptide bond, this will result in an inactive altered serpin and free enzyme. The inhibition thrombin by AT-III has been used as a model to study the mechanism of serpins.
The importance of AT-III in vivo as an anticoagulant protein is indicated by the thromboembolic complications occurring in AT-III deficient patients. AT-III has been shown to inhibit the enzyme activity of thrombin, factor Xa, factor IXa, factor VIIa, factor XIa and factor XIIa [38]. The most relevant targets of AT-III in vivo are probably thrombin, factor Xa and factor IXa as indicated by circulating complexes of these enzymes with AT-III. Factor Xa is relatively protected against inactivation by AT-III when bound to cofactor (factor Va) on a phospholipid surface [39]. Upon dissociation from the cofactor factor Xa will be inactivated by AT-III in the circulation. Factor VIIa activity and its inactivation by AT-III are only significant when the protease is bound to TF. TF "opens" the active site of factor VIIa and this alteration of the active site probably sensitizes factor VIIa to inactivation by the active site directed AT-III. Factor VIIa is unique among the proteases of blood coagulation in that it becomes only sensitive for inactivation when bound to its cofactor. This probably provides the mechanism by which traces of free factor VIIa may circulate in blood [40].

Factor II (Prothrombin) The single chain zymogen (which circulates in plasma at a concentration of 1.4 μM) is activated during coagulation to the potent serine protease α-thrombin, by the enzymatic complex termed prothrombinase, which is composed of the enzyme factor Xa, associated to its cofactor factor Va, on a membrane-surface in the presence of Ca^{2+} ions. α-Thrombin is responsible for the cleavage of fibrinogen to fibrin leading to the ultimate step in coagulation, the formation of the fibrin clot. Prothrombin deficiency is manifested as an autosomal recessive trait and is observed rarely. Several prothrombin deficiency states which are associated with hemorrhagic manifestations have been reported in the literature. Recently, a polymorphism corresponding to a G→A substitution in the 3' untranslated region of the prothrombin gene (nucleotide 20210) was associated with an increased level of prothrombin procoagulant activity. Individuals with this mutation have a two-to five-fold increase in the risk of venous thrombosis. This mutation is very common among the Caucasian population.

Factor V (Proaccelerin) Factor V is the precursor of factor Va which is the required cofactor of prothrombinase which in turn is responsible for the efficient cataly-

sis of the activation of prothrombin to α-thrombin. Incorporation of factor Va into prothrombinase increases the rate at which prothrombin is converted to α-thrombin by 300,000-fold relative to the rate of the reaction produced by factor Xa alone. A total deficiency in factor V is lethal in mice whereas, in humans individuals who are deficient in plasma factor V (< 2% factor V clotting activity) have been described. Thus, platelet factor V is extremely important for normal hemostasis.

Dahlbäck et al. [41] observed that plasma from an individual with venous thrombosis had an abnormal response to APC in a modified aPTT (activated Partial Thromboplastin Time) assay [41]. When APC is introduced into normal plasma which has been preincubated with an aPTT reagent a prolongation of clotting time which is proportional to the concentration of APC used has been observed. In plasma from some patients, higher concentrations of APC are required to obtain similar prolongation of clotting time as seen with normal plasma. This condition was called "APC-resistance". The molecular defect in individuals with APC-resistance was identified at the genetic level by Bertina et al. [42] who showed that individuals with APC-resistance have a mutation in the factor V gene (a G to A substitution at nucleotide 1691). This mutation results in an Arg^{506}→Gln mutation in the factor V molecule. This mutation is present in up to 60% of patients with venous thrombosis and in 3-5% of normal individuals. As a consequence of the mutation the abnormal molecule, called factor V^{LEIDEN}, does not possess the APC-cleavage site at Arg^{506}. When isolated from the plasma of patients homozygous for the Arg^{506}→Gln mutation, factor Va^{LEIDEN} is inactivated by APC at a rate slower than that observed for normal factor Va [43]. However, inactivation still proceeds as a consequence of cleavage at Arg^{306}. Recently it has been proposed that low concentrations of heparin could accelerate cleavage at Arg^{306} and inactivation of factor V by APC. These data together with the fact that at elevated APC concentrations, inactivation of membrane-bound factor V by APC may occur faster that activation of the procofactor (α-thrombin) would suggest an increased antithrombotic effect of APC in the presence of low doses of heparin.

Interestingly, and in contrast to the observations made with factor Va^{LEIDEN}, the APC-catalyzed rate of inactivation of factor V^{LEIDEN} is comparable to that observed with normal factor V. These results are in agreement with the data demonstrating the initial membrane-dependent cleavage site in normal factor V which leads to inactivation, occurs at Arg^{306}. The APC-catalyzed inactivation of membrane-bound factor V^{LEIDEN} also occurs through initial cleavage at Arg^{306} at a comparable rate to normal factor V, indicating that inactivation of factor V does not necessarily require cleavage at Arg^{506}.

Regulation by proteolysis for activation /inactivation of the cofactor plays a preeminent role in clinical assays for the definition of one individual's genetic status. Thus, the quality of the reagents for the assay must be always determined. For example, during execution of the APC-resistance assay, plasma is incubated with reagent that will activate factor V for a given period of time. Then APC is added together with Ca^{2+}. The clotting time of the sample is measured and compared to the clotting time in the absence of APC. In plasma from normal individuals the prolongation of clotting time is correlated with the inactivation of factor Va by APC. In plasma from individuals homozygous for the Arg^{506}→Gln mutation, a lesser prolongation of the clotting time is observed in the presence of APC. Since factor Va, not factor factor V is procoagulant active, all assays that rely on the in situ activation of factor V to support the clotting reaction are highly sensitive to the status of factor V activation in the plasma sample. Furthermore, since factor V inactivation occurs by an initial cleavage at Arg^{306} while factor Va inactivation results from sequential cleavages at Arg^{506} followed by Arg^{306} the factor V activation status can and will compromise the APC-resistance assay if the procofactor is not properly and timely activated. As a consequence a patient with thrombotic disorders who is suspected to be homozygous for the factor V^{LEIDEN} mutation and who's plasma factor V was not properly activated to factor Va because of a defective assay reagent will have a normal sensitivity to APC when compared to normal plasma assayed under similar condition since inactivation will only involve cleavage at Arg^{306} and will escape detection.

Factor VII Factor VII is the precursor of factor VIIa which is the initiator of blood clotting. Factor VIIa binds to the endothelial cell receptor tissue factor which is only exposed following injury of the vasculature or cytokine stimulation, to form the extrinsic tenase complex which is responsible for the initiation of blood coagulation. This complex is primarily responsible for the cleavage/activation of factor X and factor IX and for the production of small quantities of active enzyme (i.e. factor Xa and factor IXa).

Factor VIII (Antihemophilic Factor) Factor VIII is the precursor of factor VIIIa which is the required cofactor of the intrinsic tenase complex which in turn is responsible for the efficient catalysis of the activation of factor X to factor Xa. Incorporation of factor VIIIa into the intrinsic tenase complex increases the rate at which factor X is converted to factor Xa by approximately 200,000-fold relative to the rate of the reaction produced by factor IXa alone. Over 200 point mutations of the factor VIII gene have been reported in the literature. The importance of the corresponding phenotype varies, however, all individuals affected (all males) have bleeding problems. An examination of the second order rate constants of the activation of factor X by the factor VIIa/TF complex (extrinsic tenase) when compared to the second order rate constant of the activation of factor X by the factor IXa/factor VIIIa complex (intrinsic tenase) demonstrates that the latter complex is approximately 50 times more efficient in producing factor Xa than the former. Thus, the preferred pathway for factor Xa formation in plasma where low (physiological) levels

of factor VIIa are present, is the pathway that utilizes the intrinsic tenase complex. Thus, from a physiological point of view factor VIIIa and factor IXa are clearly identified as essential for normal hemostasis.
The importance of the correct regulation of factor Va cofactor activity is very much illustrated by the "beneficial" effect of factor V^{LEIDEN} in some individuals with severe hemophilia A. Different pathology (phenotype) has been reported in several patients with hemophilia carrying the same mutation(s). The reported mutations normally result in a phenotype which is characterized as severe bleeding disorders. However, while some hemophiliacs are classified clinically as severe hemophiliacs, several other individuals carrying the same mutation(s) were classified as mild/moderate hemophiliacs and only had minor bleeding problems. This classification is based on the level of measurable factor VIII activity in a one-stage clotting assay using factor VIII deficient plasma. The net effect of a factor VIII deficiency is a reduced α-thrombin formation rate, whereas in the case of an abnormal factor V molecule that would be resistant to inactivation by APC (i.e. factor V^{LEIDEN}) the net effect would be an increase in α-thrombin formation. The patients classified as severe hemophiliacs were carrying normal factor V whereas the other were heterozygous for the factor V^{LEIDEN} mutation. Since factor Va^{LEIDEN} is inactivated by APC with a rate slower than normal factor Va, hemophiliac patients who posses the factor V^{LEIDEN} gene may have a milder bleeding syndrome than hemophiliac patients with normal factor V because of increase rate in prothrombin activation. This hypothesis was recently confirmed *in vitro*, and the data demonstrated an increase in α-thrombin generation in individuals with hemophilia A which is proportional on the plasma level of factor V^{LEIDEN}. Thus, it appears that if an individual possesses both, a factor VIII mutation that normally results in severe bleeding and factor V^{LEIDEN} the extended lifetime of factor Va^{LEIDEN} is able to partially compensate for an abnormal or absent factor VIII molecule resulting in sufficient α-thrombin generation to provide hemostasis.

Factor IX (Christmas Factor) Factor IX is the precursor of factor IXa which is the required enzyme for the intrinsic tenase complex. Factor IXa binds to its cofactor factor VIIIa on a membrane surface in the presence of divalent metal ions, to form the intrinsic tenase complex which is responsible for efficient activation of factor X during blood coagulation.

Factor X (Stuart Factor) Factor X is the precursor of factor Xa which is the required enzyme for the prothrombinase complex. Factor Xa binds to its cofactor factor Va on a membrane surface in the presence of divalent metal ions, to form the prothrombinase complex which is responsible for efficient activation of prothrombin during blood coagulation.

Factor XI Recent research has attempted to uncover why factor XI deficiency is biologically important for normal coagulation, whereas its presumed activator, factor XII, is unnecessary for clotting in vivo. An alternate means of activating factor XI, independent of factor XII and the early contact pathway, is implied by the clinical data. Recent data demonstrated activation of factor XI by α-thrombin in a purified system. The rate of this reaction was greatly accelerated by negatively charged glycosaminoglycans such as dextran sulfate [44]. α-Thrombin activated factor XIa at femto- to picomolar levels in the absence of factor XII or negatively charged surfaces has been detected during the initiation phase of thrombin generation using sensitive assays [45]. Such observations support a role for thrombin-activated factor XI in thrombin activation and coagulation.

Factor XII (Hageman factor) Deficiency of factor XII is not associated with any bleeding tendency in vivo. Cleavage of factor XII by kallikrein is central to the initiation of the intrinsic pathway of the blood coagulation cascade. Surface bound αXIIa activates factor XI to factor XIa. Secondary cleavage of αXIIa by kallikrein results βXIIa which in turn will activate kallikrein, factor VII and the complement cascade.

Factor XIII (Plasma Transglutaminase) Factor XIII is the precursor form of the glutaminyl-peptide γ-glutamyl transferase, called factor XIIIa (fibrinoligase, plasma transglutaminase, and fibrin stabilizing factor). This is the last of the zymogens of the blood coagulation process to become activated and is the only enzyme in the system that is not a serine protease. α-Thrombin will activate factor XIII to factor XIIIa following cleavage at Arg^{36} of the NH_2-terminal portion of the α chain and exposure of the active site sulfhydryl. Factor XIIIa will catalyze the conversion of soluble fibrin to an insoluble fibrin clot by crosslinking the lysine and glutamine side chains of fibrin to form homopolymers.

Fibrinogen Fibrinogen is a multi-functional molecule possessing diverse biological activities within coagulation and hemostasis. Fibrinogen also participates in extravascular inflammatory responses. Following addition of α-thrombin to fibrinogen two different fibrinopeptide release rates will result in two separate changes in fibrinogen. Briefly, α-thrombin removes fibrinopeptide A from the fibrinogen molecule to form soluble monomers of fibrin which will polymerize to yield a double stranded protofibril. Removal of fibrinopeptide B from the protofibril by α-thrombin will result in the aggregation of the protofibrils into fibers. Finally, the γ-chains of two adjacent monomers will be cross-linked by their respective D regions in the presence of factor XIIIa, resulting in a cross-linked clot.
Albeit the greatest attention upon the transition of fibrinogen to fibrin and the polymerization of the fibrin derivative substantial work has also focused in the past on the capacity of fibrinogen to mediate platelet aggregation through the binding of the protein to the platelet glycoprotein receptor GPIIb/IIIa. This phe-

nomenon which is induced by a wide variety of physiologic and pharmacological stimuli establishes a role of potential significance for the molecule in the primary phase of hemostasis.

High molecular weight kininogen (Fitzgerald factor) Deficiency of high molecular kininogen is not associated with any bleeding tendency in vivo. The most important function of the kininogens is the release of bradykinin upon their activation. Bradykinin operates as an anti-thrombotic/profibrinolytic agent. Further, bradykinin is a potent stimulator of endothelial cell prostacyclin synthesis, an inhibitor of platelet function. Bradykinin also prevents subedothelial cell-dependent smooth muscle proliferation.

Prekallikrein (Fletcher Factor) Deficiency of prekallikrein is not associated with any bleeding tendency in vivo. Following activation the light chain of α-kallikrein reacts with circulating plasma protease inhibitors (α_2-macroglobulin, and C1 inhibitor). The interaction with C1 inhibitor results in the loss of proteolytic and amidolytic activity of the protein.

Protein C Protein C is the precursor of activated protein C (APC) which is the required enzyme for the normal inactivation of factor Va and the arrest of α-thrombin production by the prothrombinase complex. α-Thrombin bound to its endothelial cell surface receptor thrombomodulin will cleave protein C at Arg^{12} in the heavy chain to generate APC.

Protein S Protein S is an important coagulation inhibitor as evidenced by the thrombotic tendency of protein S deficient individuals. However the mechanism of anticoagulant action of protein S remains controversial. It has been reported that: a) protein S functions as a cofactor of APC; b) protein S eliminates the protection of factor Va by factor Xa against inactivation by APC; c) protein S inhibits prothrombinase (factor Xa/factor Va) and intrinsic tenase (factor IXa/factor VIIIa) activity in a APC-independent manner. The enhancement of the activity of APC by protein S ranges from 2 to 10-fold, and is rather dependent on the experimental conditions under which cofactor activity is assessed. Addition of protein S to APC results in an increase in the rate of the membrane dependent cleavages by APC in factor Va (Arg^{306}) and factor VIIIa (Arg^{336}).
The independent inhibitory activity of protein S was first hypothesized to be the result of interactions of protein S with factor Xa, factor Va or factor VIIIa. Protein S, however, inhibits all phospholipid dependent reactions. The APC-independent inhibitory action of protein S on the intrinsic tenase complex and on the prothrombinase complex is reported to correlate with the apparent phospholipid binding properties of the various protein S preparations. Based on these observations it was hypothesized that protein S inhibits these reactions by competing for the procoagulant phospholipid available in the reaction. A very significant inhibitory effect of protein S on thrombin generation initiated by TF in the presence of quiescent platelets is observed. These data demonstrate that the inhibitory effect of protein S which is only expressed when a limited number of phospholipid binding sites for the procoagulant enzyme complexes are present is potentiated in the presence of the cell surfaces usually available in vivo for clot formation and is caused by competition for negatively charged phospholipid. Whether the APC-independent effect of protein S on the procoagulant reaction is important in vivo remains a question.

Thrombomodulin Thrombomodulin is the required endothelial cell surface cofactor for the activation of protein C to APC by α-thrombin. Thrombomodulin forms a 1:1 stoichiometric complex with α-thrombin. The activation of protein C to APC is accelerated approximately by 1000-fold when compared to the activation rate of protein C by α-thrombin alone. Further, binding of α-thrombin to thrombomodulin completely alters the procoagulant activity of the enzyme. Following binding to thrombomodulin, thrombin no longer triggers platelet aggregation or clots fibrinogen. The α-thrombin -thrombomodulin complex also no longer activates factor V, or inactivates protein S.

Tissue Factor (TF) Tissue factor (TF) is the membrane-bound glycoprotein that is exposed at the site of the vascular injury and is responsible for the initiation of the coagulation process. TF is expressed by a variety of vascular and extravascular cells. Normally the endothelial cells cover the luminal side of the blood vessels and form a barrier between the flowing blood and the underlying cells. Upon exposure to the blood flow TF will bind factor VIIa. The catalytic efficiency of the enzymatic complex which initiates coagulation by activating factor X and factor IX increases by three orders of magnitude as compared with the catalytic efficiency of the enzyme, factor VIIa alone.

Tissue Factor Pathway Inhibitor (TFPI) The in vivo role of TFPI in coagulation is still under investigation since no TFPI deficient individuals are yet found. However, it has been demonstrated that complete deficiency of TFPI in mice is incompatible with birth and survival [46]. TFPI complexes with the limited quantities of factor Xa formed initially by the extrinsic tenase, and factor Xa-TFPI subsequently inhibits the extrinsic tenase via formation of a quaternary complex with TF and factor VIIa. Factor Xa-dependent inhibition of the extrinsic tenase by TFPI down-regulates further generation of factors Xa and IXa. Available factor Xa that is produced by the extrinsic tenase is therefore limited by these factors. TFPI will inactivate factor Xa by reversible binding of the second Kunitz domain to the active site of the enzyme. The following step is the inhibition of the catalytic activity of TF/factor VIIa complexes by formation of the quaternary complex TF/factor VIIa/TFPI/factor Xa. This complex formation depends on the binding of the first Kunitz domain of TFPI to the active site of factor VIIa. The quaternary complex is stable and can be dissociated by EDTA. The membrane

interaction of the Gla-domain of factor Xa in complex with TFPI seems of major importance since Gla-domain-less factor Xa does not function as a cofactor for TFPI in the inhibition of the TF/factor VIIa complex. The physiological function of TFPI seems therefore to stem primarily from its ability to regulate TF-dependent coagulation and not by its ability to inhibit FXa directly. *In vitro* studies it have shown that TFPI exerts a potent inhibition of TF/factor VIIa activity in flow models using purified human coagulation factors. The direct activation of factor X by TF/factor VIIa is blocked in time by TFPI in the absence of factor IX and factor VIII. Activation of traces of factor IX by TF/factor VIIa before inactivation by TFPI allows continuation of factor X activation by the factor IXa/factor VIIIa activity in the presence of TFPI. While the direct activation of factor Xa by TF/factor VIIa is rapidly inhibited by TFPI, the traces of factor IXa formed by the transient TF/factor VIIa activity results in sufficient factor Xa generation to sustain the hemostatic reaction.

Role in Vascular Biology

Physiological Function

While the importance of the TF pathway in coagulation is clear, a role for contact initiation is questionable. Deficiencies in factors VII, VIII, IX and X and V are invariably associated with bleeding disorders; however, hemorrhagic tendencies resulting from deficiency of prekallikrein, factor XII or high molecular weight kininogen are unknown. While the role of factor XI is suggested by several studies, the bleeding disorders associated with factor XI deficiency (Hemophilia C) are variable in their severity and frequency. Rapaport et al. [47] have shown a major and minor form of factor XI deficiency. Levels of factor XI below 20% are considered most commonly associated with significant hemorrhage upon surgical challenge.

The recent years an earlier theory of coagulation has resurfaced. This theory of coagulation has been first proposed by Paul Morawitz [1] in 1905 who postulated that the key proteins required for normal blood clotting and physiological hemostasis are thromboplastin, prothrombin and fibrinogen. The material called thromboplastin in the early days is the equivalent of the tissue factor/phospholipid used in the present days. TF (the integral membrane glycoprotein) is now accepted as the in vivo initiator of thrombin generation in the revised theory of coagulation. While the majority of proteins involved in blood coagulation circulate as inactive zymogens that require proteolytic activation in order to function, approximately 1% of the circulating factor VII molecules are active (factor VIIa) and possess an active site which is not inhibited by circulating stoichiometric protease inhibitors. Following injury to the vasculature and subsequent exposure of TF, the circulating factor VIIa molecules can bind to the exposed TF forming the extrinsic tenase complex (TF/factor VIIa) and initiate the blood coagulation process. The TF pathway proceeds by assembly of three distinct complexes. The first is the extrinsic tenase (factor VIIa and the membrane bound cofactor TF). This complex activates a fraction of the circulating zymogen factors X and IX to their respective active forms, factors Xa and IXa. but only transiently at relatively low levels as the result of stringent down-regulation of the factor VIIa/TF complex by TFPI and AT-III. This initial amount of factor Xa provides limited amounts of α-thrombin, adequate to activate the cofactors [48]. Factor IXa assembles with factor VIIIa to form the intrinsic tenase complex, which produces additional factor Xa. Free factor Xa assembles with factor Va into the prothrombinase complex on the cell surface, which is the activator of prothrombin to α-thrombin. This delayed burst of factor Xa results in a burst of prothrombinase, which in turn creates the burst of thrombin activity required for clotting and adequate hemostasis. Thrombin-activated factor XIa serves to augment this burst of factor Xa by increasing the quantity of factor IXa-factor VIIa complex. As a result there is measurable increase in the final amount of thrombin generated when factor XI participates in the reaction when compared with reactions without factor XI. The extrinsic tenase activity alone is attenuated by AT-III and tissue factor pathway inhibitor (TFPI), while the intrinsic tenase is largely under the control of AT-III. Thus, factor Xa generated by the extrinsic tenase is insufficient to maintain an ongoing hemostatic response. Under these conditions, the intrinsic tenase complex provides the additional factor Xa required to maintain thrombin generation. This is the reason that individuals which lack factor VIII (hemophilia A) and factor IX (hemophilia B) bleed profusely albeit the presence of normal concentrations of circulating factor VII. This theory of coagulation is supported by observations concerning the severity of hemophilias. In factor IX or factor VIII deficiency (hemophilias A and B), the delayed burst of thrombin is not observed, because only factor Xa supplied directly by factor VIIa/TF supports thrombin generation. Thus, this low level of thrombin is not adequate to ensure hemostasis. Studies of the platelet-fibrin plug in hemophilias A and B indicate that the platelet plug formed initially in response to injury, ruptures subsequently due to defective fibrous transformation and stabilization within the plug. This theory that supports an active involvement of factor XIa in blood clotting taken alone does not adequately explain, however, why factor XI deficiency (hemophilia C) exhibits the type of infrequent and variable hemorrhagic symptoms which accompany this defect. However recent observations have demonstrated that in factor XI deficient individuals, when very low TF concentrations are used to initiated the coagulation reaction, platelet activation, factor V activation and fibrin formation is significantly delayed [49]. Thus, factor XI deficiency will result in delayed hemostasis only at low concentrations of TF.

α-Thrombin will finally cleave fibrinogen and activate the circulating platelets which together will form the hemostatic plug. α-Thrombin participates in its own

down-regulation by binding to the endothelial cell receptor thrombomodulin, and initiating the protein C pathway, which in turn leads to the formation of APC. APC is required for efficient neutralization of factor Va and VIIIa cofactor activities which result in the inactivation of the prothrombin and factor Xa activating complexes [50]. This inactivation can only occur in the presence of the appropriate membrane surface. Thus, while following α-thrombin activation, factor VIIIa at physiological concentration (0.7 nM), is rapidly and spontaneously inactivated by dissociation of the A2 domain from the rest of the cofactor, APC is required for down-regulation of α-thrombin formation by prothrombinase. APC down-regulates the prothrombinase complex by cleaving specific peptide bonds on the heavy chain of factor Va which also results in the dissociation of the A2 domain of factor Va from the rest of the molecule. Dissociation of the A2 domain of both cofactors impairs their ability to interact with the other protein components of prothrombinase and intrinsic tenase and results in the arrest of α-thrombin formation. Interestingly, it has been demonstrated that effective down-regulation of α-thrombin generation by the protein C pathway in combination with TFPI and AT-III occurs because APC prevents the coexistence of the factor Va heavy and light chains [51].

Pathology, Clinical Relevance and Therapeutic Implications

Most of the pathology associated with the coagulation factors has been described above. However efficient hemostasis requires Ca^{2+} ions and the presence of a negatively charged membrane surface.

Common to each vitamin K-dependent zymogens relevant to coagulation is a highly-conserved NH_2-terminal "Gla-domain," which binds multiple calcium ions and contains 9-12 γ-carboxyglutamic acid residues. At the COOH-terminus is the serine protease domain, which is largely homologous to that of trypsin and chymotrypsin, with insertions which alter the macromolecular substrate specificity of each enzyme. Between the NH2- and COOH-terminal domains is a region of the protein which varies in structure among the zymogens, containing either epidermal growth factor-like domains or kringle domains.

The binding of calcium to a number of sites in the Gla-domain stabilizes the structure in this region of the protein, which is disordered in the absence of calcium. The importance of the Gla-domain to the function of these coagulation zymogens is exemplified in warfarin therapy, which blocks the vitamin K-dependent carboxylation of glutamate residues. Cooperativity is observed for the binding of calcium to prothrombin, and the calcium-bound configuration exhibits a greater affinity for negatively-charged phospholipid than the apoform. Despite the remarkable complexity of this cooperative calcium effect, no regulatory role for calcium has been elucidated in coagulation. Investigations in porcine carotid arteries demonstrated that the growing thrombus formed in response to vascular injury is a neo-tissue, with packed platelets inaccessible to the flow of oxygenated blood outside the aggregate. Within this structure, anoxia develops and interstitial calcium levels fall as a result of platelet membrane depolarization, a result which was not observed when blood clotted *in vitro*. Administration of hirudin a very potent inhibitor of α-thrombin, aided clot dissolution and reversed the calcium effects, providing the first in vivo evidence of calcium modulation within a reversibly-formed thrombus. Along with the data for cooperative calcium binding by the vitamin K-dependent proteins, these observations suggest a mechanism wherein complex-dependent coagulation may be effectively ablated by a modest reduction in the extracellular calcium concentration. These observations allows for the possibility of a regulatory role for calcium in coagulation.

In quiescent cells, distinct activities maintain lipid asymmetry with the negatively charged phospholipids (phosphatidyl serine (PS) and phosphatidyl inositol (PI)) almost exclusively located in the inner membrane. Neutral choline lipids (i.e., phosphatidyl choline (PC)) are moved negligibly or very slowly. Activation of the cell causes internal calcium release resulting in a redistribution of the internalized negatively charged lipids, the extent of which appears to depend upon the level and type of activation. With platelets, strong phospholipid redistribution is observed upon treatment with calcium ionophores (such as A23817), diamide, and a combination of thrombin and collagen, while weaker effects are detected following treatment with thrombin or collagen alone; ADP and epinephrine have little effect. In platelets, differences among the activators have been reported to correlate with the level of intrinsic tenase and prothrombinase activity observed. Formation of procoagulant microvesicles is also observed following extended activation of platelets, but PS accumulation can occur significantly in advance of microvesiculation.

Defects in platelet phospholipid reorganization have been noted in a bleeding disorder known as Scott syndrome. Scott syndrome is characterized by a failure to accumulate PS at the platelet surface. Poor binding of the cofactors results, leading to impaired tenase and prothrombinase activity. This disease has been detected in a French family, and was found to be hereditary in nature. While it is evident that the defect affects Ca^{2+}-dependent lipid scrambling, the exact molecular cause of the disease is unknown. Since the defect can be demonstrated in erythrocytes as well as platelets, a common genetic origin in various cell types is suggested. Whereas calcium ionophores induce loss of lipid asymmetry on red cells leukocytes and endothelium, α-thrombin and other platelet activators do not universally cause loss of lipid asymmetry. However, erythrocyte procoagulant lipid activity has been noted in reversible red cell sickling and diabetes. High glucose buffers also lead to a loss of phospholipid asymmetry in erythro-

cytes and apoptosis in endothelium. Apoptosis is accompanied by a loss of lipid asymmetry in a variety of cell types, including lymphocytes, vascular smooth muscle cells and endothelium.

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Collagen

Definition *Extracellular matrix protein that is a constituent of basement membranes. A large number of collagen subtypes have been described ranging from I to XVIII.*

See: →Extracellular matrix

Collagenase

Definition *Proteolytic enzymes that degrade collagen. Important in cell invasion, tissue remodeling or angiogenesis. Also identified as matrix metalloproteinases 1, 8, or 18.*

See: →Matrix metalloproteinases

Colony-Stimulating Factors

Definition *Cytokines that regulate the hematopoietic cell development and function. A number of CSF have been characterized including monocyte/macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF).*

See: →Cytokines in vascular biology and disease; →Megakaryocytes

Complement

Definition *Enzymatic cascade that mediates a number of biological functions including host defense against infection, initiation of an inflammatory response or processing and clearance of immune complexes.*

See: →Complement system

Complement S-Protein

See: →Vitronectin/vitronectin receptors

Complement System (Interaction of Vascular Cells with)

Introduction The complement system mediates a number of biological functions that participate in host defense against infection, initiation of the inflammatory reaction, processing and clearance of immune complexes and regulation of the immune response [1, 2]. Pathogens, altered host cells and immune complexes trigger complement activation, resulting in the pro-

duction of biologically active complements fragments. Except for lysis and for the non-cytolytic cellular responses elicited by the terminal C5b-9 sequence, most of the biological effects derived from complement activation depend on ligand-receptor interactions between complement proteins and specific receptors on cells. Inadequate regulation or extensive complement activation may alter the physiological functions of normal cells and contribute to pathology.

Characteristics The human complement system comprises 23 plasma components and regulatory proteins that represent 5% of the plasma protein content. Upon activation, complement components interact within distinct and finely regulated functional units (Figure 1). The classical and the alternative pathways of activation both form specific enzymatic complexes termed C3 convertases that cleave C3 and generate the major cleavage fragment C3b. A single amplification pathway exists that augments C3 cleavage once initial C3b has been generated and covalently linked to complement activating surfaces. A common effector sequence comprising C3b and components C5 to C9 generates the opsonizing, vasoactive, leukocyte-attracting, immune regulatory and cytolytic activities of complement. The classical pathway of activation comprises the C1 complex formed by one molecule of C1q, two molecules of C1r and two molecules of C1s, the components C2 and C4 and the regulatory proteins C1 inhibitor (C1-inh), C4-binding protein (C4BP) and I. The alternative and amplification pathways involve the components C3, B and D, and the regulatory proteins P, H and I. When not engaged in the assembly of a membrane-bound C5b-9 complex, the components C5-C9 bind the serum protein S, also termed vitronectin to form a fluid phase cytolytically-inactive SC5b-9 complex. Several membrane-associated proteins widely distributed on human cells, i.e. DAF, MCP and CD59 regulate the cleavage of C3 and the formation of the C5b-9 complex. Phagocytic cells and lymphocytes express specific receptors for complement proteins and their fragments, which upon interaction with their ligands, elicit the various cellular responses that initiate inflammatory process.

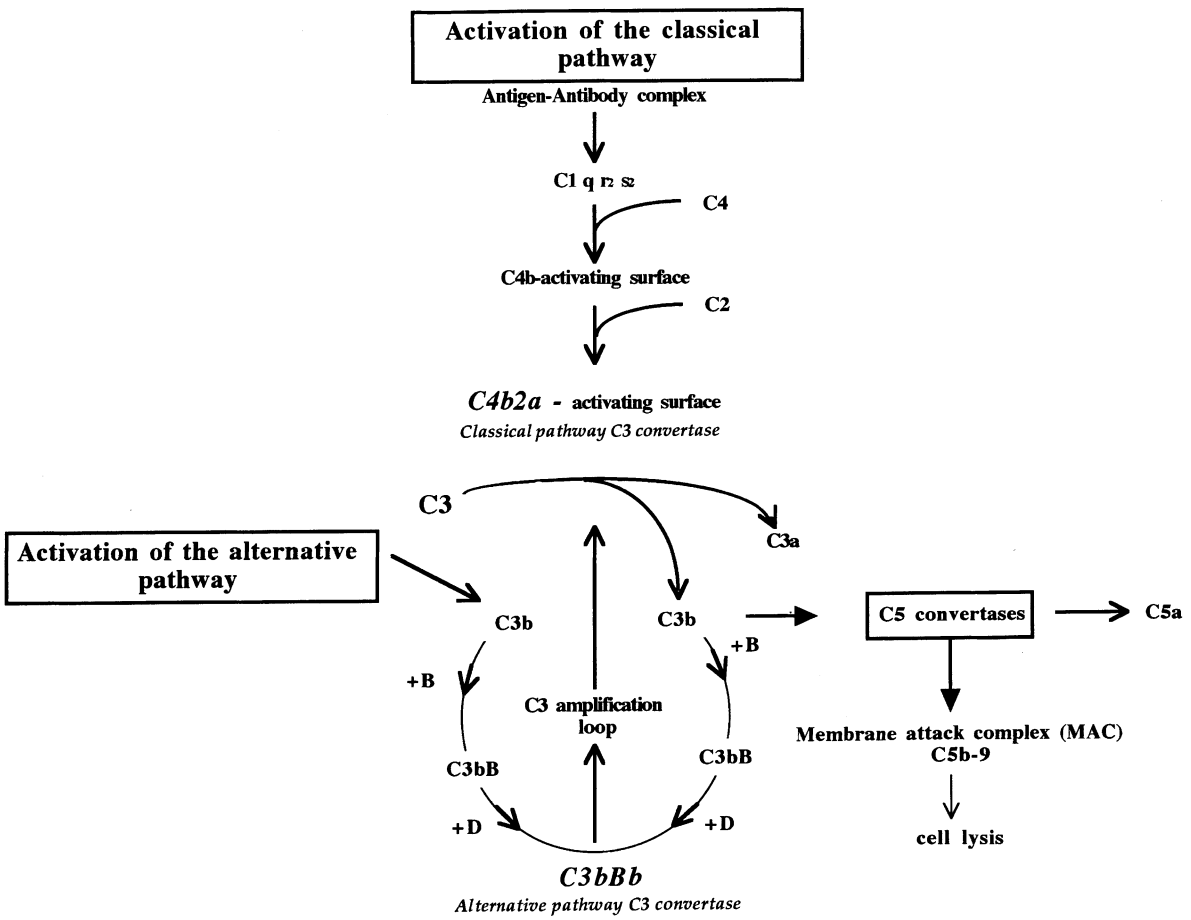

Figure 1. Activation of the human complement system

Generation of classical pathway C3 convertase Classical pathway activation by immune complexes is initiated by the binding of the globular heads of C1q to the CH2 domain of complexed IgG or IgM [3, 4]. Of the various immunoglobulin isotypes, only IgM and IgG3, 1 and 2 bind C1q to trigger complement activation. Binding of C1q to the immunoglobulin results in a conformational change in the distal portion of C1q that renders the C1r and C1s molecules within the C1 complex inaccessible to the C1 inhibitor. C1r and C1s are normally associated with C1q in a pentameric C1q, C1r2, C1s2 calcium-dependent complex. Bacteria, viruses and virus-infected cells, products of membrane and tissue damage directly bind C1q and may activate the classical pathway in the absence of antibody [5]. Activation of C1 results in expression of a proteolytic activity by C1s. The substrates for the active C1s serine protease are C4 and C2. C4 is first cleaved into a small anaphylatoxin fragment, C4a, and a large fragment, C4b, that expresses a labile binding site which mediates covalent attachment of the molecule to cell membranes or to the constant regions of immunoglobulins. Surface-fixed C4b binds C2 which is, in turn, cleaved by C1s into a small fragment C2b that is released in the fluid phase and a large fragment C2a that remains associated with C4b on the activator. The C4b,2a complex is the classical pathway C3 convertase in which the C2a fragment carries the proteolytic site for cleavage of C3. The next step in the classical pathway activation process is the cleavage of C3 by the immune-complex-bound C3 convertase, which results in the release of the anaphylatoxin C3a and the generation of C3b. As it is the case for C4b, nascent C3b transiently expresses a reactive site that allows the formation of a covalent bond with hydroxyl or amino groups on immune complexes and/or on bystander surfaces [6]. Binding of multiple C3b molecules to the target surface will then change the specificity of the C3 convertase C4b,2a to a C5 convertase denoted [C4b,2a(C3b)n] and trigger the activation of the terminal sequence C5-C9.

Generation of the alternative pathway C3 convertase
The alternative pathway represents a natural system for resistance to infection in the non-immunized host [7]. Activation by the alternative pathway displays unique features. First, antibody is not required, although it can facilitate the activation process. Second, activation proceeds both in the fluid phase and on cell surfaces. Activation of the alternative pathway is initiated by the low rate interaction in normal plasma of a "C3b like" form of C3 and factors B, D and P, resulting in the formation of an initial C3 convertase. Upon generation in the fluid phase, C3b randomly attaches to bystander surfaces following exposure of the reactive site of the molecule. Binding of C3b to an activating surface of the alternative pathway is followed by the formation of a bimolecular complex with factor B, cleavage of B by factor D, and assembly of the alternative pathway amplification convertase C3b,Bb on the target surface. Once formed, the amplification convertase C3b,Bb cleaves C3 generating molecules of C3b that bind to the activating surface. The binding of multiple C3b molecules in the vicinity of the C3b,Bb enzyme changes the specificity of the alternative pathway C3 convertase to a C5 convertase [C3b, Bb(C3b)n] and initiates activation of the terminal sequence C5-C9.

Assembly of the C5b-9 membrane attack complex
Formation of the C5b-9 complex (membrane attack complex, MAC) on target cells is initiated by the cleavage of C5 into C5a and C5b by cell-bound C5 convertases. Generated C5b is rapidly released in an inactive form in the fluid phase unless it forms a stable bimolecular C5b6 complex with C6. The C5b6 complex can reversibly bind to cell membranes. With the addition of C7-C9, it lyses unsensitized bystander cells, a process known as "reactive lysis". Binding of one molecule of C7 to C5b,6 creates a trimolecular complex which inserts firmly into the lipid bilayer of the target cell membrane. Assembly of the C5b-9 complex is further completed by the binding of one molecule of C8 and several molecules of C9. The interaction of the terminal complement components C5-C9 on the surface of susceptible cells results in the formation of protein-lined hydrophilic transmembrane channels which cause osmotic lysis of the cells [8].

Regulation

Activation of the classical and alternative pathways is subjected to fine regulatory processes, i.e. the spontaneous and rapid dissociation of enzymatic complexes and the regulatory activity of several plasma and cell-associated proteins.
Formation of the C1 complex is regulated by C1 inhibitor (C1-inh) which belongs to the family of human serum protease inhibitors (serpins). C1-inh inhibits auto-activation of C1r and C1s into native C1 [9]. The half-life of the C4b,2a enzyme is limited by the intrinsic decay of the C2a subunit and by the decay-accelerating activity of C4BP in plasma and of decay-accelerating factor (DAF, CD55) that is expressed on the membrane of a large variety of autologous cells. The formation of the classical pathway C3 convertase is also limited by the cleavage and inactivation of C4b by factor I in the presence of the cofactors C4BP or membrane cofactor protein (MCP, CD46). Cleavage of C4b generates C4c and C4d.
Regulation of the activity of the alternative pathway does not depend on the initial binding of B to C3b which is unaffected by the nature of the surface to which C3b becomes covalently attached. Discrimination between "activating" and "non-activating" surfaces of the alternative pathway depends on the relative capacity of factor H to bind to C3b, that prevents formation of the C3b,Bb enzyme and allows for inactivation of C3b by factor I. Formation of the C3b,Bb convertase is inhibited on "non-activating" surfaces by the preferential binding of factor H to C3b as compared with factor B. Sialic acid and sulfated mucopolysaccharides (e.g. heparan sulphate) modulate the ability of cell surfaces to activate

the alternative pathway by enhancing the interaction between surface-bound C3b and the regulatory protein H [10]. Several membrane proteins inhibit formation of the classical and/or alternative pathway C3 convertases on the cell surface thus contributing to the protection of host cells from damage by autologous complement. They include decay-accelerating factor (DAF, CD55), the C3b receptor (CR1, CD35) and membrane cofactor protein (MCP, CD46) which accelerate the dissociation of C3 convertases and serve as cofactors for proteolytic cleavage of C3b by factor I [11, 12]. Inactivation of C3b by factor I generates the iC3b, C3dg and C3d fragments that remain covalently linked to the target surface of complement activation but are unable to perpetuate complement activation. Each of the C3 cleavage products C3b, iC3b, C3dg and C3d may react with one or several of four types of C3 receptors CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) expressed on phagocytes and lymphocytes [11]. The critical importance of C3 in the complement cascade is evident from: (1) its position at the convergence of the classical and alternative pathways; (2) its role in the activation and amplification of the alternative pathway; (3) the fact that C3 is a major step for regulation of complement activity; (4) the multiple biological activities associated with the cleavage products of the C3 molecule.

Cytolytic damage by the membrane attack complex (MAC) is modulated by a plasma protein termed S-protein or vitronectin. The S-protein blocks the action of C5b-9 by binding to the newly-formed C5b-9 complex rendering it unable to attach to the surface of target cells. Nucleated cells which are relatively resistant to lysis possess the ability to repair complement-mediated damage by both endocytosis and exocytosis of the C5b-9 channels. MAC formation is also inhibited on cells by two membrane proteins, CD59 and C8 binding protein (C8bp) that inhibits homologous complement-mediated lysis and thus acts as homologous restriction factor (HRF) [13]. CD59 that is anchored to the membrane via a glycosyl phosphatidyl inositol linkage, binds to neoepitopes on C8 and C9 that are exposed during MAC assembly [14].

Molecular Interactions

Complement-mediated processing of immune complexes The transient occurrence of circulating immune complexes is now regarded as a physiological phenomenon. The role of complement in handling immune complexes in the circulation and at a site of a tissue lesion is decisive in that it may favor the removal of immune aggregates or mediate a clinically overt inflammatory process [15]. Activation of the classical or the alternative pathway by immune complexes results in the covalent binding of C4b and C3b to the antigen and/or to the immunoglobulin, that further allow an optimal interaction of the immune complexes with cellular complement receptors. Complement activation is also involved in maintaining immune complexes in solution and in the transport of C3-bearing complexes to phagocytes in the spleen and/or the liver. The interaction of classical pathway proteins with immune complexes during the formation of the complexes prevents immune precipitation [16], whereas incubation of preformed complexes with serum (i.e. with complement proteins) leads to their disaggregation and solubilization through an alternative pathway-mediated mechanism [17]. Once immune complexes have been opsonized with complement, their clearance from the circulation depends on the interaction with C3b receptors (CR1) on erythrocytes and on the functional state of the reticuloendothelial system. Erythrocyte CR1 is the major source of CR1 in whole blood. CR1 endows the cells with the capacity for repeated uptake and release of C3b-bearing immune complexes which allows the erythrocyte to transport the complexes and deliver them through the portal circulation to the liver [18]. In the liver, the complexes are stripped from the cells and transferred to Kupffer cells. An impaired clearance of immune complexes in the liver has been found in hypocomplementemic monkeys in whom complexes appear widely distributed throughout the body.

Receptors for complement proteins Complement activation results in the generation of diffusible biologically-active peptides and the deposition of complement fragments on target surfaces of complement activation. Both diffusible anaphylatoxins and target-bound activation fragments may interact with receptors on effector cells to trigger specific cellular responses [11]. In addition, the insertion of C5b-9 into the lipid bilayer of cell membranes may directly induce lysis of target cells. Although nucleated cells are relatively resistant to membrane attack by complement, sublytic amounts of C5b-9, C5b67 and C5b678 mediate signal transduction in various cell types [19]. These include the mobilization of Ca^{2+} from intracellular stores, stimulation of arachidonic acid metabolism, release of prostaglandins and leukotrienes and the production of oxygen metabolites by leukocytes that induces activation of endothelial cells.

Activation of complement results in the rapid generation of the anaphylatoxins C3a, C4a and C5a through cleavage of the α-chains of C3, C4 and C5. The peptides diffuse into plasma and extracellular fluids where their binding to specific receptors mediate a number of biological functions. Thus, the anaphylatoxins cause smooth muscle contraction and increased vascular permeability. C5a is more active than C3a, and C4a has little activity. In plasma, the C-terminal arginines of C3a and C4a are removed physiologically by carboxypeptidase N, converting the anaphylatoxins in stable biologically inactive C3a-desArg and C4a-desArg derivatives. C3a binds to receptors expressed on mast cells, basophils, monocytes, smooth muscle cells, lymphocytes and platelets. A receptor for C3a on macrophages has recently been cloned [20]. C3a has also been reported to suppress immunological functions such as polyclonal and antigen specific humoral immune responses. In addi-

tion to being the most potent anaphylatoxin, C5a at nanomolar concentration is chemoattractant for leukocytes causing directed migration of neutrophils, eosinophils, basophils and monocytes against the gradient of concentration that occurs when the peptide diffuses away from the site of complement activation. C5a may have systemic effects due to its relative resistance to cleavage by carboxypeptidase N and by the retention of significant biological activity of C5a-desArg. The C5a receptor belongs to the rhodopsin superfamily and is coupled to a G protein [21]. Triggering of the C5a receptor on specific target cells (i.e. neutrophils, mastocytes, basophils, monocytes) results in receptor phosphorylation and activation of intracellular signal transduction pathways. The recent cloning of C5a receptor allowed the demonstration that its expression extends to non-myeloid cells such as lung vascular smooth muscles and endothelial cells [22].

The C3b receptor (CR1, CD35) is a single chain polymorphic glycoprotein (Table 1) [23]. The most common allotype has a molecular weight of 250 kDa. CR1 functions as a high-affinity receptor for C3b that is covalently bound to particles, cells or immune complexes. C3b is thus presented as a multivalent ligand for the receptor. CR1 also binds with lower affinity the surface-fixed fragments iC3b and C4b. CR1 is expressed on erythrocytes, neutrophils, monocytes, eosinophils, mastocytes, B lymphocytes, a subset of T lymphocytes and thymocytes, follicular dendritic cells, Kupffer cells and glomerular podocytes. Soluble and membrane-associated CR1 efficiently regulate alternative pathway and classical pathway activation by impairing the formation of the C3 convertases and promoting cleavage of C3b and C4b by factor I. CR1 is a potent anti-inflammatory agent since the administration of soluble recombinant CR1 prevents tissue damage induced by complement activation in experimental models of myocardial reperfusion injury and xenograft rejection [24]. CR1 expresses additional functions that differ with the cell type that carries the receptor (see below).

CR3 (the iC3b receptor, CD11b/CD18) is a member of the β_2-integrin family, which also includes the leukocyte adhesion molecules LFA-1 (CD11a/CD18) and p150,95 (CD11c/CD18)[25]. All three molecules are heterodimers sharing a common β_2 subunit associated with a specific α-chain. The α and β chains of CR3 are of respective Mr 165 and 95 kDa. CR3 may bind multiple ligands. The divalent cations Ca^{++} and $Mg^{++}$are essential for the stabilization and function of the α/β complex since both subunits contribute to ligand binding. CR3 is present in an inactive state on circulating leukocytes. Acquisition of an active state when cells are triggered by inflammatory mediators, reinforces the binding reaction of iC3b to CR3 and allows for expression by CR3 of binding sites for other ligands including coagulation factor X, fibrinogen, intercellular adhesion molecule (ICAM-1, CD54), and betaglycans. The p150/95 (CD11c/CD18) protein may also be considered as a C3 complement receptor (CR4), since it binds iC3b and C3dg. The cellular expression of CR3 is restricted to monocytes, macrophages, neutrophils, eosinophils, basophils and natural killer (NK) cells. In resting monocytes and granulocytes, 90% of CR3 is stored as intracellular pools localized in peroxydase-negative granules. In response to a variety of stimuli including chemoattractants, leucotrienes, cytokines, the intracellular pools of CR3 translocate and fuse with the plasma membrane. Most stimuli that upregulate membrane expression of CR3 also induce qualitative changes allowing CR3-mediated biological functions. In addition to phagocytic function, CD11b/CD18 is essential for spreading, adhesion and transendothelium migration of leukocytes during the inflammatory process.

The CR2 receptor (CD21), a 145 kDa type I transmembrane glycoprotein, is the cellular receptor for the iC3b,C3dg, C3d cleavage fragments of C3 and for Epstein Barr virus (EBV) [23]. More recently, CD21 was also shown to bind CD23 that serves as the low-affinity receptor for IgE [26]. CD21 is only expressed on B lymphocytes, approximately 50% of peripheral blood T lym-

Table 1. Cellular receptors for target-bound C3 activation fragments

Receptor	Molecular Weight (kD)	Ligands	Cellular Distribution
CR1 (CD35)	160-250	C3b (C4b, iC3b)	erythrocytes, monocytes/macrophages, neutrophils, eosinophils, basophils, B lymphocytes, subset of T lymphocytes, NK cells, follicular dendritic cells, Kupffer cells, glomerular podocytes
CR2 (CD21)	145	iC3b, C3dg, C3d CD23, EBV	B lymphocytes, subset of T lymphocytes and thymocytes, follicular dendritic cells
CR3 (CD11b, CD18)	α chain: 165 β chain:* 95	iC3b fibrinogen, ICAM-1 factor X	monocytes/macrophages, neutrophils, eosinophils, basophils, follicular dendritic cells, Kupffer cells
CR4 (CD11c, CD18)	α chain: 150 β chain:* 95	iC3b, C3dg	macrophages, neutrophils, Kupffer cells

* CR3 and CR4 share common β chain of β2 integrins

phocytes, a subpopulation of immature thymocytes and on follicular dendritic cells. CR2 plays an important role in antigen-induced B cell proliferation. The interaction of CR2 with CD23 contributes to the regulation of IgE production, germinal center B-cell survival, and B cell-presentation of soluble antigen to T cells.
The biological relevance of cellular receptors for C1q is as yet poorly understood. Lymphocytes, monocytes, neutrophils, platelets express a C1q receptor which binds to C1q that remains fixed to classical pathways activators following the dissociation of macromolecular C1 by C1inh. Human umbilical vein endothelial cells express receptors for both the collagen-like and the globular domain of C1q, which may have implications for the role of C1q in vascular inflammatory and thrombotic lesions [27].

Cells and Cellular Interactions Complement mediates a number of important host defense reactions including the recognition of pathogens and altered host cells, the production of an acute inflammatory response, opsonization, phagocytosis and cytolysis which facilitate the elimination of pathogens. Endothelial cells normally provide a barrier to the egress of proteins and cells from blood vessels, maintain an anticoagulant environment intravascularly and remain not adherent for leukocytes under physiological conditions. Generation of anaphylatoxins modify vascular permeability, attract inflammatory cells and modify their adhesion properties to the endothelium. Uncontrolled complement activation may occur following the entrapment of bacterial antigens in chronic inflammatory sites, during immune complexes-associated diseases or, e.g., following intravascular complement activation during hemodialysis. In such pathological situations complement may directly induce vascular inflammatory injury by the generation of anaphylatoxins and the membrane attack complex. Complement may also induce vascular injury in an indirect fashion by generating chemotactic peptides which activate leukocytes inducing their adherence to target tissues and the secondary release of inflammatory mediators and toxic oxygen products.
Endothelial cells and complement
C5a causes the rapid expression of P-selectin, the secretion of von Willebrand factor and adhesion of human neutrophils to human umbilical vein endothelial cells, indicating that C5a behaves as an important inflammatory mediator for early adhesive interactions between neutrophils and endothelium [28]. Formation of the membrane attack complex directly influences the integrity of the vascular endothelium. The sequential association of C5b with complement components C6-C9 establishes pores on endothelial cell surfaces. The pores when present in sufficient numbers, mediate cell lysis. In sublytic amounts, the membrane attack complex leads to modification of coagulant status and to activation of endothelial cells. Thus, the assembly of C5b-9 triggers von Willebrand factor production and induces membrane cell vesiculation, leading to the expression of a prothrombinase complex [29]. By inducing upregulation of P selectin and the synthesis of the chemokines IL-8 and MCP-1 [30], C5b-9 promotes the adhesion of phagocytes to endothelial cells and amplifies the recruitment of leukocytes at the site of inflammation. The expression of the complement regulatory proteins DAF, MCP and CD59 by endothelial cells limits the insertion of autologous MAC [31]. The inhibitory capacity of these membrane-associated regulatory components has been extensively documented in discordant xenograft models. The use of organs from transgenic pigs expressing human DAF and CD59 revealed striking protection from complement-mediated injury of xenogenic endothelial cells [32]. Finally, the synthesis of the alternative pathway components B and C3 by endothelial cells *in vitro* has been shown to be enhanced by the pro-inflammatory cytokine IL-1, suggesting that complement protein secretion may take an active part in the local deposition of C3 fragments on endothelial cells [33].

Leukocytes and complement Neutrophils and monocytes express receptors for anaphylatoxins and receptors for C3 fragments. Neutrophil stimulation by C5a results in respiratory burst, enhances neutrophil turnover of arachidonic acid and stimulates cellular production of 5-HETE and leukotriene B4 [34]. These lipids are neutrophil chemotactic factors. C5a causes degranulation of leukocytes, inducing the release of lysosomal enzymes from neutrophils and the release of ECP and EPO by eosinophils which may mediate changes in the integrity of blood vessels. For example, the release of proteases from neutrophils induces rapid cleavage of heparan sulfate from the surface of endothelial cells that may enhance their capacity to activate the alternative pathway. Neutrophil activation by C5a increases the membrane expression of CR1 and CR3 which are stored in secondary granules, allowing for a better attachment of the cells to targets opsonized with C3b and iC3b [35,36]. C5a and C5a-desArg also enhance the adhesiveness of neutrophils to foreign surfaces and endothelial cells and reversibly aggregate the cells *in vitro* and *in vivo* [37]. The latter effect is secondary to enhanced expression of the adhesion-promoting molecule CR3. CR3 mediates the intravascular aggregation of leukocytes which causes leukopenia in individuals undergoing hemodialysis with membranes that activate complement. This mechanism is probably essential for the pathogenesis of pulmonary endothelial damage in the adult respiratory distress syndrome (ARDS). Recent data indicate that oxygen-derived free radicals released from sequestrated neutrophils play a major role in endothelial cell damage. Triggering of C5a receptor leads to production of IL-1 by monocytes and IL-8 chemokine by eosinophils, pro-inflammatory cytokines which, in turn, can activate endothelial cells.
The interaction of CR1 and CR3 with complement-opsonized targets will cause particles to adhere to monocytes and neutrophils [38]. The main function of

CR1 on these cells is to enhance phagocytosis of IgG-coated particles and mediate the internalization of small ligands bearing C3b. The binding of fibronectin or laminin to C3b-coated particles confers the ability to ingest the opsonized targets in the absence of antibody on both monocytes and C5a-stimulated neutrophils. The enhancing effect of connective tissue proteins on phagocytosis may be particularly relevant to the pathogenesis of vascular lesions where extracellular matrices become exposed. The interaction of bound C3b with CR1 has also been shown to induce enzyme release by neutrophils, trigger the oxidative metabolism and activate the arachidonic acid pathway in neutrophils and monocytes. The expression of CR1 as that of CR3 is increased by IL-1 and chemoattractants and decreased by IFNγ. CR3 is probably the most important receptor for phagocytosis of opsonized bacteria. Triggering of C3 receptors on monocytes with polymeric C3b and iC3b has also been reported to result in intracellular accumulation and release of IL-1. As mentioned earlier, CR3 is a member of the b2 integrin family which is involved in adhesion events. By enhancing the expression of CR3 and by activating the receptor, C5a and other chemoattractants (PAF acether or leukotrienes) increase the adhesiveness of the cells to vascular endothelium and pathogens. It should be mentioned here that activation of endothelial cells with cytokines such as IL-1 upregulates the expression of ICAM-1, which serves as an additional ligand for CR3 that may facilitate the adhesion of monocytes and granulocytes to the vascular endothelium.

Additional Features Complex interactions occur between complement and proteins of the coagulation and fibrinolytic systems. The serine esterase enzymes and the serine protease inhibitors of the systems are structurally related. *In vitro* plasmin generation may directly act on C1 to activate the complement system or inactivate C1inh, thereby releasing C1 from inhibition. Plasmin and kallikrein also cleave C3 and C5 *in vitro*, although the cleavage efficiency is low compared to that normally obtained with physiological convertases, raising the question of the physiological relevance of this interaction.

Clinical Relevance There are several mechanisms by which antigen-antibody complexes may form and/or deposit in the vascular wall: (1) local interaction between antigen and pre-formed antibody (as is the case in the Arthus reaction); (2) deposition of circulating immune complexes; and (3) the interaction of circulating antibody with an antigen *in situ*, whether the antigen is a constitutive antigen of the vascular wall or whether it is an exogenous "planted" antigen. The deposition or *in situ* formation of immune complexes and the subsequent activation of complement mediate immunologically-induced vascular injury [39]. A critical function of complement with regard to the pathogenesis of vasculitis is its ability to prevent immune complex precipitation and to prepare optimally solubilized complexes for their intravascular transport to the sites of removal in the reticulo endothelial system. Thus, inherited deficiencies of proteins of the classical pathway are associated with an increased incidence of autoimmune and immune complex-mediated diseases [40,15]. Patients with homozygous C4 or C2 deficiencies have a high incidence of systemic lupus erythematosus.

Systemic small vessel vasculitides, polyarteritis nodosa and related syndromes, pulmonary endothelial cell injury in the adult respiratory distress syndrome and vascular hyperacute rejection of transplants are examples of immunologically-mediated vascular damage where complement is involved. For diagnostic purposes, antibodies to complement proteins and to neoantigens expressed by activation products of complement may be used to detect and characterize deposits of complement in pathological tissues by indirect immunofluorescence. The availability of monoclonal and polyclonal reagents to C5b9 has allowed for the detection of terminal complexes in the skin, vessels and kidney lesions in SLE, in vasculitis, and in ischaemic areas of myocardial infarction, bullous pemphigus and synovial tissues in rheumatoid arthritis. Deposits of C3 and of C5b-9 have been found in association with immune complexes along the glomerular capillary walls and in the mesangium in a variety of glomerular diseases (Figure 2).

Because the complement system has both protective and autoaggressive potential, therapeutic modulation of complement is difficult to design and conduct unless it may be targeted in the future to the sites of complement activation. At the present time, no therapeutic intervention specifically aimed at down-regulating complement is used in vascular diseases where complement activation is involved. Yet, clinical trials of recombinant CR1, the most promising inhibitor of complement in the fluid phase, have recently been initiated.

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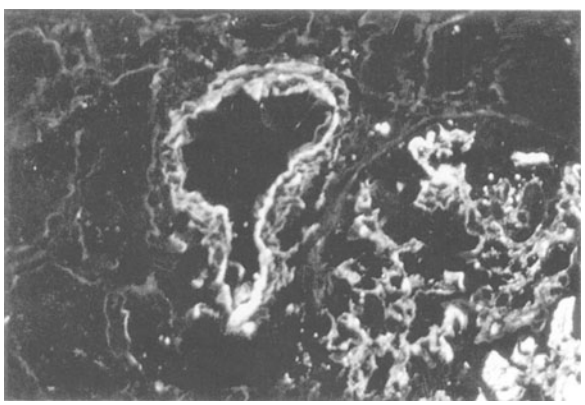

Figure 2. Nephritis during systemic lupus erythematosus. Staining of adjacent glomerulus and artery with anti-C5b-9 monoclonal antibody. x 450

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Contractility

Definition *Contractility, the shortening of cells through the sliding action of actin-myosin and Ca^{2+} in smooth muscle cells. Extracellular factors of vasoconstriction are angiotensin II and endothelin.*

See: →Smooth muscle cells

COX-1

Definition *Cyclooxygenase-1*

See: →Cyclooxygenase; →Bleeding disorders; →Prostacyclin; →Prostaglandins; →Thromboxanes

COX-2

Definition *Cyclooxygenase-2*

See: →Cyclooxygenase; →Bleeding disorders; →Prostacyclin; →Prostaglandins; →Thromboxanes

CPAE

Definition *Calf pulmonary artery endothelial cell*

See: →Angiogenin; →von Willebrand Factor

CR1, 2, 3 and 4

Definition *Complement receptor 1, 2, 3 and 4*

See: →Complement system (interaction of vascular cells with)

CSF

Definition *Colony-stimulating factor*

See: →Colony-stimulating factors; →Atherosclerosis

Cyclic Nucleotides

Definition *Cyclic adenine monophosphate (cAMP) is involved in vascular smooth muscle relaxation. Cyclic guanosine monophosphate (cGMP) binds to type I (smooth muscle cells) or type II G-kinases (epithelial cells). Production of cyclic nucleotides is under the control of cyclases and phosphodiesterases.*

See: →Platelet stimulus-response coupling; →Vasomotor tone regulation, molecular mechanisms of

Cyclooxygenase (Cox)

Definition
Same as PGH synthase-1 (Cox-1) and PGH synthase-2 (Cox-2), enzyme involved in the generation of prostaglandins

See: →Platelet stimulus-response coupling; →Prostacyclin; →Prostaglandins

Cytokines in Vascular Biology and Disease

Introduction Interactions between immune mediators and vascular cells regulate basic functions of the immune system such as lymphocyte migration, regional immunity, and inflammatory responses. Immune mediators also modulate cardiovascular functions controlling both systemic and regional hemodynamics. In

this review, we explore current evidence for a role of cytokines in the development of cardiovascular diseases. In particular, we will focus on how cytokines regulate cell surface receptors on vascular cells and modulate the functions of vascular endothelial and smooth muscle cells, with emphasis on two basic vascular activities: vascular tone and hemostasis. We also discuss the involvement of cytokines in thrombosis and the stability of atherosclerotic plaques. Finally, the crucial role of signal transduction pathways in mediating the function of cytokines will be examined.

Characteristics (of atherosclerotic vascular disease)
A chronic pathologic process in the intima of large arteries, atherosclerosis starts when cholesterol-bearing low density lipoproteins (LDL) infiltrate the arterial intima in such amounts that the normal elimination mechanisms are exceeded [1, 2]. In the intima, LDL adheres to proteoglycans of the extracellular matrix and is oxidized by free oxygen radicals and enzymes produced by vascular endothelial cells and macrophages [3]. Thies leads to the inflammatory proliferative disease of atherosclerosis.
The atherosclerotic plaque is the site of a local immune activation. In its fibrous cap, approximately 20 % of the cells are T lymphocytes [3] and nearly half of these show signs of activation [5, 6]. Cytokines produced by T cells, macrophages and smooth muscle form a local network that plays a pivotal role in the development of cardiovascular diseases. The expression of cytokines is tightly controlled in the producing cells, and one of the most important regulatory steps is the control of gene transcription. Several lines of evidence indicate that transcription factors are crucial in determining changes in cell function, growth and differentiation, leading to characteristic patterns of gene expression during inflammation. The final stage of plaque development is characterized by the appearance of fissures, thrombi, and actual plaque rupture [7-9].
Vascular contractility plays an important role in the pathophysiology of atherosclerosis. Vascular contractility is determined by interaction between actin and myosin filaments in smooth muscle cells. Some of the filament-forming proteins such as α-SM-actin are specific for smooth muscle cells but the filaments themselves are not as well developed as in striated muscle. The contractile process is dependent on energy provided by ATP derived from mitochondrial respiration. It is regulated by several different soluble factors including α-adrenergic agonists from local nerve endings and from the blood, circulating angiotensin, and endothelium-derived factors such as endothelin and nitric oxide (NO).

Regulation

Molecular Interactions

Cytokines in fatty streak formation and antigen presentation Oxidized LDL is a ligand for the macrophage scavenger receptors (MSR), which bind proteins and particles with clustered negative charges [10]. Receptor-ligand complexes are internalized and degraded intracellulary, and fragments of the ligands can associate with MHC molecules and may be presented as T cell antigens [11, 12]. LDL cholesterol entering the macrophage via MSR accumulates in cytoplasmic droplets; the increasing intracellular cholesterol accumulation gradually transforms the macrophage into a foam cell, which is the hallmark of atherosclerosis [13]. Foam cells form fatty streaks, i.e. lesions of the arterial intima which predispose to atherosclerosis. Two macrophage surface receptors, CD36 (a class B scavenger receptor) and the SR-A macrophage scavenger receptor (a class A scavenger receptor), have been identified as major receptors that bind and internalize OxLDL [3]. Recently, the importance of MSR in the formation of fatty streaks has been further addressed by targeted disruption of the SR-A gene in mice [14]. Deletion of SR-A results in a reduction in the size of atherosclerotic lesions in animals deficient in apolipoprotein E. Expression of both class A and class B MSR in monocyte/macrophages is dependent on the differentiation state as well as exposure to soluble mediators (cytokines and growth factors).
Macrophage colony-stimulating factor (M-CSF) is a differentiating factor for monocyte/macrophage development as well as a regulator of lipoprotein uptake. M-CSF, which is produced by plaque cells, increases SR-A expression on the mRNA level and enhances foam cell formation [15–17]. Thus, M-CSF can be expected to have profound effects on atherosclerosis. Its importance was recently confirmed by the observation that *op/op* mice, which carry a defective M-CSF gene, only form diminutive fatty streak lesions when fed an atherogenic diet, even when bred onto the highly atherosclerosis-prone, apolipoprotein E-knockout background [18, 19]. In addition to M-CSF, an S-100-like chemotactic cytokine has also been shown to control scavenger receptor expression [20].
Proinflammatory cytokines and endotoxins may exert effects on MSR opposite to those of M-CSF. Both interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) downregulate SR-A expression and inhibit foam cell formation [21–23]. In the case of IFN-γ, modulation is accomplished by destabilization of cytoplasmic mRNA, whereas TNF-α inhibits transcription of the SR-A gene [22, 24]. Endotoxin has similar effects on SR-A expression as TNF-α and appears to act by inducing autocrine TNF-α secretion that in turn inhibits SR-A gene transcription [22]. The importance of proinflammatory cytokines for SR-A expression is supported by the *in vivo* observation that areas of atherosclerotic plaques with inflammatory activity and T cell activation show reduced expression of SR proteins [23]. This suggests that macrophage-T cell interactions may be decisive for the development of the fatty streak.
Apart from its role in scavenging oxLDL leading to forming fatty streak, macrophages are professional antigen presenting cells that may initiate immune reactions in atherosclerotic plaques. This has been addressed in a

recent review [25]. Finally, recent studies suggest that the vascular endothelium not only plays an important role in the cytokine-regulated recruitment of leukocytes from blood to tissues, but also performs a series of other functions relating to immunity, inflammation, hemostasis, and permeability, which can all be modulated by cytokines.

Cytokines in the transition into atherosclerosis RT-PCR and immunohistochemical analyses have shown that the T cell cytokine, IFN-γ is produced in the plaque [5, 23]. Furthermore, smooth muscle cells, endothelial cells, and macrophages all express high levels of MHC class II proteins (HLA-DR), suggestive of cellular responses to IFN-γ [26]. In addition to the specific T cell cytokines, proinflammatory and macrophage-stimulating cytokines as well as chemokines are also produced in the atherosclerotic plaque. TNF-α and IL-1 are both found in plaques and can be produced both by endothelial cells (preferentially IL-1), smooth muscle cells, and macrophages [27–29]. They may be important as regulators both of smooth muscle cell growth and the activation of endothelial cells, macrophages, and T cells. Finally, chemokines such as monocyte chemoattractant protein-1 (MCP-1) [30, 31] and IL-1 [32] could be important for the recruitment of inflammatory and immunocompetent cells into the plaque. Thus, cytokines produced by inflammatory cells in the atherosclerotic plaque form a network that may direct the progression of the disease. Recent studies of disease development in murine models of atherosclerosis support a role for immune factors. Thus, immunosuppressive treatment aggravates fatty streak formation in C57BL/6 and immunodeficient MHC class I knockout mice of the C57BL/6 background develops significantly larger fatty streaks than wild type C57BL/6 mice [33]. These observations imply that immune factors are anti-atherogenic. They are also supported by observations in rabbits implying an anti-atherogenic role of immune factors [34] and an anti-fatty streak effect of treatment with the cytokine, LIF (leukemia inhibitory factor) [35].
The results of similar studies in atherosclerosis-prone ApoE-knockout mice are in apparent contrast to these findings. Double-knockouts lacking both ApoE and RAG-1 (recombinase-activating gene-1) exhibit a reduction of lesion size by approximately 40% compared to the ApoE single knockout [36]. Similarly, double-knockouts lacking ApoE and the interferon-γ receptor show a lesion reduction by 60% [37]. Finally, transplant atherosclerosis induced by allgrafting of blood vessels was found to be dependent on CD4+ T cells, MHC class II, and B cells [38]. To interpret these findings, we must bear in mind that the immune system is a complex defense system that contains counterbalancing activities. For instance, activation of proinflammatory Th1 responses is counteracted by the Th2 and T suppressor cells, and many of the transcriptional effects of inflammatory cytokines such as TNF-α and IFN-γ are counteracted by TGF-β and IL-10. Therefore, not only the extent but also the type of immune activation is likely to be decisive for the immunopathogenesis of a disease. In atherosclerosis of ApoE-knockout mice, recent data show that the balance between Th1 and Th2 responses is determined by serum cholesterol levels [39]. Lack of IL-10 increases fatty streak formation in S578L/6 mice [194] while treatement of apo E-/- or LDL receptor-/- mice with immunomodulating antibodies reduce atherosclerosis [195-196]. More information will therefore be needed to understand the conditions promoting proatherogenic and antiatherogenic immune responses.

Role of transcription factors in cardiovascular diseases Among transcription factors defined to date, NF-κB (nuclear factor-κB), AP-1 and STATs (signal transducers and activators of transcription) have been intensively studied. NF-κB is a homo- or heterodimeric combination of Rel-related proteins. Members of this family are p65 (RelA), RelB, p50 (NF-κB1), p52 (NF-κB2) and c-Rel [40-45]. They all share a conserved domain, the Rel homology domain, that participates in DNA binding and dimerization. In most cell types, NF-κB resides in the cytoplasm in a latent form that is associated with the inhibitor IκB. Three major IκB molecules, IκBα, IκBβ and IκBε, have been identified [46–49], which retain NF-κB in the cytoplasm by masking its nuclear localization signal domains. In response to multiple stimuli, including cytokines, phorbol esters, bacterial lipopolysaccharide (LPS), and viral products, the inhibitor is proteolyzed and NF-κB translocates into the nucleus, where it activates its target genes [50-53]. The NF-κB family of transcription factors plays a crucial role in the expression of a large array of genes essential to the immune response in the atherosclerotic lesion, including the genes encoding IL-1β, TNF-α, tissue factor, VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1), iNOS (inducible NO synthase), MCP-1and M-CSF (for reviews, see [54, 55]).
Human vascular smooth muscle cells express predominantly RelA, p50 and p52 precursor (p100) as well as its inhibitors including at least IκBα and IκBβ [56, 57]. Activation of NF-κB in vascular smooth muscle cells involves two phases. In the absence of stress or cytokine stimuli, vascular smooth muscle cells display a constitutive NF-κB activation, which seems to be composed of a p50 homodimer [56]. This constitutive NF-κB signal is serum-dependent, and does not initiate transcription of inflammatory genes. The constitutive activation of NF-κB may be needed for driving cell proliferation (our unpublished data and [56]). Upon cytokine stimulation, an inducible activation of NF-κB will be triggered, leading to strong translocation of a p65/p50 heterodimer into the nucleus. This inducible signal exerts a critical regulatory role in the transcription of inflammatory genes such as the iNOS gene in smooth muscle cells and macrophages [57-59]. Interestingly, intimal smooth muscle cells, a population of cell isolated from the intima of injured rat arteries, show a different NF-κB activation than normal medial smooth muscle cells. Compared with medial smooth muscle cells, intimal smooth muscle cells have a high constitutive NF-κB activity and

a hyperinducible activation of NF-κB ([58], and our unpublished data). This special feature is in agreement with a high ability for iNOS transcription in the intimal smooth muscle cells, and may be the reason why inflammatory genes are expressed preferentially by intimal smooth muscle cells in injured vessels and in atherosclerotic lesions.

Although the importance of NF-κB in cytokine expression has been well established *in vitro*, the work to unravel the role of NF-κB in the development of atherosclerosis has just started. Brand and colleagues have recently reported that NF-κB is activated in macrophages, smooth muscle cells and endothelial cells in human atherosclerotic lesions, but not detected in vessel free of atherosclerosis [60]. Using double immunostaining, they have further shown that activation of NF-κB correlates with expression of tissue factor and ICAM-1, two NF-κB dependent inflammatory genes. Bourcier et al have also found that activated NF-κB is preferentially found in smooth muscle cells of human atherosclerotic lesions [57]. These findings suggest that NF-κB is important in the regulation of cytokine networks during the development of atherosclerosis.

Because of its pivotal role in the regulation of cytokine production, the NF-κB signal transduction pathway is an appealing target for therapeutic intervention in cardiovascular disorders. Antioxidants such as PDTC (pyrrolidone derivative of dithiocarbamate) and NAC (*N*-acetyl cysteine) are effective inhibitors of NF-κB activation *in vitro* [61, 62]. In an experimental septic model, pretreatment of rats with PDTC compromises the activation of NF-κB, and prevents expresssion of inducible NO synthase in lung tissue [63]. Although PDTC has to be applied before endotoxin treatment and the toxic effects as well as the possibility for long term application of the substance are uncertain, prevention of inducible NF-κB activation is a promising area for future research. In agreement with this study, Böhrer and his colleagues injected mice intravenously with a plasmid construct encoding IκBα, and achieved a 20–35% transfection efficiency for monocytes/ macrophages and endothelial cells *in vivo*. They report that pretransfection of mice with IκBα attenuated NF-κB activation and tissue factor expression in renal tissue and significantly increased survival rate following LPS treatment [64]. No high-selective, non-toxic inhibitor of NF-κB is yet available, but Morishita et al have developed an alternative strategy, using a DNA fragment containing the consensus for the NF-κB binding site to block NF-κB driven transcription. By infusion of the decoy DNA into rat coronary arteries, they successfully prevented NF-κB activation and inhibited myocardial infarction [65, 66]. The therapeutic benefit seen with antioxidants and DNA constructs against NF-κB activation in diseased state such as myocardial infarction and septic shock, together with the rapidly increasing knowledge of the signalling processes involved, will certainly encourage the search for more selective and effective low molecular drugs in the near future.

Obviously, research on the role of NF-κB in inflammatory and immune cardiovascular disorders is still at an early stage. Many questions remain to be answered. For instance, two studies recently reported that oxidized LDL, generally regarded as a major pathogenic initiator of atherosclerosis, inhibits NF-κB activation in macrophages and smooth muscle cells [67, 68], raising the question as to which stimuli and mechanisms are responsible for the regulation of NF-κB signalling in atherosclerosis.

STATs comprise another family of transcription factors. To date, seven STAT genes have been identified [69]. As originally defined, activation of STATs involves ligand-dependent activation of a particular class of receptor-associated tyrosine kinases, the JAK proteins, which phosphorylate themselves and receptor components, creating recruitment sites for STATs. The STATs are phosphorylated, dissociate from the receptor-JAK complex, and then translocate to the nucleus where they participate in transcriptional gene activation. The JAK-STAT mediated cytokine-response has been more precisely defined than the NF-κB transduction pathway. One example is the interferon-induced signalling pathway. During the response to interferon-α (IFN-α), JAK1 and Tyk2 tyrosine kinases are activated, leading to activation of STAT1 and STAT2. Activated STAT1 and STAT2 assemble together with ISGF3 (interferon-stimulated gene factor 3), and translocate into the nucleus where they bind to interferon-stimulated response elements in the promoter of IFN-α stimulated genes. The response to IFN-γ is mediated by activation of JAK1 and JAK2 associated with IFN-γ receptors, leading to phosphorylation of STAT1. The activated STAT1 forms homodimers, the so-called γ-activated factors, translocate into nucleus, and bind the γ-activated site (GAS) element in the promoters of IFN-γ induced genes [70, 71]. The JAK-STAT pathway is crucial for cytokine-mediated gene responses and a central determinator of their specificities (reviewed in [69, 72]). Therefore, this transcription pathway attracts considerable attention in the study of inflammatory and immune diseases.

It is now evident that infiltration of T cells occurs in the initial stage of atherosclerosis, and also happens in the development of transplant arteriosclerosis [5, 25, 73, 74]. Studies of cytokine expression suggest a Th1 predominant pattern in these conditions ([75] and unpublished results). However, the molecular mechanisms responsible for regulating the differentiation of Th1 and Th2 cells are less well defined.

Several lines of evidence have suggested that STATs may participate in the Th1 development. In STAT4 knockout mice, IL-12 function is impaired, resulting in multiple deficiencies including impaired induction of IFN-γ, reduced proliferation and cytolytic function of natural killer cells as well as hampered defective Th1 differentiation [76-79], indicating that STAT4 is required for mediating IL-12 regulated functions. Furthermore, exposure of murine CD4+ T cells to IFN-γ or human CD4+ cells to IFN-α maintained expression of the IL-12 receptor β2

chain, which is specifically expressed by Th1 cells but not Th2 cells [80-82]. Since the function of IFN-γ and IFN-α both depend on the activation of STATs, these data again suggest that differential regulation of STATs may determine T cell differentiation, resulting in a specific cytokine pattern in a local environment.
Although it is well recognized that transcription pathways have a central role during the development of cardiovascular diseases, the crosstalk between different signalling pathways in determining the response of cells to stimuli and ultimately controlling cytokine networks is unclear. Future studies should gain a more detailed insight into the various mechanisms that activate the different signalling pathways.

Cells and Cellular Interactions

Fatty streak formation and antigen presentation One of the most important, cytokine-regulated endothelial functions is the capacity of these cells to activate immunocompetent T cells by presenting foreign antigens. Endothelial cells cannot present antigens under baseline conditions but stimulation with the immune-regulating cytokine, IFN-γ, renders the endothelium capable of antigen presentation. This activity was discovered by Hirschberg, Thorsby and their colleagues, who showed that cultures of umbilical vein endothelial cells can activate allogeneic T lymphocytes in mixed cultures [83]. Pober et al. unveiled the cytokine-dependent mechanism that endows antigen-presenting capacity to the endothelium. IFN-γ induces transcriptional expression of MHC class II molecules, which can bind fragments of internalized peptide antigens in an endosomal compartment. The MHC-oligopeptide fragments are transported to the cell surface, where they can be recognized by antigen-specific CD4+ T lymphocytes [84-87]. Although IFN-γ alone is required for induction of MHC class II expression by endothelial cells, several other cytokines modulate the IFN-γ induced MHC expression, including TNF-α , IL-1, and IL-3 [84, 88].
Antigen presentation to CD4+ T cells depends not only on MHC expression but also on the ability of the antigen-presenting cell to internalize and process antigen, secrete IL-1, and express costimulatory adhesion molecules such as ICAM-1, LFA-3, and B7. The IFN-γ activated endothelial cell must therefore also be able to carry out these functions [84] . Similar and in part identical molecular interactions operate in endothelial antigen presentation via the synthetic route to HLA class I-restricted CD8+ T lymphocytes [89].
In vivo, endothelial cells of the microvasculature are often seen to express MHC class II molecules [84] and such expression can also be observed in large vessel endothelium under pathological conditions such as chronic rejection of organ transplants [90, 91]. Due to its great surface area, the endothelium should have a huge potential for antigen presentation and probably represents an important amplification loop for immune activation.
An interesting functional difference exists between endothelial cells, which respond to IFN-γ by MHC class II expression and antigen-presenting capacity, on the one hand, and fibroblasts and smooth muscle cells, which also respond to IFN-γ by MHC class II expression but which cannot *de novo* activate resting T cells. This difference could be due to differences in the capacity to express costimulatory molecules and/or antigen processing capacity [92, 93].

Cytokines in the transition into atherosclerosis Cell culture studies have shown that proinflammatory and immune-regulatory cytokines modify proliferative responses in vascular cells. IL-1 has a weak, growth-promoting effect on smooth muscle cells by upregulating PDGF receptors and increasing autocrine PDGF production [94]. This is, however, counteracted by its induction of the growth-inhibitory prostaglandin, PGE1 [94]. *In vivo*, it is likely that cellular growth and differentiation is regulated in a complex fashion by integration of signals derived from cytokines, growth factors, and autocoids [25, 95, 96].
Interferons are potent growth inhibitors for cultured smooth muscle cells [93, 97-100]. This is due to a direct inhibitory effect on growth factor-induced progression through the first part of the G1 phase of the cell cycle [98, 101]. IFN-γ also inhibits α-actin and collagen production by smooth muscle cells [97, 102], which could contribute to its drastic effects on arterial scar formation (see below). The inhibitory effect of IFN-γ on smooth muscle cell replication is, however, dependent on the simultaneous presence of growth factors in the extracellular milieu. Thus, IFN-γ may upregulate PDGF receptors, leading to a paradoxically increased growth factor sensitivity in a serum-free environment. The divergent effects of IFN-γ in growth factor-poor vs. growth factor-rich cell culture environments might reflect a differential regulation of vascular cell proliferation by IFN-γ producing T and NK cells depending on the local availability of growth factors in different stages of inflammation *in vivo*.
Animal experiments using the rat carotid artery injury model have revealed that IFN-γ is an *in vivo* modulator of smooth muscle cell proliferation and tissue accumulation in the arterial intima. Injection of recombinant IFN-γ inhibits smooth muscle cell proliferation and reduces the size of intimal hyperplastic lesions [103, 104]. Administration of IFN-γ during the first week after injury causes persistent growth inhibition and reduces lesion size even 10 weeks later [103]. This suggests that interferon-mediated growth inhibition during the early phase of the response may determine the final outcome of the lesion.
Removal of IFN-γ producing T lymphocytes using cytolytic antibodies, in contrast, increases smooth muscle cell proliferation and lesion formation in the injured artery [104]. Similarly, restenotic lesions become significantly larger in T cell deficient, homozygous rnu/rnu rats compared to T cell competent, heterozygous rnu/+ littermates [104]. Together, the results from genetically T cell defective, T cell depleted, and IFN-γ treated rats suggest

that the Th1 response inhibits vascular and other connective tissue repair processes.

It appears likely that growth factors govern the formation of the fibrous cap but the precise molecular mechanisms are not fully known [2, 95]. It is, however, clear that plaque smooth muscle cells express receptors for PDGF and FGF and that plaque macrophages and endothelial cells produce mRNA for PDGF [105]. Since endothelial dysfunction and damage is often observed at this stage, it is possible that microthrombi formed on denuded plaque surfaces release PDGF that stimulates smooth muscle cell immigration and the formation of the cap.

Apoptosis of smooth muscle cells has recently been demonstrated in human atherosclerotic plaques, indicating that death of vascular smooth muscle cells also influences the final composition and tensile strength of the plaque [106-108].

Among the cytokines existing in atherosclerotic lesions, TNF induces apoptosis by a mechanism that is at least partly clarified. This process is dependent on the binding of TNF to TNF receptor-1 which is associated with a "death domain" in the cytoplasmic region [109, 110]. Other cytokines including IL-1, IL-2 and IFN-γ can induce apoptosis directly as well as indirectly through induction of TNF from target cells, including smooth muscle cells. TNF and some other cytokines are also able to induce nitric oxide production, which depresses smooth muscle cell function and can induce apoptosis [111-113]. As growth hormone is able to inhibit the production of proinflammatory cytokines in many cell types, it may also play an important role in the regulation of apoptosis induced by these cytokines. Whether a specific cytokine inhibits or suppresses apoptosis depends on their effects on cell death regulatory genes such as bcl-2 and iap family members, Fas receptor, and others. Finally, the intracellular pathways of cytokine receptor-mediated control of apoptosis have begun to be unravelled, implicating specific intracellular receptor domains and protein kinases in the regulation of apoptosis [114-116].

Smooth muscle cells produce the extracellular matrix of the vessel wall and therefore have a high capacity to synthesize structural proteins such as collagens, elastin, basement membrane components, and core proteins of proteoglycans. This is reflected in the ultrastructure, which is dominated by endoplasmic profiles together with contractile filaments [117]. In fact, the proportion between these two components can be used to determine the phenotypic state of the smooth muscle cell [118]. Thus, contractile filaments dominate in contractile smooth muscle cells of the media, which regulate vascular tone, while endoplasmic reticular structures are abundant in "synthetic" smooth muscle cells found in the intima and in cell culture systems.

Agents such as PDGF, which induce smooth muscle cell proliferation, also affect differentiation and matrix formation [119, 120]. Vice versa, agents that degrade the extracellular matrix modulate smooth muscle cell phenotype. This implies that these phenomena are linked and that smooth muscle cell growth and differentiation could be regulated by cell-matrix interactions. Studies on cytokine effects on smooth muscle cells support this notion.

The extracellular matrix of the vessel wall is controlled by inflammatory cytokines. Interferon-γ is a potent inhibitor of collagen synthesis [102] and also inhibits production of α-actin, the major component of contractile filaments, and DNA synthesis in smooth muscle cells [97]. IL-1 and TNF may exert important control of the extracellular matrix by inducing metalloproteinases that degrade matrix components [121, 122]. Proteolytic degradation of the matrix reduces adhesive interactions between smooth muscle cells and their microenvironment. This, in turn, causes the smooth muscle cells to dedifferentiate and proliferate in response to growth factors [123, 124].

The antiinflammatory cytokine, TGF-β, exerts effects on smooth muscle cells that are opposite to those of the proinflammatory ones. Thus, TGF-β stimulates collagen [125, 126] and α-actin production [127, 128] and induces fibrotic hyperplasia when transfected into the arterial wall [129]. Consequently, antibodies to TGF-β inhibit neointimal hyperplasia after mechanical injury [130, 131]. TGF-β also modulates growth of smooth muscle cells but its effects are complex, dependent on the phenotypic state, and mediated via expression of other growth factors and their receptors [132, 133].

Plaque complications It has been suggested that macrophage activation causes the appearance of fissures, thrombi, and actual plaque rupture by secreting proteases that degrade the extracellular matrix [134-137]. Mast cells, which are also present at sites of plaque rupture [138, 139], can exert similar functions [140]. Interestingly, leukocyte elastase and mast cell chymase activate TGF-β [141]; this may counteract tissue destruction by inducing a fibrotic antiinflammatory response.

Proinflammatory cytokines (TNF-α, IFN-γ) could play a role in this process by virtue of their macrophage-activating and matrix-inhibiting activities [142]. In addition, such cytokines (TNF-α, IL-1, IFN-γ) enhance PAI-1 (plasminogen activator inhibitor-1) and downregulate tPA (tissue plasminogen activator) expression by endothelial cells (see above); this would be expected to tip the balance towards thrombus formation on the arterial surface.

Under normal conditions, the endothelial cells constitute a non-thrombogenic surface due to their production of antithrombotic factors such as prostacyclin [143], heparin-like molecules [144], nitric oxide [145, 146], and an activator of the fibrinolytic system, tissue-type plasminogen activator (t-PA) [147]. However, endothelial cells are also able to produce procoagulant substances, e.g. von Willebrand factor [148], tissue factor [149] and inhibitors of the fibrinolytic system, i.e. plasminogen activator inhibitor-1 (PAI-1) [150] and plasminogen activator inhibitor-2 (PAI-2) [151].

TNF-a and IL-1 have been shown to increase the expression of PAI-1 [152-154]. The effect of TNF-α on PAI-1

expression has been confirmed *in vivo* in healthy volunteers [155,156]. TNF-α also increases the expression of u-PA, urokinase plasminogen activator, [157, 158] and PAI-2 in cultured endothelial cells [159]. These effects are modulated by IFN-γ: the TNF-α-induced expression of u-PA and PAI-1 is antagonized by IFN-γ [160-162], while the TNF-α-induced expression of PAI-2 is synergistically increased by IFN-γ [160]. The net effect of TNF-α and IFN-γ is antifibrinolytic in cultured endothelial cells due to increased expression of PAI-1 and PAI-2. Increased expression of PAI-1, t-PA and u-PA has been demonstrated in smooth muscle cells and macrophages of atherosclerotic arteries. The expression of PAI-1 exceeds that of the plasminogen activators, suggesting that fibrinolysis is inhibited [163-167].

Cytokine-induced expression of plasminogen activators and their inhibitors may have a role in the development of the plaque as well as in the induction of thrombotic complications. t-PA and u-PA could induce degradation of the extracellular matrix by activating matrix metalloproteinases (MMP). These enzymes degrade extracellular matrix and can be activated by plasmin. Their activation is inhibited by plasmin inhibitors, tissue inhibitors of metalloproteinases, and PA inhibitors [168-170] . PAI-1 may limit MMP activation and thus protect a developing plaque from uncontrolled matrix degradation. In advanced atherosclerosis, increased expression of MMPs and matrix degrading activity has been detected in vulnerable regions of the plaque [136, 171] and it is possible that cytokine-induced MMPs may promote destabilization and rupture of the plaque.

Studies in mice with targeted gene inactivation of t-PA, u-PA, PAI-1, the urokinase receptor (u-PAR), and plasminogen revealed that vascular injury-induced neointima formation is reduced in mice lacking u-PA-mediated plasmin proteolysis, unaltered in t-PA- or u-PAR-deficient mice, and that PAI-1 plays an inhibitory role in vascular wound healing and arterial neointima formation after injury [172, 173]. The plasminogen system could therefore exert several effects in advanced atherosclerotic lesions. By inhibiting MMP activation, it may protect the plaque against rupture. However, if the plaque is already destabilized, PAI-1 may prevent thrombosis from occurring on the plaque surface. The role of proinflammatory cytokines in these processes is intriguing and deserves further clarification.

Thrombosis on atherosclerotic plaques is thought to be the cause of myocardial infarction and stroke [174]. Tissue factor (TF) is the cellular receptor for coagulation factor VIIa and is generally viewed as the primary physiological initiator of blood coagulation. In atherosclerotic plaques, TF has been found in smooth muscle cells, macrophages, endothelial cells, and in the lipid-rich necrotic core [175-177], implicating a role for TF in mediating thrombosis associated with atherosclerosis. Induction of the TF gene in human monocytic cells and endothelial cells exposed to bacterial lipopolysaccharide or cytokines is mediated by a distal enhancer (-227 to -172 bp) containing two AP-1 sites and a κB site. TNF-α induces a procoagulative state of endothelial cells by stimulating their production of tissue factor. Additionally, procoagulant activity of atherosclerotic lesions is also induced by a T cell-derived cytokine, macrophage procoagulant-inducing factor (MTIF).

Regulation of vascular tone Proinflammatory cytokines (TNF, IL-1, IFN-γ) modulate smooth muscle cell contractility on several levels of this regulatory system. The most long-lasting effect on smooth muscle cell contractility is caused by direct interference with the production of contractile filaments. For example, IFN-γ down-regulates expression of the α-SM-actin gene [97]. A more rapid effect on vascular contractility is accomplished by cytokine regulation of NO production (Reviews: [178, 179]). As mentioned, NO is normally released by endothelial cells. It diffuses over to smooth muscle cells, where it nitrosylates the heme group of guanylyl cyclase, resulting in an activation of the enzyme to produce cGMP. The elevated cGMP level activates the myosin kinase cascade, resulting in dissociation of myosin from actin and a relaxation of the smooth muscle cells. The artery is normally under modest NO-dependent vasodilation, and systemic administration of NO synthase inhibitors results in increased vascular tone and elevated blood pressure [178].

Endothelial NO synthesis is accomplished by the enzyme, endothelial NO synthase (eNOS or NOS-3). It is constitutively expressed as a protein but requires activation by Ca^{++}/calmodulin [180]. This occurs after stimulation of the endothelial cells by bradykinin, acetylcholin and other stimuli. Since NO synthase inhibitors increase vascular tone under baseline conditions, the normal vessel wall is probably in a state of NO-dependent, partial relaxation [178, 181].

Smooth muscle cells do not normally produce NO but can be stimulated to do so by proinflammatory cytokines. Both TNF-α, IL-1, lipopolysaccharide (LPS), and IFN-γ induce the production of large amounts of NO in cultured smooth muscle cells [182-185]. The cytokine-inducible NO synthase isoform, iNOS or NOS-2, binds calmodulin with high affinity immediately after translation and is therefore independent of Ca^{++} for its activity [186, 187]. This results in a high capacity for NO synthesis, which lasts until the enzyme protein is degraded [187]. The iNOS expressed by smooth muscle cells appears to be identical to the one expressed by cytokine-activated macrophages and is highly conserved between species [188].

The dichotomy of inflammatory signalling is also reflected in the regulation of iNOS. While transcription of this gene is induced by IL-1, TNF-α and IFN-γ, it is inhibited by IL-4. Furthermore, TGF-β downregulates iNOS by suppressing transcription as well as reducing mRNA and protein stability [189, 190].

NO produced by cytokine-stimulated smooth muscle cells acts as an auto- and paracrine mediator to control contractility and metabolism of the cells. Moderate cytokine stimulation induces sufficient NO to nitrosylate

smooth muscle cell heme proteins, including guanylyl cyclase [191]. At higher levels of cytokine stimulation, the output of NO is sufficient to react with iron atoms in the iron-thiol groups of many intracellular enzymes, which therefore lose their activity [191]. This causes inhibition of mitochondrial respiration, ATP deficiency and a switch to anaerobic glycolysis in the cell [184, 191]. The combined effects of guanylyl cyclase activation and reduced energy levels are likely to explain the vasodilation of inflammation [184].

In vivo, the iNOS gene is activated in smooth muscle cells during the response to vascular injury. De-endothelializing balloon catheter injury to the rat carotid artery causes rapid expression of iNOS in the underlying smooth muscle cell layer [192]. iNOS expression is maintained in the smooth muscle cells for more than a week, probably due to cytokine stimulation. Enzyme expression is, however, induced so rapidly after deendothelialization (hours) that it is unlikely to be caused by *de novo* production of IFN-γ, TNF-α and/or IL-1 from infiltrating leukocytes. Instead, there may be release of extracellularly deposited cytokines or activation of transcription through cytokine-independent mechanisms. iNOS activity is likely to be important by dilating vessels during inflammation, modulating platelet deposition and thrombus formation on the injured vessel, and controlling smooth muscle cell proliferation during the response to injury [145, 192, 193].

Clinical Relevance see under the different subsections

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DAF

Definition *Decay-accelerating factor*

See: →Complement system (interaction of vascular cells with)