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Crosstalk Between Activated Platelets and the Complement System

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Abstract

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Several studies have shown that complement and thrombotic events co-exist. Platelets have been suspected to act as the bridge between the two cascade systems.

To study the platelet-induced complement activation we developed a system in which platelets were activated by thrombin receptor activating peptide (TRAP) in platelet rich plasma (PRP) or whole blood anti-coagulated using the specific thrombin inhibitor, lepirudin.

TRAP-activated platelets induced a fluid-phase complement activation measured as generation of C3a and sC5b-9, triggered by released chondroitin sulphate-A (CS-A) which interacted with C1q and activated the complement system through the classical pathway.

Complement components C1q, C3, C4 and C9 were also shown to bind to TRAP-activated platelets but this binding did not seem to be due to a complement activation since blocking of complement activation at the C1q or C3 levels did not affect the binding of the complement proteins. The C3 which bound to activated platelets consisted of C3(H_2O), indicating that bound C3 was not proteolytically activated. Binding of C1q was partially dependent on CS-A exposure on activated platelets. The abolished complement activation on the surface of activated platelets was suggested to be dependent on the involvement of several complement inhibitors. We confirmed the binding of C1INH and factor H to activated platelets. To this list we have added another potent complement inhibitor, C4BP. The binding of factor H and C4BP was shown to be dependent on exposure of CS-A on activated platelets.

The physiological relevance of these reactions was reflected in an elevated expression of CD11b on leukocytes, and increased generation of platelet-leukocyte complexes. The platelets were involved in these events by at least two different mechanisms; generation of C5a which activated leukocytes and binding of C3(H_2O)/iC3(H_2O), a ligand to the intergrin CD11b/CD18 on their surface.

These mechanisms add further to the understanding of how platelets interact with the complement system and will help us to understand the role of the complement system in cardiovascular disease and thrombotic conditions.

Keywords: platelets, activated platelets, TRAP, chondroitin sulfate, C1q, factor H, C4BP, C3, Compstatin, complement, platelet-leukocyte complexes, platelet microparticles

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

ٱقْرَأْ بِٱسْمِ رَبِّكَ ٱلَّذِى خَلَقَ ۝١ خَلَقَ ٱلْإِنسَـٰنَ مِنْ عَلَقٍ ۝٢ ٱقْرَأْ وَرَبُّكَ
ٱلْأَكْرَمُ ۝٣ ٱلَّذِى عَلَّمَ بِٱلْقَلَمِ ۝٤ عَلَّمَ ٱلْإِنسَـٰنَ مَا لَمْ يَعْلَمْ ۝٥

صَدَقَ اللّٰهُ الْعَظِيْمِ

To the memory of my Father and Mother: I miss you both.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Hamad OA., Ekdahl KN., Nilsson PH., Andersson J., Magotti P., Lambris JD., Nilsson B. (2008) Complement activation triggered by chondroitin sulfate released by thrombin receptor-activated platelets. *J Thromb Haemost*, (6):1413-1421.

II Hamad OA., Nilsson PH., Wouters D., Lambris JD., Ekdahl KN., Nilsson B. (2010) Complement component C3 binds to activated normal platelets without preceding proteolytic activation and promotes binding to complement receptor 1. *Journal of Immunology* 184(5):2686-2692

III Hamad OA*., Nilsson PH*., Lasaosa M., Ricklin, D., Lambris JD. Nilsson B+., Ekdahl KN+. Contribution of chondroitin sulfate A to the binding of complement proteins to activated platelets. *submitted*

IV Hamad, OA., Ekdahl, KN., Nilsson, B. Non-proteolytically activated C3 promotes binding of activated platelets and platelet-derived microparticles to leukocytes via CD11b/CD18. *manuscript*

*,+ The authors contributed equally to this work.

Contents

Abbreviations

ADP	Adenosine diphosphate
aHUS	Atypical hemolytic uremic syndrome
AP	Alternative pathway
APC	Activated protein C
C1INH	C1 inhibitor
C3aR	C3a receptor
C4BP	C4b-binding protein
C5aR	C5a receptor
C5aRA	C5a receptor antagonist
CAM	Cell adhesion molecules
CD40L	CD40 ligand
COX-1	Cyclooxygenase-1
CP	Classical pathway
CR	Complement receptor
CR1	Complement receptor type 1
CRIg	Complement receptor of the immunoglobulin superfamily
CRP	C-reactive protein
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
D-GalNAc	N-acetylgalactosamine
D-GlcA	D-glucuronic acid
D-GlcNAc	N-acetyl-D-glucosamine
DAF	Decay acceleration factor
DS	Dermatan sulfate
ECM	Extracellular matrix
GAG	Glycosaminoglycan
Gal	Galactose
GP	Glycoprotein
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
Ig	Immunoglobulin
HA	Hyaluronic acid
HAE	Hereditary angioedema
HAGG	Heat aggregated gamma globulin
HRGP	Histidine-rich glycoprotein
HS	Heparan sulfate

IBMIR	Instant blood-mediated inflammatory reaction
IC	Immune complex
KS	Keratan sulfate
LMWDS	Low molecular weight dextran sulfate
LP	Lectin pathway
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP	MBL-associated serine proteases
MBL	Mannan-binding lectin
MCP	Membrane cofactor protein
PAF	Platelet activating factor
PAR-1, 4	Protease activated receptors 1 and 4
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PG	Proteoglycan
PMN	Polymorphonuclear
PMP	Platelet microparticle
PMSF	Phenylmethylsulfonyl fluoride
PNH	Paroxysmal nocturnal hemoglobinuria
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RT	Room temperature
sC5b-7	Soluble C5b-7
sC5b-9	Soluble C5b-9
SCR	Short consensus repeats
sCR1	Soluble CR1
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
TAT	Thrombin anti-thrombin complexes
TCC	Terminal complement complexes
TF	Tissue factor
TNF-α	Tumor necrosis factor α
TRAP	Thrombin receptor-activating peptide
TSP	Thrombospondin
TxA2	Thromboxane A2
vWF	von Willebrand factor
Xyl	Xylose

INTRODUCTION

INTRODUCTION

Introduction

The Complement System

Overview

The complement system is a part of the innate immune system and is one of the main effector mechanisms of antibody-mediated immunity. As many as 30 soluble and membrane-bound glycoproteins are involved in the complement system [1-3], whose central protein is complement component 3 (C3).

Complement activation is a target surface-oriented process that centers around the activation of C3. Activation of C3 can be achieved by three different pathways: the classical pathway (CP), which responds to specific immunoglobulins (Igs); the alternative pathway (AP), which is triggered by interaction with bacteria, viruses, or immune complexes (ICs); and the mannan-binding lectin pathway (LP), which responds to the presence of certain carbohydrates on microbial surfaces (Fig 1). Once activated, the complement system mediates at least three traditionally described major functions: opsonization of pathogens, and thus enhancement of phagocytosis; attraction of phagocytes (chemotaxis) to the site of inflammation; and lysis of foreign pathogens by damaging their cell membranes. Other more recently described functions of the complement system are elimination of apoptotic cell debris, enhancement of humoral immunity [4], modification of T-cell responses [5], and regulation of tolerance to self-antigens [6].

Complement component 3

C3 is the central molecule in the complement cascade and the most abundant complement protein in blood. C3 is present in plasma at a concentration of about 1 g/L [7], but since it is an acute phase protein, its level may be rapidly elevated during inflammation and infections [8].

C3 is a member of the macroglobulin superfamily that also includes C4 and C5. C3 consists of two chains, an α-chain (115 kDa) and a β-chain (75 kDa), that are linked through a disulfide bridge and noncovalent forces [9].

Upon activation of C3, C3a (9 kDa) is released from the α-chain as a result of proteolysis by the C3 convertases, which are activated by any of the three activation pathways [10, 11]. The remaining portion of the C3 molecule (C3b) is conformationally changed and becomes bound to the cell sur-

face [12, 13]. The ability of C3 to bind to cell surfaces is due to a thioester bond, which is sensitive to nucleophilic attack. In native C3, the thioester bond is protected by a hydrophobic pocket. When C3 is cleaved by any of the C3 convertases, the bond is exposed, and nucleophilic groups on cell surfaces attack the thiol ester, resulting in covalent binding of C3b to the surface [14].

The thiolester bond in C3 can also be spontaneously hydrolyzed. This autohydrolyzation creates a new form of C3: C3(H_2O). C3(H_2O) has C3b-like properties but still contains the C3a moiety. Generation of this form of C3 occurs naturally in the human plasma in small amounts due to hydrolysis in a process called "tick-over" and it is believed to be an initiator of the AP [15]. Surface-bound C3b further contributes to activation of the complement system by the AP. The C3a that is released is a strong anaphylatoxin and enhances inflammation by recruiting polymorphonuclear leukocytes (PMNs) and monocytes [16].

Interaction of C3b with factor I and H leads to further conformational changes and proteolytic cleavage, generating iC3b, which is unable to participate in complement activation [17]. Further interaction with factor I and H results in the formation of two different fragments, soluble C3c and surface-bound C3d,g. Thus, the cleavage of C3b by factor I and H represents a regulatory mechanism for the complement activation.

C1q

C1q is a 420-kDa glycoprotein with a hexameric structure that consists of six identical subunits arranged to form a central core and symmetrically projecting arms [18]. The molecule is composed of 18 peptide chains in 3 subunits (A-, B-, and C-chains) forming six globular heads connected by a triple helix collagen-like stalk [19]. The triple helices of the collagen-like region begin close to the N-terminus of each polypeptide chain and continue to about residue 89. The remaining ~131 residues of each chain fold to form the globular head domains [20]. The protein structure of the C1q has been described as a "bundle of tulips" and has a multivalent binding capacity for the complement fixation sites of Igs [21].

C1q is the target recognition molecule of the CP of complement. A large portion of the C1q is free [22] and the rest circulates as part of the C1 complex with two each of the C1r and C1s subunit proteins in a calcium-dependent association [23]. The collagen-like regions of C1q interact with C1s and C1r (the proteases of the CP) and form the C1 complex. Binding of two or more globular heads of the C1q molecule to the Fc region of IgG or IgM leads to enzymatic activation of C1r and C1s [24]. To avoid misdirected activation of the complement system, IgG and IgM in their monomeric forms have only a weak affinity for C1q in the circulation. The affinity of monomeric IgG for C1q has been estimated as between $4x10^3$ and $5x10^4$ M

but increases up to 1000-fold when IgG is present in the aggregated form. This high avidity is believed to reflect the molecular structure of C1q [25].

In addition to Igs, C1q binds directly to a variety of substances, including a number of different proteins, polyanions, cell structures, DNA, and many different cell types, including platelets. A group of antibody-independent complement activators, including serum amyloid P (SAP), C-reactive protein (CRP) [26], DNA [27], and β-amyloid fibers [28], and the polyanion chondroitin sulfate (CS) have all been shown to interact with C1q. These activators share a common property: they all have repeating negative charges and have been demonstrated to bind in close vicinity to or directly to the globular heads of C1q.

C1q circulates in plasma at a concentration of 80-180 μg/mL [29]. C1q is believed to contribute to the pathogenesis of several pathological conditions, including systemic lupus erythematosus (SLE) [30], hypocomplementic urticarial vasculitis syndrome (HUVS) [31], and hypogammaglobulinemia [32]. In SLE, for instance, low levels of C1q appear to result from an increased catabolism. C1q also takes part in the clearance of apoptotic bodies, with hereditary C1q deficiency causing SLE as a result of an impaired clearance of apoptotic cells [33].

The classical pathway (CP)

The CP was the first complement pathway to be described. This pathway is initiated when IgG and IgM antibodies bind to surface antigens [34] (Fig 2). Binding of C1q to the Fc portion of Igs activates the serine proteases C1r and C1s of the C1 complex [35]. C1s in the activated C1 complex cleaves C4 into C4b, which binds to a surface close to the site of activation, and the weak anaphylatoxin C4a. C2 binds to C4b, and the activated C1 complex cleaves C2 into C2a and forms a complex with C4b, while C2b is released. This C4bC2a complex is the C3 convertase of the CP [36]. The CP can also be activated by agents such as C-reactive protein (CRP), by binding to C1q [37, 38].

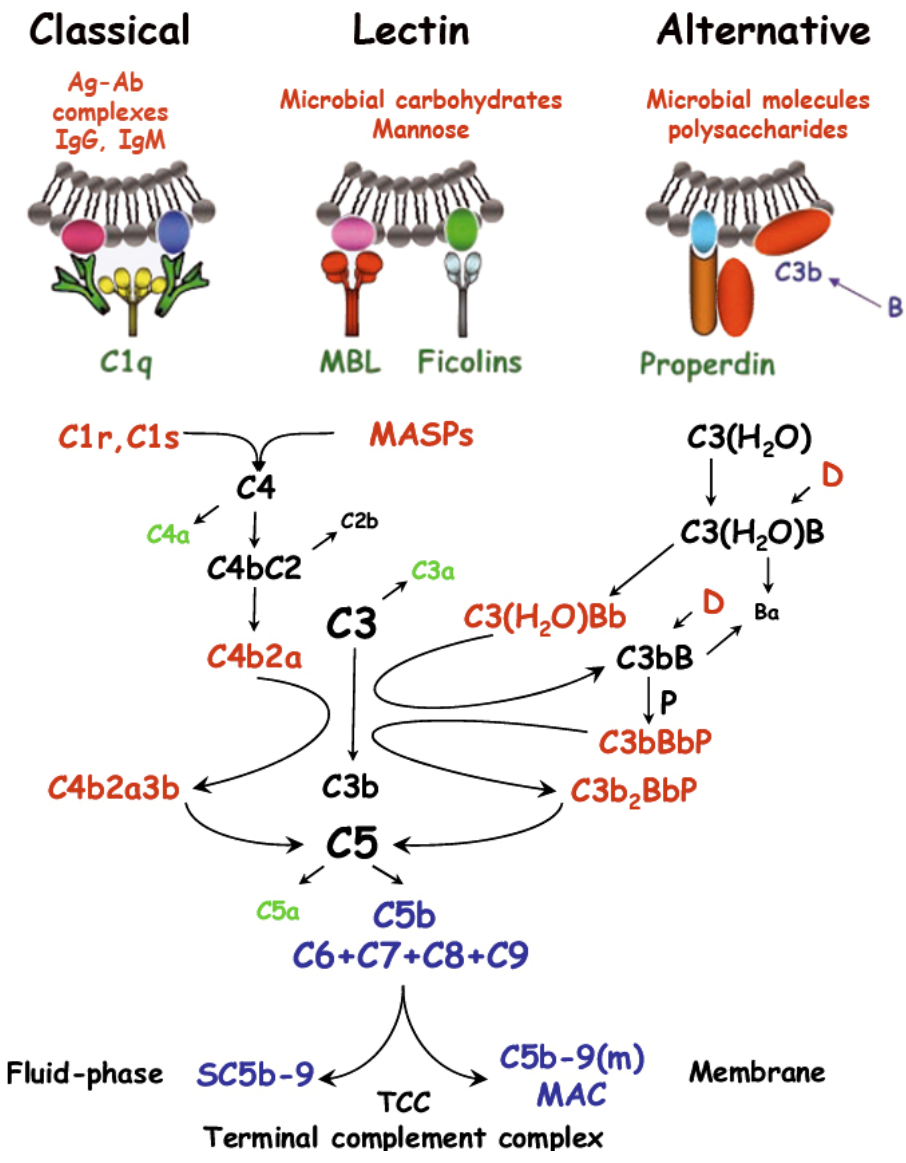

Figure 1: Overview of the complement system. The complement system can be activated by three different pathways: the classical, the lectin, and the alternative pathway. These pathways converge in the common terminal pathway.

The lectin pathway (LP)

The LP is the most recently discovered pathway but phylogenetically the oldest, and is thus far the least well characterized. LP is activated by certain carbohydrates on the surfaces of microorganisms. Activation occurs through recognition of these carbohydrates by mannan-binding lectin (MBL) or ficolins [39] (Fig 2). Upon binding of MBL to the carbohydrates, the MBL-associated serine proteases (MASP 1-3), homologous to C1r and C1s in the C1 complex [40], are activated [41]. Once activated, MASP-2 cleaves both C4 and C2, generating the CP C3 convertase C4bC2a [41]. The downstream portions of the LP and CP are identical; the CP C5 convertase is formed when a C3b molecule is bound to the C3 convertase.

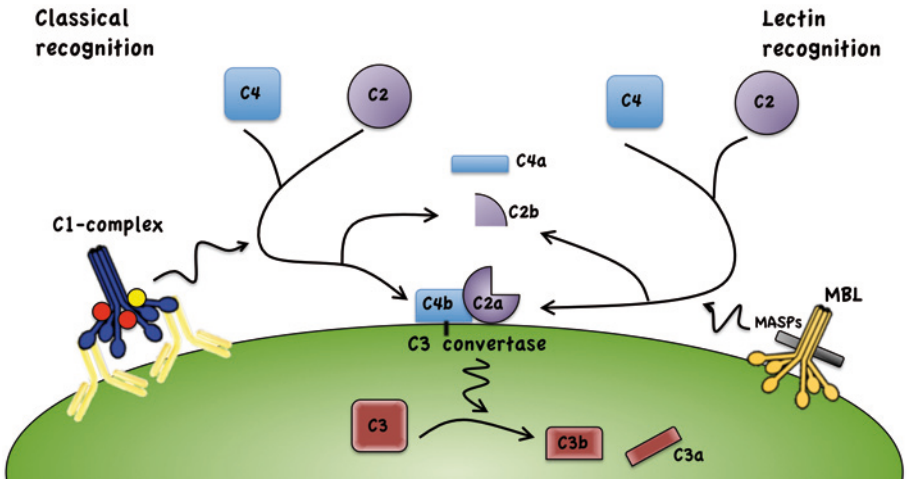

Figure 2: Schematic illustration of the activation of the complement system via the classical and the lectin pathways and the formation of the classical pathway C3-convertase, C4bC2a.

The alternative pathway (AP)

The AP was discovered by Pillemer and colleagues [42] and was believed to be an alternative way to activate the complement system. The AP was initially called the properdin system or the antibody-independent system [43]. It was found to be an important part of the innate immune system, since it could differentiate between self and non-self [44].

The AP is triggered by C3b, generated by the CP classical convertase, or by a soluble convertase containing C3(H_2O) generated by the tick-over process [45-47] (Fig 3). Factor B binds to C3b or C3(H_2O) and changes its conformation. Factor B is then cleaved by factor D to soluble Ba and Bb; Bb remains bound to C3b or C3(H_2O) and forms the C3bBb complex, the AP C3 convertase, which cleaves C3 into C3a and C3b and thus serves as an amplification loop for the other two activation pathways [48, 49].

The C3bBb complex is unstable and has a short half-life (approximately 90 μs) but is stabilized by properdin, thereby enhancing the amplification properties of the AP [50]. Recently, it has been demonstrated that properdin also acts as a recognition molecule that can directly initiate AP activation [51, 52]. Thus, the AP should be regarded as a recognition pathway acting through properdin, corresponding to the CP acting through C1q and the LP through MBL and ficolins [53]. The C3bBb complex can bind an additional C3b and form the C3bBbC3b complex, which is the AP C5 convertase [54].

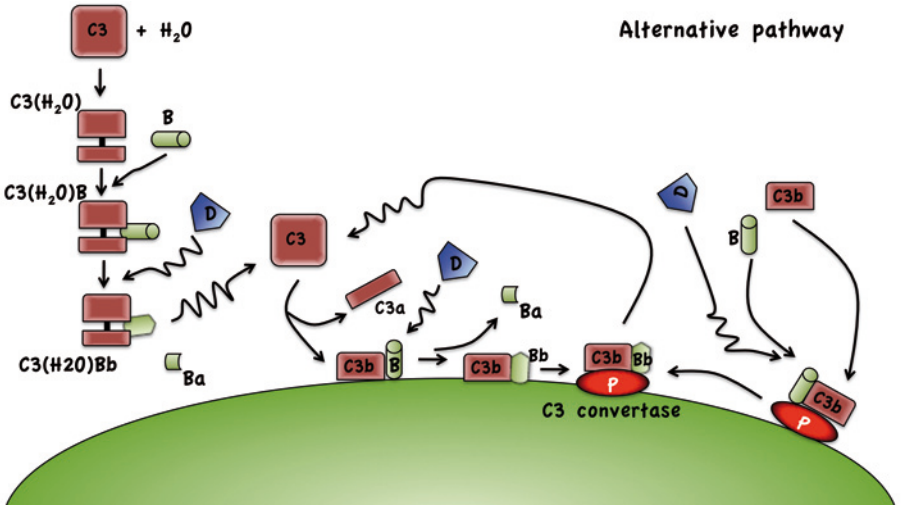

Figure 3: Schematic illustration of the activation of the complement system via the alternative pathway and the formation of the alternative pathway C3-convertase, C3bBb(P).

The common terminal pathway

The C5 convertases, generated by any of these three pathways, bind and cleave C5 into C5a and C5b. C5a is released into the fluid phase and exhibits stronger anaphylactic properties than C3a. C5b remains bound to its convertase and binds C6 and C7, forming a metastable hydrophobic complex. The complex is then released from the convertase and inserted into the lipidic cell membrane [55]. C8 binds to the C5b-7 complex, followed by the binding of multiple copies of the C9 molecule to form a pore-like structure (the membrane attack complex, MAC) (Fig 4) that is able to mediate cell lysis [56]. Not all C5b-9 complexes are inserted into the membrane of the microorganism or the activating surface. Some are released and bind to vitronectin (S-protein), and circulate in the blood in the form of soluble C5b-9 (sC5b-9), which serves as an indicator of complement activation [57]. Soluble C5b-9, also called the terminal complement complex (TCC), contributes to the inflammation process by activating platelets and leukocytes [58].

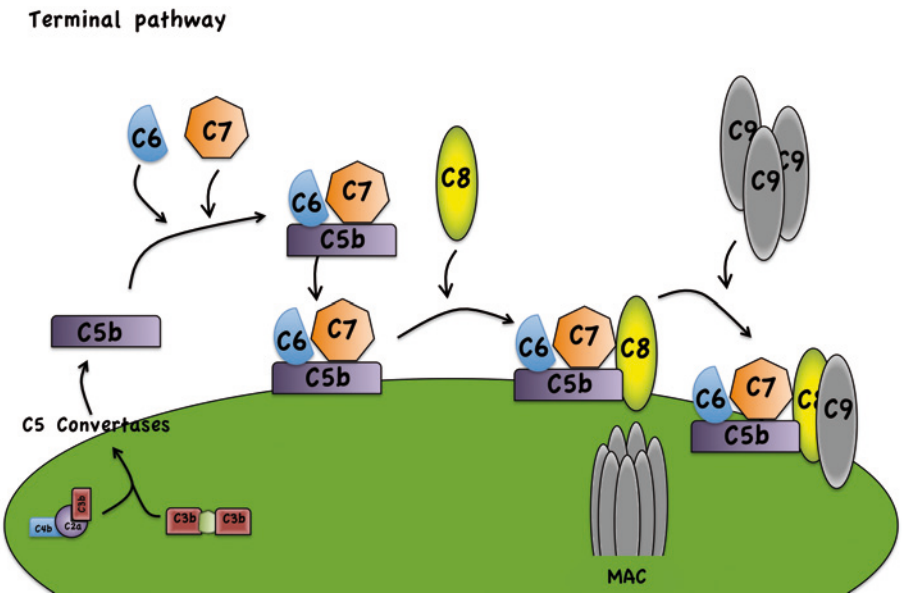

Figure 4: Formation of the membrane attack complex (MAC, C5b-9). The C5-convertases cleave C5 to C5a and C5b. C5a is released and is a potent anaphylatoxin, while C5b binds C6 and C7 and the formed complex binds to the surface. C8 binds to the attached C5b-7 complex and incorporates into the membrane. Several molecules of C9 bind and start to polymerize into a pore that causes lysis of the cell.

Complement regulation and regulators

Deficiencies in complement regulation can cause tissue damage as a result of uncontrolled inflammation and can contribute to the pathology of many diseases. Therefore, the complement system is tightly regulated by various types of control mechanisms at numerous points in the cascade [2, 3]. These mechanisms can be divided into five main subgroups: (1) protease inhibition; (2) decay acceleration, i.e., dissociation of the convertase complexes; (3,4) cofactor activity to factor I proteolytic cleavage of activated C3 and C4; and (5) MAC inhibition. These mechanisms of inhibition are summarized in Table 1.

Table 1: Summary of mechanism of action of complement regulators.

Protease Inhibition	Decay Acceleration	Cofactor Activity	Proteolytic Cleavage	MAC Inhibition
C1INH	CR1	CR1	Factor I	CD59
	Factor H	Factor H		Vitronectin
	DAF	MCP		Clusterin
	C4BP	C4BP		

The complement regulators are either soluble or membrane-bound proteins (Fig 5). The soluble proteins include C1INH, factor H, C4BP, and factor I, which will all be described in greater detail, as well as vitronectin and clusterin. The membrane-bound complement regulators are CR1, CD59, membrane cofactor protein (MCP), decay accelerating factor (DAF) [2, 59], and CRIg [3, 60, 61]. Membrane-bound regulators are generally found only on host cells, which makes the complement system more specific and able to differentiate between self and non-self tissue. However, many microorganisms deceive the system by expressing regulators on their surfaces [62].

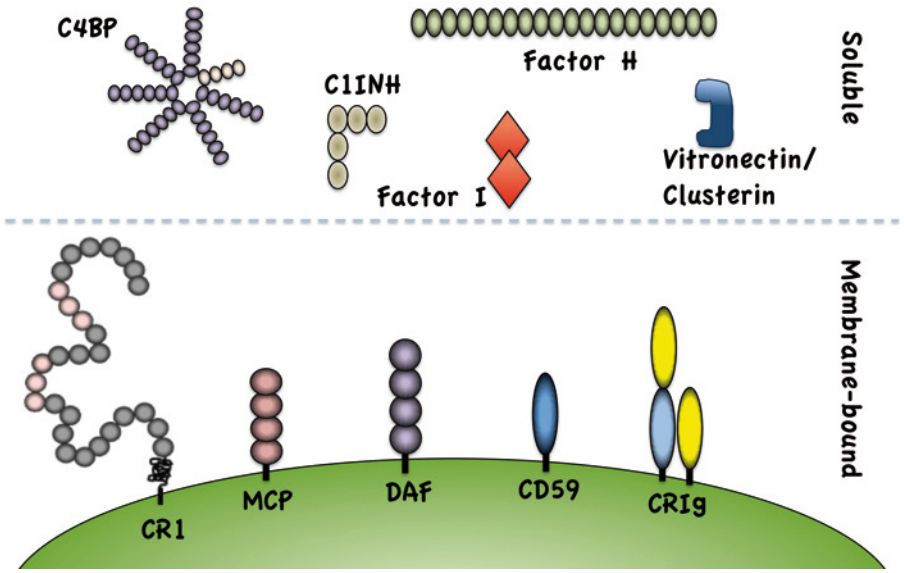

Figure 5: Schematic illustration of the soluble and the membrane-bound complement regulators. C4BP, C1INH, factor I, factor H, vitronectin and clusterin are soluble proteins. The main cell membrane-bound inhibitors are CR1, MCP, DAF, CD59, and CRIg.

C1INH is a highly glycosylated single-chain glycoprotein belonging to the family of serine protease inhibitors known as serpins. C1INH is the only plasma protease inhibitor that regulates the CP of complement [63]. C1INH inactivates the serine proteases C1r and C1s, dissociating them from C1q [64] (Fig 6). It also regulates the LP by binding and inactivating the MBL-associated serine protease MASP-2 [65].

C1INH has also been shown to possess a biological activity independent of the earlier-described protease inhibitor activity: It has been found to inhibit the AP via an incompletely defined mechanism that does not require protease inhibition [66]. The mechanism seems to involve C1INH interacting with C3b and thereby inhibiting factor B binding [67]. C1INH is also the primary inhibitor of both plasma kallikrein and coagulation factor XIIa [68].

Factor I is a serine protease that circulates in plasma in active, rather than proenzymatic form. It is not inhibited by any of the protein protease inhibitors in plasma and has a very restricted substrate range, i.e., C3b and C4b, which are produced only when the complement system is activated. Before C3b or C4b can be cleaved by factor I, they must bind to one of several complement control proteins (cofactors) to form a noncovalent complex (Fig 6). There are two major soluble cofactors: C4b-binding protein (C4BP), which forms a complex with C4b; and factor H, which binds to C3b, thereby regulating the CP and the AP, respectively. In addition, two cell surface-expressed cofactor proteins, complement receptor type 1 (CR1, CD35) and membrane cofactor protein (MCP, CD45), have the same function [69].

Factor H is the second most abundant complement protein in plasma and regulates the complement system in plasma as well as on cell surfaces [70]. Factor H is composed of 20 so-called short consensus repeats (SCR) organized as a string of beads [71]. Factor H is mainly a regulator of the AP and acts through two different mechanisms [2]: It inactivates C3(H_2O) and C3b by acting as a cofactor for factor I in the cleavage of C3b to iC3b. Upon binding to C3b, it competes with factor B for binding to C3b [59] (Fig 6). Thus, factor H has a decay-acceleration activity and can displace Bb from the C3bBb complex, thus abrogating the formation of the AP C3 and C5 convertases. Its binding to C3b seems to be dependent on the properties of the surface to which C3 binds. On host cells, factor H binds to C3b and prevents complement activation. However, it does not bind to foreign surfaces, thereby allowing the binding of factor B and progression of complement activation [72, 73].

The complement-regulatory domain of factor H has been pinpointed to SCRs 1-4, where the major C3b binding and cofactor activity sites are located [70]. The C-terminus of factor H (SCRs 18–20) mediates surface binding and target recognition [74, 75]. This C-terminal region includes binding sites for several ligands, such as C3b, C3d, heparin, cell surface glycosaminoglycans and microbial virulence factors [76]. In the factor H molecule there are at least two heparin binding sites [77], that allow it to bind to negatively charged surfaces and molecules such as glycosaminoglycans and proteoglycans.

In the presence of factor I and a cofactor, C3b and C4b can be cleaved. This proteolysis occurs in the α-chain of the proteins, with the α-chain of C3b being cleaved into two polypeptide chains of 68 kDa and 43 kDa. The inactivated molecule remains bound to the surface and is called iC3b [70]. Factor I (acting with a cofactor molecule) also has an affinity for C3(H_2O) but not for native C3. Factor I and factor H cleave the α-chain of C3(H_2O) in the similar manner as they cleave C3b [78].

C4 binding protein (C4BP) is a large (≈570 KDa) glycoprotein that inhibits the CP convertase C4bC2a. C4BP acts as a decay-accelerating factor by binding C4b and displacing C2a from the C4bC2a complex. C4BP, like factor H, possess cofactor activity for factor I. C4BP and factor I cleave C4 into C4c and C4d [2] (Fig 6). C4BP consists of seven identical α-chains and one β-chain linked together by disulfide bridges. Each α-chain contains seven SCRs, but the β-chain contains only three [79]. The large octopus-like structure of C4BP occupies multiple C4b binding sites, facilitating a strong interaction with C4b, particularly when it is bound to surfaces [59]. C4BP has also a high affinity for anticoagulant vitamin K-dependent protein S [80, 81]. Binding of protein S to C4BP results in a decreased cofactor function of protein S for anticoagulant activated protein C (APC) in the degradation of coagulation factors Va and VIIIa [82]. A more recently described function of the Protein S – C4BP complex is that, this complex enhances the binding of C4BP to macrophages. C4BP bound to macrophages ensure no unwanted inflammatory response around the macrophages [83].

In addition to these fluid-phase complement inhibitors, there are several membrane-associated complement regulators, including MCP (CD46), DAF (CD55), CR1 (CD35), and CD59 [84] (Fig 6).

Furthermore the complement system is also naturally regulated by spontaneous decay of the convertases, and convertase complexes dissociate within minutes. Also, the complement activation is regulated in time and space. The time of activation must be limited to avoid excessive consumption of complement components in one reaction. Less than 5 minutes is required to deposit several million copies of C3b on a target cell and to release an equal quantity of C3a. Restriction of activation in space is also needed, since the reaction must be focused on the target surfaces and not be allowed to spread to the autologous tissue [2].

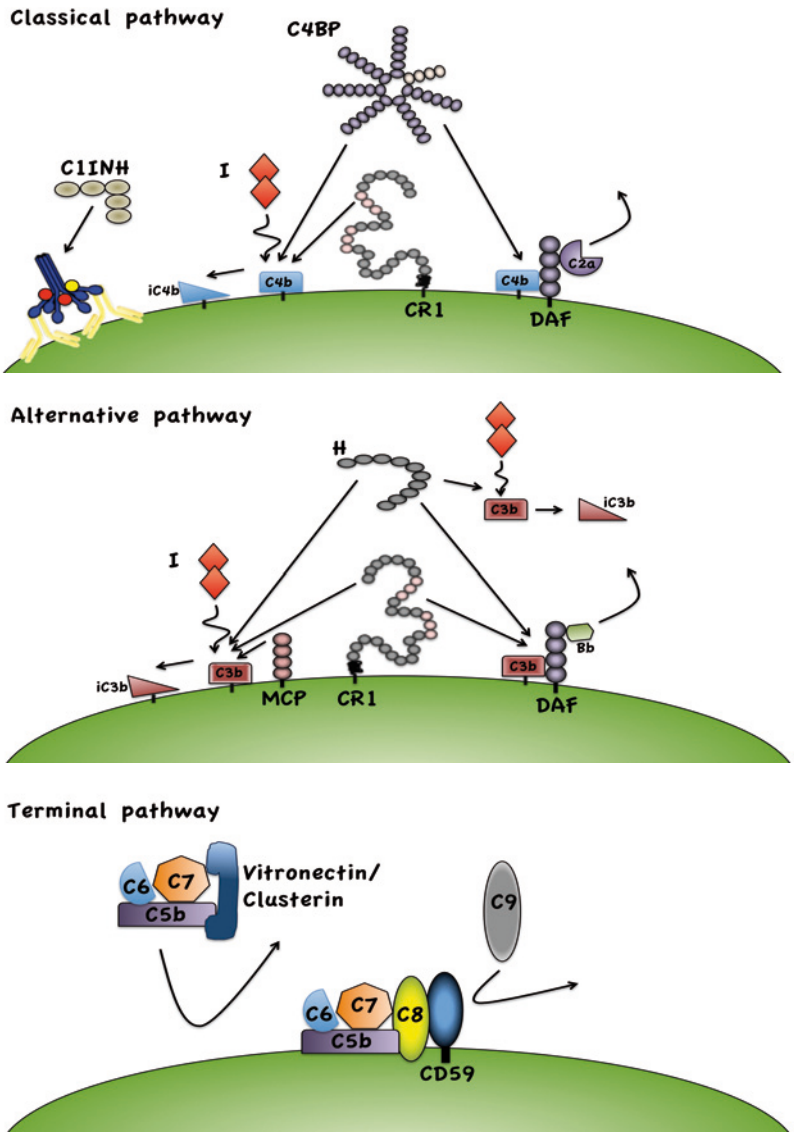

*Figure 6: Schematic illustration of the complement inhibitors and their mechanisms of action. Initiation of **classical pathway** activation (top panel) is controlled by the C1 inhibitor (C1INH). Membrane bound C4b is digested to iC4b by factor I (I) with complement receptor 1 (CR1) or fluid phase C4 binding protein (C4BP) as cofactor. Decay of the classical pathway C3 convertase complexes (C4bC2a) is accelerated by either decay acceleration factor (DAF) or C4BP. Control of the **alternative pathway** (middle panel) is achieved by factor I (I) mediated cleavage of membrane bound C3b to iC3b with membrane cofactor protein (MCP), CR1 or fluid phase factor H (H) as cofactor. Decay of the alternative pathway C3 convertase complexes (C3bBb) is accelerated by either decay acceleration factor (DAF), CR1 or H. Formation and insertion of MAC complexes into the cell membrane by activation of the **terminal pathway** (lower panel) is inhibited by the actions of vitronectin, clusterin and cell bound CD59.*

Complement receptors

One of the most important functions of the complement system is to mediate the clearance of pathogens, ICs, and apoptotic cells. Complement fragments deposited on a particle surface serve as targets for complement receptors present on phagocytic cells. There are several types of receptors for complement molecules that are specific for bound C3 fragments, anaphylatoxins, or C1q (Fig 7).

C1q contributes to phagocytosis and the clearance of apoptotic cells through several different mechanisms: First, by binding and activating the complement system through the CP, leading to the generation of C3b and C4b fragments, which enhance phagocytosis [85, 86]. It is also believed that free C1q (i.e., C1q not complexed with C1r and C1s) binds to receptors and affects the phagycytosis of apoptotic cells and ICs without activating the complement system. The latter mechanism is mediated mainly through the binding of C1q via its collagen-like stalks [87]. There are at present three known receptors for the C1q molecule, which are expressed on a wide range of cells: A 60-kDa cC1q receptor (the collectin receptor) is expressed on almost all blood cells except erythrocytes [88]. A closely related receptor is the 100-kDa cC1q-R, which is expressed on monocytes and neutrophils. Both of these receptors bind to C1q via its collagen-like domain, allowing it to continue to interact with Igs. A third C1q receptor is gC1q-R, which binds C1q via its globular heads [89]. The gC1q-R is expressed on almost all blood cells except erythrocytes [90]. On platelets, it is believed to bind C1q and activate the complement system via the CP [91].

Deficiencies of C1q are associated with defective phagocytosis and a decreased clearance of apoptotic cells and ICs, contributing to the pathophysiology of autoimmune diseases such as SLE [30, 92].

The apoptotic cells, ICs, and pathogens opsonized by C3 or C4 fragments are rapidly eliminated by phagocytosis through a receptor-ligand response [93]. These opsonized elements can be recognized by three gene superfamilies of complement receptors: the SCR modules coding for complement receptor 1 (CR1, CD35) and CR2 (CD21), the β_2 integrin family members CR3 and CR4 (CD11b,c/CD18), and the immunoglobulin superfamily member CRIg [60].

CR1 (CD35), a glycoprotein of 200 to 250 kDa consisting of 30 SCRs, is specific for C3b and iC3b. CR1 is found on a variety of cells, including erythrocytes, neutrophils, monocytes, B cells, and some T cells [94, 95]. On neutrophils and monocytes, activated CR1 enhances the phagocytosis of C3b- and C4b-opsonized particles [60]. On erythrocytes, CR1 captures C3b-coated ICs and transports them to the liver for clearance [96].

CR2 (CD21) is structurally similar to CR1. It is present on follicular dendritic cells and B cells and binds mainly membrane-bound C3d, C3dg, and iC3b. CR2 facilitates B-cell activation and maturation, providing a clear link between the innate and adaptive immune systems [97, 98].

The integrin CD11b/CD18 (also known as Mac-1, CR3, and $\alpha_M\beta_2$) is the predominant β_2 integrin on neutrophils, macrophages, and monocytes and mediates pro-inflammatory functions in these cells [99]. CD11b/CD18 recognizes the complement fragment iC3b, fibrinogen, and ICAM-1 as ligands, among others. CD11b/CD18 has been implicated in many inflammatory and autoimmune diseases, such as ischemia-reperfusion injury (including acute renal failure and atherosclerosis), tissue damage, stroke, neointimal thickening in response to vascular injury, and in the resolution of inflammatory processes [100-103]. CD11b/CD18 plays also an important role in complex formation between platelets and monocytes or neutrophils.

CD11b is commonly used as a leukocyte activation marker, since its expression is usually increased in response to stimuli such as C5a [104], RANTES [105], lipopolysaccharides (LPS) [106], P-selectin [107], and ICs [108]. Studies have also shown that the binding capacity of CD11b/CD18 is not constitutive but is induced in response to various stimuli, and an iC3b-binding capacity has been described for the CD11b domain. This binding has been confirmed by the use of various inhibitory monoclonal antibodies against CD11b, which block the binding of iC3b-coated particles to PMNs and monocytes [109].

Although the cellular distribution and functions of CD11c/CD18 (CR4) are similar to those of CD11b/CD18, CR4 is also found on neutrophils and platelets and may facilitate the accumulation of both neutrophils and platelets at sites of IC deposition [93].

The recently identified complement receptor CRIg is expressed by a subset of tissue macrophages (e.g. Kupfer cells) [61, 110, 111]. It binds to the C3c domain of complement fragments C3b and iC3b and is required for efficient binding and phagocytosis of C3-opsonized particles [61]; it has also been reported to regulate T-cell activation and maturation [112]. Other function that has been assigned CRIg is its ability to function as a selective complement regulator of the AP [61]. CRIg has no decay or co-factor activity like other complement regulators. Instead, CRIg inhibits convertase activity by inhibiting binding of the substrates C3 and C5 to the convertases [60].

The anaphylatoxins C3a and C5a that are produced in inflammatory reactions exert their effects by binding to specific receptors. (The term “anaphylatoxins” refers to the ability to stimulate histamine release from mast cells, leading to anaphylactoid reactions involving smooth muscle contraction and

an increase in vascular permeability.) C3a and C5a also possess chemotactic properties and are able to recruit inflammatory and immunological cells to the site of inflammation.

C3a mediates its effects on cells by binding to the C3a receptor (C3aR). The C3aR is a 95- to 105-kDa G-protein-coupled receptor [113] that is expressed on a wide variety of cells.

C5a is the most potent anaphylatoxin produced during an immune response. There are two receptors known to bind C5a, the C5a receptor (C5aR) (CD88) and C5L2 (GPR77). Both are 7-transmembrane proteins that bind C5a with very high affinity [114]. The C5aR is widely expressed on both immune and nonimmune cells. It is now established that the C5aR is expressed on neutrophils, eosinophils, basophils, monocytes, mast cells, vascular endothelial cells, cardiomyocytes, renal glomerular mesangial cells, neural stem cells, and hepatocytes [114]. C5L2 is not as widely expressed as the C5aR, but it is expressed at least on neutrophils, macrophages, and some other nonimmune cell types.

C3a-C3aR and C5a-C5aR interactions are believed to mediate a wide range of immunological responses, including cell activation, chemotaxis, and the release of histamine and cytokines. These responses contribute to the pathogenesis of many diseases, including asthma and allergy [115, 116], sepsis [117], glomerulonephritis [118], ischemia/reperfusion injury [119], atherosclerosis [120], and SLE [121].

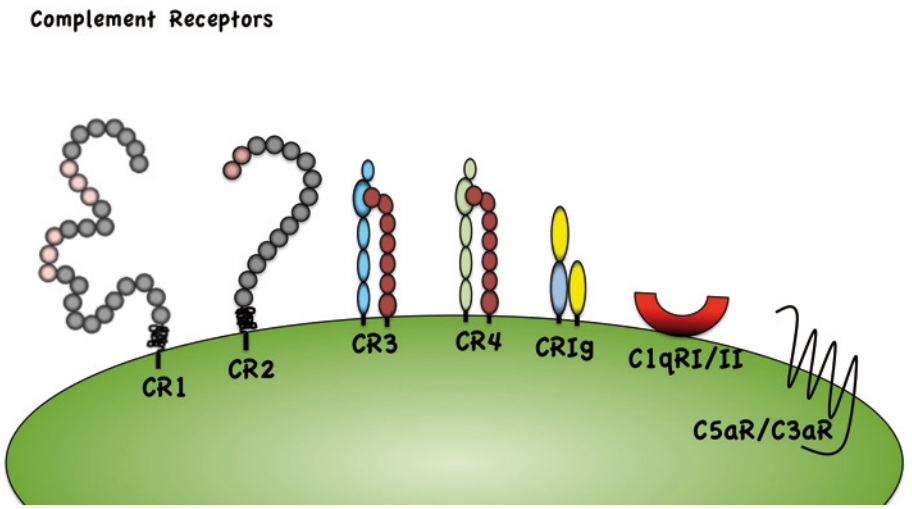

Figure 7: Schematic illustration summarizing the complement receptors, complement receptor 1-4 (CR1-4), the recently described CRIg, C1q receptors I and II (C1qRI/II), and the anaphylatoxin receptors C5aR and C3aR.

Platelets

Morphology of platelets

Platelets are the smallest corpuscular components of human blood. They are 2 to 4 μm in diameter, and 150,000 to 400,000/μL are present in the blood. Platelets are anucleated cells that originate from megakaryocytes in the bone marrow [122]. They normally circulate for ~10 days in the blood [123].

The typical shape of resting platelets is discoid; upon activation, they undergo a change in shape, to a globular form with pseudopods. Platelets contain a number of preformed, morphologicaly distinguishable storage granules: α-granules, dense granules, and lysosomes, the contents of which are released upon platelet activation [124] (Fig 8).

Apart from the traditional view of platelets as mediators of hemostasis, evidence is emerging that indicates that platelets and platelet-derived microparticles (PMPs) focus complement activation on the site of vascular injury. Thus, it is not surprising that activated complement components have been demonstrated in many types of atherosclerotic and thrombotic vascular lesions [125]. Platelets are also often found to be involved in many of the inflammatory diseases that are mediated by complement dysregulation.

Platelet activation

Platelets are extremely sensitive cells that respond to minimal stimulation and become activated when they contact any thrombogenic surface, such as injured endothelium and subendothelium, or artificial surfaces such as stents, vascular grafts, and cardiopulmonary and hemodialysis equipment [126]. Platelets also respond to stimulation by other physiological agonists, including thrombin, ADP, collagen, platelet activating factor (PAF), and thromboxane A2. In these situations, platelet activation is initiated by the interaction of an extracellular stimulus with receptors at the platelet surface [127].

Activation of platelets results in a series of well-characterized responses. These include: (1) the secretion and release of the contents of platelet granules into the microenvironment of the platelets, and (2) the release and expression of P-selectin on the platelet membrane after α-granule secretion. P-selectin is important in mediating the adhesion of platelets to endothelial cells, monocytes, neutrophils, and a subset of lymphocytes [128]. (3) Activation of platelets is also associated with a shape change from the normal discoid shape to a globular form with pseudopods. This drastic shape change promotes platelet aggregation, and during this mechanism a trans-bilayer flipping of the membrane phospholipids occurs so that the platelet membrane is effectively inside-out [129]. (4) During platelet activation, large amounts of PMPs are formed through exocytotic budding from the surface membrane. PMPs are rich in procoagulant factors and can activate leuko-

cytes [130]. PMPs have also been found to bind complement components and activate the complement system on their surface [131]. (5) The platelet eicosanoid pathway is initiated, and as a result, arachidonic acid is released from platelet phospholipids, and there is increased synthesis and release of prostaglandins, thromboxane B_2, and thromboxane A2, which are necessary for the recruitment and activation of adjacent platelets [132].

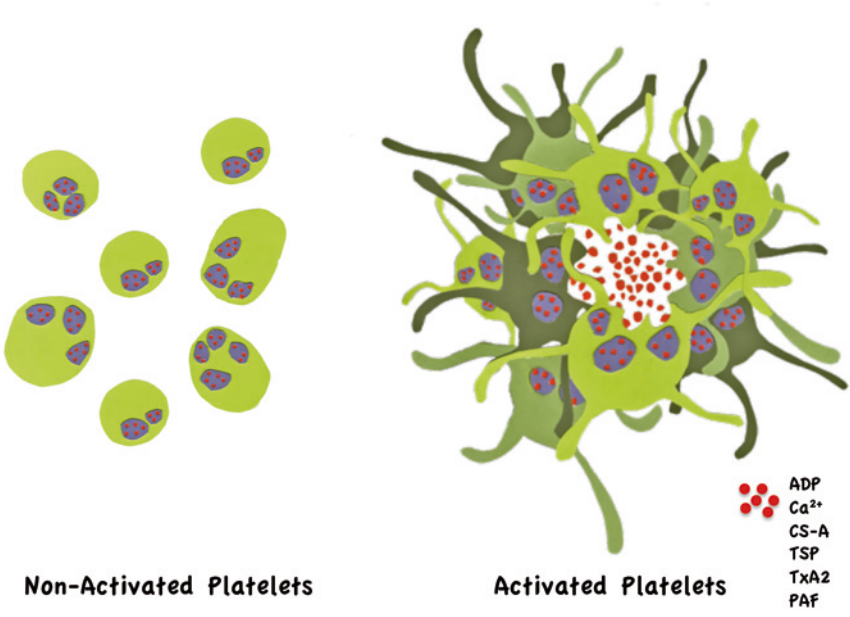

Figure 8: Non-activated and activated platelets. In a resting state platelets circulate in a discoid form. Upon contact with different stimuli, platelets rapidly become activated. This mechanism involves several steps, including change of the shape and release of different types of granules.

Platelet function

Platelets normally circulate in a quiescent state and are prevented from premature activation by the presence of the endothelial cell monolayer [133]. It is only when these barriers are overcome that platelets can become activated. Activation can occur after local trauma or in response to rupture of an atherosclerotic plaque. Platelet plug formation requires a coordinated series of events that can overcome local resistance to platelet activation long enough for bleeding to stop. This is not a trivial task, particularly if unwarranted platelet activation is to be avoided.

Platelet activation in the human body normally occurs upon contact with a disrupted vascular wall and exposed subendothelium, which contains collagen, von Willebrand factor (vWF), and fibrinogen (Fig 9). Platelets adhere to the subendothelium by binding vWF to the glycoprotein receptor (GP) Ib/IX/V complex present on the platelet surface [134]. Binding of vWF is principally mediated through the receptor GPIb [135]. The binding of collagen and vWF to the receptors activates an intracellular signaling pathway that results in an increase in cytosolic Ca^{2+}, release of thromboxane A_2 (TxA_2), the previously mentioned changes in the platelet's shape, and finally the release of storage granules [136, 137]. The key event in the extension of platelet aggregates is the presence of receptors on the platelet surface that can respond directly to some of the released agents, for example, thrombin, ADP, and TxA_2 [138]. Aggregation requires a change in the conformation of the integrin GPIIb/IIIa on the platelet surface, mediated mainly by ADP and TxA_2, which increases its affinity for fibrinogen [139]. Fibrinogen binds to platelets and forms bridges between adjacent, stimulated platelets.

When platelets are stimulated by agonists such as collagen that induce the secretion of granule contents, a trans-bilayer flipping of the membrane phospholipids occurs that brings procoagulant phospholipids to the platelet surface. The exposed phospholipids greatly accelerate the tenase (FIXa/FVIIIa) and prothrombinase (FXa/FVa) reactions of the coagulation pathway, resulting in the generation of thrombin, the most potent platelet agonist [140, 141]. Thrombin induces further platelet stimulation, aggregation, and secretion. Thrombin also converts fibrinogen to fibrin, which is deposited around the mass of aggregated platelets and confers stability on the formed hemostatic plug.

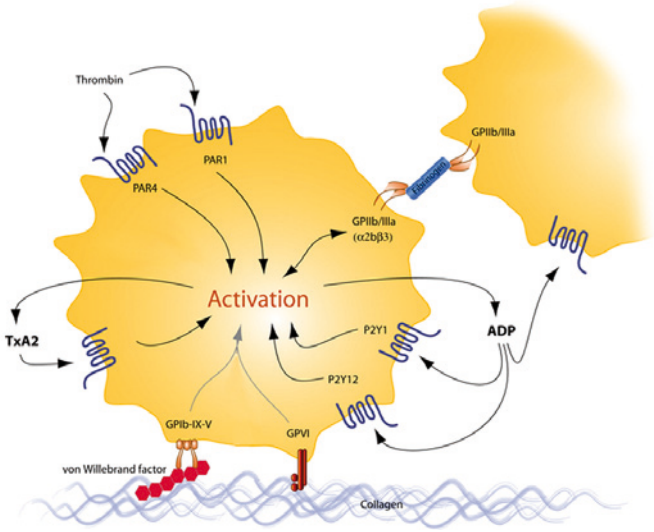

Figure 9: Overview of platelet function. Normally, in the human body, platelets are activated when they come in contact with a disrupted vascular wall. The platelets come in contact with collagen and vWF in the subendothelium and bind via different integrins. Platelets become activated and release ADP and TxA_2, which further activate the platelet and recruit adjacent platelets. The activated platelets activate the coagulation system and fibrinogen is cleaved to fibrin, which stabilizes the formed platelet plug. Thrombin generated via coagulation activation also activates the platelets by proteolytic cleavge of the PAR1 and PAR4 receptors. Illustration by Lars Faxälv (haemostasis.se), with some modifications.

Platelet secretion and degranulation

As mentioned earlier, platelets contain three morphologically distinguishable types of storage granules: dense granules, α-granules, and lysosomes. These granules differ in their molecular composition, kinetics of exocytosis, and responses to different stimuli [142]. The granules have already developed in megakaryocytes, the progenitor cells of platelets. The α-granules are the largest (200 to 500 nm) and most abundant granules in platelets [143]. α-granules contain platelet-specific proteins, growth factors, coagulation factors, adhesion molecules, cytokines, angiogenic factors [144], and proteoglycans. The proteoglycans include a chondroitin sulfate-containing protein, serglycine, and a histidine-rich glycoprotein (HRGP). Albumin and thrombospondin (TSP) are the two most abundantly released proteins from activated platelets [145]. As is true for classical exocytotic vesicles, the α-granule membrane is integrated into the platelet membrane following the secretion process, resulting in the expression of α-granule membrane proteins on the activated platelet surface. Among these proteins are P-selectin (CD62P) [146], GPIIb/IIIa, GPIV (CD36), and the platelet endothelial cell adhesion molecule (PECAM) [147].

The dense granules in human platelets are 250 to 300 nm in diameter and have the highest density of any cellular organelle. In general, dense granules contain large amounts of adenosine and guanosine diphosphates and triphosphates (ADP, ATP, GTP), divalent cations (Ca^{2+}, Mg^{2+}), and serotonin [147].

The third type of granule released from the platelets is the lysosome. Lysosomes contain a variety of proteolytic enzymes that are active under acidic conditions. Lysosomes are intermediate in size between the dense granules and α-granules. They contain glycosidases, proteases, and cationic proteins with bactericidal activity; the presence of collagenase and elastase has also been reported [147].

The process of platelet degranulation described above takes 10 to 120 seconds, depending on the strength of the stimulus and which secreted substance is being monitored [124]. Secretion of acid hydrolases requires a higher agonist concentration and occurs more slowly than does secretion of the contents of the dense granules [142, 148, 149]. Several agonists cannot evoke secretion of acid hydrolases, although they readily trigger secretion of the contents of the α-granules and the dense granules.

The physiological agonists of platelets can be divided into strong (thrombin, trypsin, collagen), intermediate (thromboxane A2), and weak (ADP) according to their ability to release the various kinds of storage granules.

Platelet-activating agents and their receptors

Thrombin

Human platelets express two protease-activated receptors (PAR) that are activated by thrombin, PAR1 and PAR4. PAR1 responds to thrombin levels of approximately 1 nmol/L, while for PAR4 a 10-fold higher thrombin concentration is needed to provoke a response [138]. Thrombin activates platelets by cleaving and activating PAR1 and PAR4. Thrombin-mediated activation [150] involves binding to the ectodomain of the PAR molecule and proteolytically cleaving it between Arg^{41} and Ser^{42}. This cleavage reaction exposes a new amino-terminus, which acts as a "tethered ligand" to activate the receptor. It should be emphasized that a very low concentration of thrombin (1.7 nmol/L) is sufficient to induce all platelet responses mentioned earlier [151]. Synthetic thrombin receptor-activating peptides (TRAP) such as SFLLRN, derived from the deduced sequence of the new amino-terminus of the cleaved thrombin receptor, can mimic thrombin receptor activation and act as full agonists for platelet activation [152]. TRAP acts by binding to PAR1 and mimicking the N-terminal ectodomain of the receptor, thereby activating it without the proteolytic action of thrombin.

Immune complexes

Immune complexes (IC) consist of antibodies that associate with their respective antigens, which may be present in soluble form or expressed on microparticles derived either from activated platelets or apoptotic cells. ICs are potent activators of the complement system and thus contribute to the appearance of acute and chronic inflammation that may result in tissue damage. Deposited and circulating ICs may be partially responsible for the pathogenesis of a number of autoimmune diseases, such as rheumatoid arthritis and SLE.

ICs are believed to be strong platelet activation agonists, capable of inducing at least four of the five responses mentioned earlier. ICs bind to the Fc receptors FcγRII (CD32) and FcγRIII (CD64) expressed on the platelet surface [153-155]. The FcRγ-chain of CD32 is a common signal transducer with GPVI and CD36, which are receptors involved in collagen-induced platelet activation. The platelet activation response to IC via FcγRII is therefore expected to be the same as for collagen-mediated activation.

Platelets also express receptors for other activators, such as ADP and collagen.

Platelet cell-surface molecules

P-selectin

P-selectin (CD62P) is a member of the selectin family of cell adhesion molecules (CAM), which also includes L-selectin and E-selectin [156] [157]. P-selectin is a large (140 kDa) transmembrane glycoprotein that is expressed on activated endothelial cells and platelets [158]. It is stored in the α-granules of platelets [128] and in the Weibel-Plade bodies of endothelial cells [159] and is translocated to the cell surface upon activation by various agonists [128]. The selectins all share a common domain structure, and this common structure is reflected in their function as adhesion molecules that support the interactions of platelets or endothelial cells with leukocytes during thrombosis and inflammation [160]. Thus, P-selectin is an important contributor to the interaction of activated platelets with stimulated endothelial cells and a subset of leukocytes [161]. The main ligand for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is expressed mainly on leukocytes [158] but also to some extent also on platelets [162]. P-selectin-PSGL-1 interactions have been shown to be important in leukocyte rolling under flow [163], in thrombus formation, and in aggregation, in which it stabilizes the GPIIb/IIIa-fibrinogen interactions [164]. It is also an important contributor to inflammatory reactions and recruitment of leukocytes [156, 165].

CD40 ligand

CD40 ligand (CD40L) is preformed and stored in the cytoplasm of resting platelets, then translocated to the cell surface upon activation [166]. CD40L binds to CD40 expressed on endothelial cells or monocytes, leading to secretion of chemokines and upregulation of adhesion molecules [167] and resulting in recruitment of leukocytes to the site of injury. Shedding of CD40L from platelets can also occur, producing soluble CD40L molecules that are proinflammatory for endothelial cells and have procoagulatory effects, since they induce tissue factor (TF) expression on monocytes [168].

GPIIb/IIIa

GPIIb/IIIa (CD41/CD61) is the most abundant receptor expressed on resting platelets. There are about 40,000 to 80,000 copies of GPIIb/IIIa on the surface of each activated platelet. Another 20,000 to 40,000 copies of GPIIb/IIIa are present inside the platelets in the α-granule membranes and in the membranes lining the open canalicular system; these molecules are translocated to the platelet membrane during the release reaction [126].

In the case of resting platelets, GPIIa/IIIb is present in an inactive form, and upon platelet activation, a conformational change occurs that leads to the exposure of high-affinity binding sites for soluble fibrinogen. Binding of fibrinogen leads to platelet aggregation as well as platelet-leukocyte aggre-

gates. Recently, factor H has been shown to bind to activated platelets via GPIIb/IIIa [169, 170].

GPIb-IX-V

GPIb (CD42) is a leucine-rich glycoprotein receptor that is constitutively expressed on the surface of the platelets, with about 25,000 copies per platelet [126, 171]. GPIb is complexed in an equimolar ratio with GPIX and GPV [171]. The GPIb-IX-V complex mediates platelets interaction with vWF and adhesion to exposed subendothelium at the site of injury. GPIb-IX-V is also important for platelet-leukocyte interactions, in which it binds to CD11b/CD18 on the leukocytes [172]. Thrombin also binds to GPIb-IX-V, but the significance of this binding is not clear.

Glycosaminoglycans

Overview

Glycosaminoglycans (GAGs) are linear polysaccharides containing repeating disaccharide units of an amino sugar, either N-acetyl-D-glucosamine (D-GlcNAc) or N-acetyl-galactosamine (D-GalNAc), and a uronic acid, either D-glucuronic (D-GlcA) or L-iduronic acid (L-IdoA) [173]. There are four structurally distinct GAG families: heparan sulfate (HS)/heparin, chondroitin (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronic acid (HA) [174, 175].

CS and DS are often designated galactosaminoglycans because they contain a galactosamine unit, whereas heparin and HS, which contain a glucosamine, are called glucosaminoglycans [176]. Thus, the galactosamine in CS and DS is substituted with a glucosamine in heparin and HS. GAGs are strong polyanions because they carry negatively charged carboxyl groups and sulfate groups on most of their sugar residues.

All the glycosaminoglycans show variation in their degree and pattern of sulfation. Heparin is the most heavily sulfated GAG, followed by HS, CS, and DS [177]. Among the glycosaminoglycans, very small differences are found in the basic sugar backbone; subsequent modifications such as sulfation, deacetylation, and epimerization distinguish individual GAGs and are critical for their roles and activity [175].

The GAGs are synthesized by membrane-bound enzymes in the Golgi system that successively add a series of monosaccharide units to a protein core acceptor. They are usually attached to the protein cores via a serine residue, creating proteoglycans (PGs) [173].

Proteoglycans

In nature, all glycosaminoglycans except HA are covalently linked to a core protein to form a PG (Fig 10). The linkage of GAGs to the protein core involves a specific trisaccharide composed of two galactose (Gal) residues and a xylose (Xyl) residue. The saccharide residues are coupled to the protein core through an O-glycosidic bond to a serine residue [176]. The substituted serine residues in the core protein are adjacent to glycine, and the Ser-Gly dipeptide seems to be a basic requirement for recognition by xylosyl transferase enzymes [173]. The number of GAG chain susbstituents on a protein core may vary from one to over 100, thus producing wide variation in the type and function of proteoglycans [174].

Almost all mammalian cells produce PGs and either secrete them into the extracellular matrix (ECM), insert them into the plasma membrane, or store them in secretory granules [178]. The biological roles of PGs are highly diversified, and most of their effects depend on binding of proteins to the

GAG chains. Certain functions, such as the anticoagulant activities of heparin/HS, are attributed to free GAG chains [174]. However, most biological activities attributed to PGs depend to some extent on the presence of the protein core, which may contribute in various ways.

Serglycin is the PG that are most commonly found in hematopoietic cells [179]. In the various types of blood cells, CS is the major GAG, with chondroitin 4-sulfate as the dominant form [173, 179]. There are also blood cell types that synthesize chondroitin 6-sulfate, chondroitin 4,6-sulfate, and heparin [173]. Serglycin is stored in the granules of hematopoietic cells, where it is believed to be involved in the generation of the storage granules [180-182]. However, serglycin is secreted to the ECM or associated with cell membranes during cell activation [177]. In a recent study, Woulfe and coworkers have demonstrated defects in platelet function and aggregation in serglycin knockout mice [183].

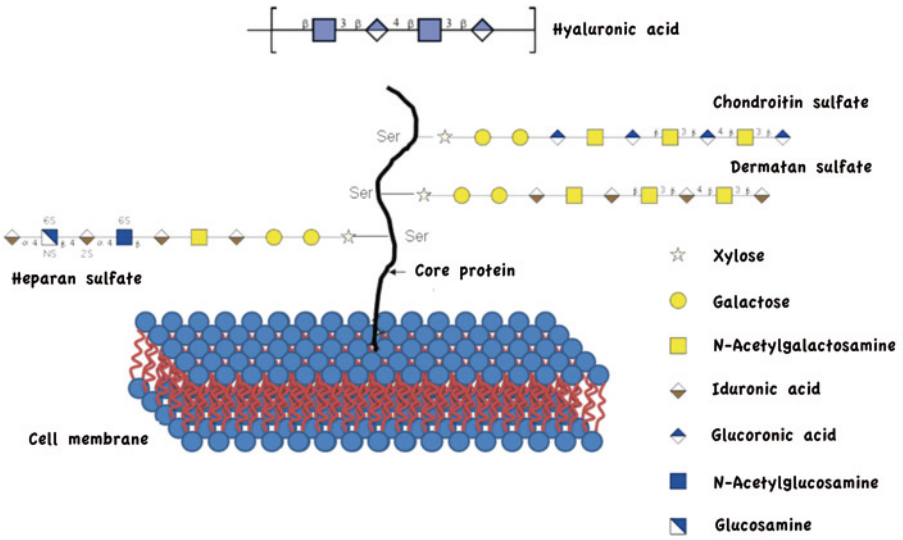

Figure 10: Overview of different GAGs and their linkage to a core protein, which creates a proteoglycan. Chondroitin sulfate, dermatan sulfate, and heparan sulfate are linked to the core protein via a serine residue. Hyaluronic acid is not associated to any core protein and is present in the body as a GAG.

Chondroitin sulfate (CS)

CS consists of the repeating disaccharide units D-GalNAc and D-GlcA. DS differs from CS by a frequent epimerization of glucuronic acid to iduronic acid [173]. Both CS and DS may be sulfated at carbon 2 of the uronic acid and at carbon 4 and/or 6 of the amino sugar [174]. The pattern of sulfation is usually used to name the CS GAG: CS mono-sulfated at carbon four is designated CS-4 or CS-A, and CS sulfated in carbon six is named CS-6 or CS-C. CS disulfated at carbon 4 and 6 is referred to as CS-4,6 or CS-E. DS, formerly known as CS-B, is sulfated at carbon 4 of the galactosamine and 2 of the uronic acid [173].

CS is the most abundant GAG in human plasma (70-80% of all GAGs), with CS-A being the major component [184]**.** It has also been well established that CS-A is the predominant GAG in platelets [185]. CS-A is stored in the α-granules of platelets and is released during the activation and degranulation process [186, 187]. CS-A has also been found to be rapidly expressed on the surface of activated platelets [188]. The release of CS-A from platelets in response to different agonists, including ADP, collagen, and thrombin, is very rapid and occurs within 3 minutes after platelet activation and raises the concentration of plasma CS to as much as 2 μg/mL [189]. Unlike the CS in blood plasma, the CS present in platelets is fully sulfated, and its molecular mass has been estimated as ~28 kDa [185]. The high degree of sulfation is thought to be linked to immune-related processes and inflammatory conditions [190].

CS has been shown to bind and interact with C1q [191, 192]. It has been suggested that this binding is mediated through the globular heads of C1q and involves ionic interactions [193]. Many studies have pointed to CS as a C1q inhibitor [192, 194, 195].

Clinical significance of GAGs and proteoglycans

The proteoglycans were previously believed to be mainly structural components of the cell. However, it is now well established that GAGs play a major role in cell signaling and development, angiogenesis [196], axonal growth [197], tumor progression [198, 199], metastasis [200], and anticoagulation [201, 202]. GAGs and proteoglycans are believed to be of major importance for cell proliferation because they act as co-receptors for many growth factors.

These macromolecules also play a pivotal role in the inflammation cascade that leads to the activation of leukocytes and endothelial cells, and ultimately to the extravasation of leukocytes and leukocyte migration into inflamed or diseased tissue. GAGs have important roles in these processes, as adhesion ligands in leukocyte extravasation and as carriers/presenters of chemokines and growth factors [203].

Platelet-complement interactions

A link between complement activation and the expression of platelet procoagulant activity has long been suspected, based on the frequent association of vascular thrombosis with complement activation. For example, clinical conditions such as sepsis [204] and SLE [205] have been shown to evoke platelet activation in parallel with high complement activity. On the other hand, platelets also promote several inflammatory conditions by interacting with T and B cells, by releasing pro-inflammatory cytokines [206], and by recruiting leukocytes to the site of injury.

It has been suggested that platelets have a significant role in the activation and regulation of complement. In agreement with this concept, several studies have shown that complement components bind to platelets [207-209]. Platelets have also been found to store, secrete, and express complement proteins and regulators upon activation. It was recently reported that C3 is present in lysates of platelets [210]. In 1981, Kenny et al. showed that platelet homogenates could inhibit the formation and accelerate the decay of the C3bBb convertase as a result of the presence of factor H [211]. Factor H was found to bind to washed human platelets via TSP [170] or GPIIb/IIIa [169].

Del Conde et al. [210] and Peerschke et al. [91] have reported that complement is activated on the platelet surface by the AP and the CP, respectively. However, these observations are in conflict with the previously demonstrated expression of DAF [212], MCP [213], and CD59 [214] on platelet membranes. Expression of these membrane-associated complement regulators and interactions of soluble regulators as C1INH [215], clusterin [216], and factor H with the activated platelet surface are believed to ensure a well-controlled complement activation on the surface of platelets.

Complement-induced platelet activation has been studied by Sims and coworkers, who since the early 1980s have demonstrated an increased procoagulant activity on platelets as a result of the insertion of sC5b-9 complexes [58]. These observations suggested that the sC5b-9 complex mimics platelet stimulation by thrombin and other agonists. However, it has been reported that platelet activation by sC5b-9 is under the regulatory control of the complement regulatory protein CD59 present on the surface of platelets [217]. Blocking of CD59 with an antibody augments the sC5b-9-mediated procoagulant response of platelets.

A potential role for the complement system in the thrombotic episodes associated with paroxysmal nocturnal hemoglobinuria (PNH) has been suggested by the increased sensitivity of platelets to activation by sC5b-9 complexes, as a result of the diminished surface expression of CD59 on platelets [218].

Platelet activation and a lowering of platelet counts has also been noted in atypical hemolytic uremic syndrome (aHUS). aHUS may be associated with mutations in the C-terminus of factor H [219]; this relationship would sug-

gest that dysregulation of the complement system plays a crucial part in the pathogenesis of aHUS. Ståhl et al. have recently shown that aHUS patients with a mutated factor H have higher levels of deposition of C3 and C9 and of complement activation on platelets compared to healthy controls [220]. Combining aHUS patient sera containing mutated factor H with normal platelets results in complement activation and the activation and aggregation of platelets. This complement deposition and platelet activation is abrogated when platelets are preincubated with normal factor H or when normal serum is used [220]. Karpman et al. have suggested that the binding of factor H to human platelets can protect them from complement activation [221].

Leukocytes

Leukocytes, or white blood cells, are a very important part of our immune system and defend the human body against infectious diseases, invading microorganisms, and foreign materials. Leukocytes are nucleated cells and can be divided into granulocytes and monocytes, which belong to the innate immunity system, and lymphocytes, which make up the acquired immune system. They are all produced and derived from a multipotent cell in the bone marrow, the hematopoietic stem cell. The leukocytes comprise around 1% of the blood cells in a healthy individual but increase rapidly in number in the course of various inflammatory conditions.

Granulocytes

Granulocytes, also known as polymorphonuclear cells (PMNs) because of the variable shape of their nuclei, can be divided in three subgroups: neutrophils, basophils, and eosinophils. Neutrophils represent 50 to 60% of the total circulating leukocytes. The bone marrow of a normal healthy adult produces more than 10^{11} neutrophils per day, and more than 10^{12} per day under different inflammatory conditions. Neutrophils have an average diameter of 12-15 μm and a very short half-life in the circulation. Upon being released from the bone marrow into the circulation, the cells are in a nonactivated state and have a half-life of only 4 to 10 h in the bloodstream; thereafter, they migrate to tissues and become activated, where they survive for 1 to 2 days. The main function of neutrophils is to participate in our defense against invading microorganisms, principally in the tissues before the microbes enter the bloodstream. Neutrophils in the bloodstream are attracted to the sites of injury (following released anaphylatoxins and chemokines), captured by molecules up-regulated on the endothelium as a result of tissue injury and become activated. They then migrate toward the site of inflammation and engulf their opsonized targets by phagocytosis, thereby eliminating the invading microorganisms [222-224].

Monocytes

Monocytes comprise 10% of the leukocytes in human blood and are distinct from PMNs, which also belong to the innate arm of the immune system. Blood monocytes develop in the bone marrow from a dividing common myeloid progenitor that is shared with granulocytes. Monocytes are subsequently released to the peripheral circulation as non-dividing cells. The half-life of a circulating monocyte has been estimated to be ~3 days. The short half-life of the monocytes in the circulation indicates that they are not functional there. Instead, they migrate into tissues and differentiate into macrophages; once the monocytes have migrated to various tissues, they do not re-

enter the circulation but instead reside in the tissues and exert their function there. Macrophages are approximately 20 μm in diameter and are found in all tissues of the human body. They play an important role in engulfing and destroying a wide range of pathogens and foreign materials. In addition, they play an important role in processing and presenting antigens to lymphocytes, thereby linking innate immunity to the adaptive immune system [223, 225].

Granulocytes, followed by monocytes, are the most rapid responders to complement activation. Both cell types possess various types of complement receptors as well as Fc receptors. The cellular distribution of the complement receptors is described in the chapter “Complement receptors”.

Platelet-leukocyte interactions

When platelets are activated in whole blood by various agonists, the platelets form complexes with leukocytes, both granulocytes and monocytes [226]. The interaction between platelets and leukocytes appears to be of pathological significance. The complexes are often detected in patients with stroke [227], unstable angina pectoris, and mycordial infections [228]. The mechanisms by which these platelet-leukocyte complexes are formed are still partially unknown. Platelets and leukocytes interact via direct cell-cell contact or indirectly via soluble mediators, following activation of either platelets or leukocytes [229].

This complex formation between activated platelets and leukocytes is believed to occur via P-selectin, which is expressed on the surface of activated platelets, and its ligand on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1). The interaction of P-selectin with PSGL-1 mediates the initial tethering of leukocytes to activated platelets through a mechanism resembling the interaction of leukocytes with endothelial cells [230]. This P-selectin-mediated interaction induces the activation and up-regulation of β_2-intergrin ($\alpha_M\beta_2$; CD11b/CD18) on leukocytes [231]. Firm attachment is subsequently mediated by the interaction of CD11b/CD18 with various ligands and adhesion molecules on platelets [232]. GPIIb/IIIa expressed on platelets is often considered to be essential for platelet-leukocyte interactions [233]. It has been proposed that GPIIb/IIIa interacts with leukocytes through two different mechanisms: by direct binding to CD11b/CD18 or through fibrinogen, which binds to GPIIa/IIIb on platelets and CD11b/CD18 on leukocytes [234]. Other proteins that are exposed on platelets and believed to take part in platelet-leukocyte complex formation are CD40L, which interacts with CD40 on leukocytes [233], and GPIbα, which binds to CD11b/CD18 [235]. However, studies by Konstantopoulos et al. [236] and Ruef et al. [229] have shown that platelet-leukocyte attachment occurs independent of GPIIb/IIIa. Thus, the ligand(s) of the leukocyte CD11b/CD18 integrin are still unclear.

Activation of platelets results in the formation of microparticles that are shed from the platelet plasma membrane. These platelet-derived microvesicles (PMP) have a high procoagulant activity because of the negatively charged phospholipids exposed on their surface. Various receptors that are present on the surface of activated platelets are also present on the surface of PMPs, enabling them to interact with other cells [237, 238]. It has been well established that platelets and PMPs contain tissue factor (TF), which is the most important initiator of intravascular coagulation [239-241]. TF acts as receptor for the coagulation factor VII/VIIa, and formation of this complex facilitates the cleavage of factor X. Under physiological conditions, TF is not exposed to the bloodstream, and cells in the circulation do not normally ex-

press TF; however, leukocytes (monocytes and granulocytes) and endothelial cells can be induced to express TF in response to various stimuli. Activation of complement and generation of C5a [242] and sC5b-9 complexes [243] have been shown to induce TF expression on leukocytes and endothelial cells. Other proinflammatory mediators, such as immune complexes, tumor necrosis factor α (TNFα) [244], and platelet-derived growth factor (PDGF) [245], are also known to induce leukocytes to express TF. However, the expression of TF on leukocytes is still a matter of debate. It has been well established that tethering of platelets and PMPs to monocytes and granulocytes via P-selectin-PSGL-1 and CD40-CD40L interactions causes TF expression on leukocytes [233]. This means that most of the TF expressed on leukocytes may be acquired by binding platelets or TF containing PMPs [233, 246].

Current Investigation

Aims of the studies

The general aim of these studies was to elucidate the mechanism by which the activated platelet acts as an innate immunity cell by activating the complement system during inflammatory and thrombotic reactions. In addition, the contribution of CS-A for binding of activators and inhibitors of complement activation, on activated platelets, was investigated.

The specific aims of the papers included in the thesis are as follows:

Paper I

- To determine whether platelet activation triggers activation of the complement system.
- To elucidate the mechanism(s) by which activated platelets trigger complement activation in the fluid phase.
- To investigate the role of CS-A, which is released by activated platelets for complement activation.

Paper II

- To reinvestigate the binding of complement proteins to activated platelets.
- To determine whether the binding of complement proteins to TRAP-activated normal platelets is a result of complement activation.
- To characterize the forms of C3 that bind to activated platelets, and to analyze its possible function(s).

Paper III

- To identify the plasma proteins that bind to CS-A.
- To characterize the contribution of CS-A for the binding of C1q, C1INH, C4BP, and factor H to the surface of activated platelets.

Paper IV

- To test the hypothesis that non-proteolytically activated C3 bound to activated platelets and platelet-derived microparticles (PMPs) can act as a ligand for CD11b/CD18 (CR3) and contribute to platelet-leukocyte complex formation.

Materials and Methods

The materials and methods used in the present investigations are described in papers I – IV, which are included in this thesis. The experimental protocols will be briefly discussed and some additional aspects of the methods will be made:

Blood sampling and platelet activation

Blood was collected from healthy volunteers who had not received any medication for at least 10 days. It was particularly important to avoid the intake of substances containing NSAID (non-steroid anti-inflammatory drugs), which can affect platelet function. In most studies, blood was drawn into vacutainer tubes with the addition of the specific thrombin inhibitor, lepirudin. The presence of lepirudin does not affect the activation of the complement system, but it stops the clot formation and amplification of the coagulation system mediated by thrombin. Thus, lepirudin ensures that the activated platelets will not be trapped in any clots that are formed and are thus be available for investigations.

Platelets were activated with thrombin receptor activating peptide (TRAP). TRAP is a hexapeptide (SFLLRN) that mimics the N-terminal exodomain of the protease activated receptor (PAR)-1. TRAP binds to and crosslinks the receptor and activates the platelets without the proteolytic effect of thrombin, thereby avoiding the formation of a fibrin network.

Complement activation

Platelets in PRP were activated with TRAP at 37°C for up to 60 min. EDTA was added to stop the complement activation, the samples were centrifuged, and the supernatants were stored at -70°C until analyzed for the presence of the complement activation products C3a and sC5b-9. Some experiments were performed in the presence of Compstatin, EDTA, or EGTA. Compstatin is a potent complement inhibitor at the C3 level. EDTA blocks complement activation totally by chelating Mg^{2+} and Ca^{2+}, whereas EGTA-Mg^{2+} blocks only the CP since only $Ca^{2+}$is affected. Other experiments were performed in the presence of the C5a receptor antagonist (C5aRA). C5aRA is a small molecule that blocks the effects of the potent anaphylatoxin C5a on its effector cells. Monoclonal antibody Anti-C1q-85 was also used to block complement activation. Anti-C1q-85 blocks the binding of the globular heads of C1q, which affects binding to IgG and CRP [247].

We hypothesized that platelets may release complement activators and that one of these might be CS. In order to test this hypothesis, plasma was incubated with CS-A or with supernatants from washed and TRAP-activated platelets, as an alternative source for CS. In some experiments, CS-A and

platelet supernatants were digested with chondroitinase ABC, which enzymatically cleaves CS-A, CS-B, and CS-C.

Platelet preparation

Platelets were activated in PRP or first isolated and washed, and then activated. Activation in PRP was performed to study the effect on complement activation of substances released by the platelets, but also to study the binding of complement proteins to the surface of activated platelets.

To examine the binding of a single complement protein in a more controlled milieu, platelets were first purified from PRP by centrifugation in order to remove the plasma. Then, purified complement proteins were added, and the binding detected by flow cytometry.

Quantification of CS released from activated platelets

The release of CS from activated platelets was determined by a colorimetric method using the basic dye toluidine blue, which can bind and polymerize on polyanionic substrates.

Immobilization of CS-A and binding of complement proteins

Immobilization of CS-A in microtiter plates

CS-A was immobilized in microtiter plates to allow us to study the interaction between complement proteins and CS-A. Plasma or serum was incubated in microtiter plates, and binding of C1q and C3 fragments was determined. By using a C1q-depleted serum, we were able to determine the significance of C1q in this system. The immobilization was verified by anti-CS-A mouse monoclonal antibody (mAb) CS-56 (Sigma-Aldrich). This system was also used to titrate various reagents used to block the binding of C1q, factor H, C4BP, and C1INH to CS-A.

Immobilization of CS-A on Sepharose

CS-A-Sepharose was generated by covalently coupling CS-A via primary amines in the protein core to CNBr-activated Sepharose, which was then packed into columns. These affinity columns were then used to identify which plasma proteins that bound to CS-A. Fresh human plasma/serum and C1q-depleted serum were passed through CS-A-Sepharose columns, and after several washing steps, the bound proteins were eluted with 1.0 M NaCl. The eluted proteins were subjected to SDS-PAGE followed by Western blotting and MALDI-TOF MS for identification. A Sepharose column without CS-A was used as control.

Immobilization of CS-A onto a sensor chip

CS-A was immobilized onto an HC500 carboxylated hydrogel sensor chip. The chip was activated using a sulfo-NHS amine coupling kit, and CS-A was injected onto the chip. After the injection of CS-A, the rest of the active NHS-amine groups were quenched with ethanolamine. The immobilization was verified by reactivity with anti-CS-A mouse mAb CS-56 (Sigma-Aldrich).

MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) was used to identify proteins that had been eluted from CS-A columns after separation on SDS-PAGE. After the SDS-PAGE was run, the gel was stained with Coomassie brilliant blue, bands were cut out, and the proteins were digested with trypsin (in-gel digestion) and subjected to MALDI-TOF MS; the proteins were identified by peptide-mass fingerprinting.

Surface plasmon resonance

Real-time surface plasmon resonance analysis was performed on a BIAcore X biosensor. For this purpose, sensor chips were prepared as described earlier. The BIAcore was used to further study the interactions between complement proteins and CS-A. The sensograms were used to estimate the binding kinetics and binding affinities of C1q, factor H, and C4BP for CS-A.

Flow cytometry

Paper I

To elucidate the physiological relevance of platelet-triggered complement activation, we studied the up-regulation of CD11b on monocytes and granulocytes and the formation of platelet-monocyte/granulocyte conjugates. Whole blood anticoagulated with lepirudin was activated with TRAP and then stained with anti-CD11b. Alternatively, double-staining was performed to measure the platelet-monocyte/granulocyte complexes, using anti-CD42a as a marker for platelets, anti-CD14 for monocytes, and anti-CD16 for granulocytes.

Papers II and III

Flow cytometry was used to study the binding of complement proteins to activated platelets. As described in paper II, binding of complement proteins to activated platelets was examined in two different ways: First, binding of complement components was assessed in a more physiological environment

in which platelets were activated in PRP. Second, binding of single components was studied under more controlled conditions, in which platelets were first isolated from PRP by centrifugation and then washed before activation. Purified complement proteins were added, and the binding was detected with labeled antibodies. Attempts to block the binding of complement proteins were made using Compstatin, anti-C1q-85, EDTA, and EGTA. In other experiments using purified proteins, binding was blocked using soluble CS-A, low molecular weight heparin (LMWH), and monoclonal antibodies to CS-A (CS-56 and 2H6).

To eliminate the detection of non-specifically bound proteins, platelets were washed before and after labeling with antibodies. The washing procedure can potentially contribute to the activation of the platelets. Therefore, a comparison was always made between activated and non-activated platelets that had been subjected to the same experimental procedure. P-selectin expression was always used as a marker for platelet activation.

Paper IV

The study described in paper IV was principally conducted to investigate whether C3 bound to activated platelets and PMPs can act as a ligand for CD11b/CD18 and contribute to the formation of platelet-leukocyte complexes. For this purpose, blood cells were washed and depleted of plasma proteins. C3 was added, and the leukocytes were activated with TRAP in order to allow binding of C3 to the platelet surface and to upregulate P-selectin (CD62P). Leukocytes were activated by the addition of C5a so that CD11b/CD18 would be upregulated and activated. To inhibit complex formation, C5a-activated leukocytes were preincubated with monoclonal antibodies (mAb) against CD11b and CD62P, individually or in combination.

To study the complex formation of leukocytes with PMPs, blood cells from EDTA-treated blood were depleted of both platelets and plasma proteins by repeated washing and centrifugation steps. PMPs, isolated from fresh serum, were added to the washed blood cells in the presence or absence of C5a and incubated for 30 min. To inhibit the leukocyte-PMP complexes, C5a-activated leukocytes were preincubated with the CD11b and CD62P mAbs, individually or in combination, prior to the addition of the PMPs. The complex formation was assessed by flow cytometry as described above (Flow cytometry, paper I). However, for studying the leukocyte-PMP complexes, anti-CD41a was used instead of anti-CD42a, since this would enhance the levels of detected PMPs [248].

Western blotting

Western blot analysis was performed in paper II to confirm the presence of platelet surface-associated C3 and to further characterize this C3. Activated and non-activated platelets were washed and then incubated with PBS con-

taining Tween-20 to dissociate the surface-bound C3. The potent protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was present to prevent any non-specific cleavage of C3 by proteases that might leak from platelets during the experimental procedure. Thereafter, the supernatant was boiled with SDS-reducing sample buffer and subjected to SDS-PAGE. Western blot analysis was also performed in paper IV as described above, in order to examine the structure of C3 bound to PMPs.

SDS-PAGE and Western blotting were also used in paper III to identify the plasma proteins that bound to CS-A-Sepharose columns.

ELISAs

Activation of the complement system in the fluid phase was investigated by measuring the generation of C3a and the sC5b-9 complex by ELISA as described in paper I.

Results

Paper I

It has previously been reported that coagulation and inflammation interact in several ways. Here we showed that clotting activated by either the TF pathway or the contact activation system triggers complement activation. Activation of platelets in PRP and blood by treatment with thromboplastin or by incubation in glass tubes was able to induce complement activation, as measured by the generation of C3a and sC5b-9. The level of complement activation correlated well with the generation of thrombin, as indicated by the formation of thrombin-antithrombin (TAT) complexes. Since only background activation was seen in PPP, platelets are believed to have played a significant role in the mechanism of complement activation in clotting blood.

Platelets activated with TRAP in lepirudin-anticoagulated PRP and blood also induced complement activation in the fluid phase, as reflected by the generation of C3a and sC5b-9. Addition of Compstatin decreased the generation of C3a and sC5b-9 to background levels. CS released from activated platelets was thought to be the trigger for this complement activation. To measure the released CS, we activated PRP with TRAP and measured the release of CS into the supernatant using a colorimetric assay. The concentration of released CS was estimated to be 4 μg/mL. To demonstrate the ability of CS to activate complement, PPP was incubated with exogenously added CS-A, either as commercially available CS-A or in the form of platelet supernatants. Generation of C3a and sC5b-9 increased in a dose-dependent fashion, and digestion of both CS-A and the platelet supernatants with chondroitinase ABC totally abrogated the reaction. Addition of Compstatin, EGTA, or EDTA inhibited the activation and the generation of C3a and sC5b-9.

To further investigate the role of CS-A in the activation of complement, we immobilized CS-A on microtiter plates and added plasma or serum to the wells. Binding of C1q, but not of C3 or AT, was seen when EDTA-plasma had been incubated in the wells. Binding of C1q was verified using purified C1q, and a dose-dependent binding was observed when serial dilutions of C1q were incubated in the wells. As a functional verification of this binding reaction, we quantified the complement activation that occurred in C1q depleted-serum, with or without the addition of purified C1q, by measuring the binding of C3 fragments. No binding of C3 fragments was seen in the absence of C1q, but binding was restored by the addition of purified C1q.

These results suggest that complement is activated via the CP and that C1q serves as the recognition molecule for CS-A.

In addition, CS-A -triggered complement activation induced an activation of monocytes and granulocytes, as indicated by the expression of CD11b and the formation of complexes between platelets and monocytes/granulocytes. CD11b expression on monocytes and granulocytes as well as platelet-monocyte and platelet-granulocyte conjugate formation were significantly inhibited by the addition of the complement inhibitors Compstatin or C5aR antagonist.

Paper II

In paper I, we had demonstrated the occurrence of a fluid-phase complement activation triggered by CS-A released from TRAP-activated platelets. In the studies described in paper II, we wanted to reinvestigate the previously reported complement activation on the surface of activated platelets, since a contradiction exists between these reports and papers that have described the presence of multiple complement regulators and inhibitors on the surface of platelets.

Platelets were activated in lepirudin-treated PRP or whole blood, isolated and washed as in paper I. Binding of complement proteins to the surfaces of non-activated and TRAP-activated platelets was then analyzed by flow cytometry. C1q, C4, C3, and C9 were found to bind to TRAP-activated platelets in PRP and to the same extent in whole blood. Binding of C1q and C3 to activated platelets was confirmed by using purified proteins and washed TRAP-activated platelets. Both C1q and C3 bound to washed TRAP-activated platelets to the same extent as in PRP and whole blood. The observed binding of C1q and C3 despite the absence of a complete complement system suggested that this binding is independent of complement activation. Addition of Compstatin or a C1q (globular head) inhibitory monoclonal antibody (Anti-C1q-85) did not affect the binding of C3 or C9. Activation of platelets in PRP in the presence of EDTA or EGTA or dilution of PRP by 1:10 (a dilution at which complement activation by the AP is minimal) prior to activation did not affect the binding of C3.

Western blot analysis of platelet surface-associated C3 and flow cytometry using monoclonal antibodies specific for different conformational forms of C3 showed that the bound C3 consisted mainly of C3(H_2O), with no C3b present. This result indicated that the bound C3 was non-proteolytically activated and that no complement activation takes place on the surface of activated platelets under physiological conditions. This form of C3 bound to soluble CR1 (CD35), indicating that it may act as a receptor ligand.

Paper III

In paper I, we studied the complement activation in the fluid phase as a result of the activation of platelets with TRAP and release of CS-A. We identified C1q as the recognition molecule, since it bound directly to CS. In paper II, we studied the binding of complement components to TRAP-activated platelets in lepirudin-treated PRP and blood and demonstrated that complement is well regulated on the surface of activated platelets. In this third paper, we characterized the binding of complement component C1q to CS-A immobilized on various matrices and exposed on the surface of activated platelets.

We also analyzed the binding of the complement regulators C1INH, C4BP, and factor H. C1q was shown to be the main protein that specifically bound to CS-A. After depletion of C1q from the serum, other complement proteins also bound specifically to the CS-A, such as C4BP and factor H; however, C1INH did not. C1q, C4BP, and factor H were also shown to bind to activated platelets. This binding was inhibited by a CS-A-specific mAb, thereby linking the binding of C1q, C4BP, and factor H to exposure of CS-A on activated platelets. CS-A-bound C1q was also shown to amplify the binding of model ICs to both microtiter plate-bound CS-A and to activated platelets, suggesting a role for activated platelets in IC diseases.

Paper IV

As mentioned earlier, in paper I we assessed the physiological relevance of platelet-triggered complement activation in the fluid phase by monitoring the expression of CD11b on leukocytes, as well as the formation of conjugates between platelets and monocytes/granulocytes, both of which were increased in TRAP-activated whole blood. In paper II, we demonstrated that the C3 bound to platelets consisted of C3(H_2O), which was partially cleaved by factor I to iC3(H_2O). It is well known that these forms of C3 interact with C3 receptors such as CR1 (CD35) and CR3 (CD11b/CD18). Platelet-leukocyte conjugate formation has been studied by others and been shown to reflect interactions between platelet-exposed P-selectin and its ligand PSGL-1 on the leukocytes. CD11b/CD18 (Mac-1) has been shown to be involved in this reaction, but the ligand for the Mac-1 complex was not identified. In the current paper, we tested the hypothesis that C3 bound to activated platelets may act as a ligand for CD11b/CD18.

Blood cells were depleted of plasma proteins, and after several washing steps, C3 was added, and the leukocytes were activated with C5a and the platelets with TRAP. We found that C3 alone, in the absence of any complement activation, enhanced the complex formation between platelets and monocytes/granulocytes. The complexes were also inhibited by ~50% by the

addition of anti-CD11b mAb. This result is in agreement with what we had seen earlier in Paper I: that inhibition of complement with Compstatin or C5aR antagonist decreases the conjugate formation by ~50%.

Upon activation of platelets, microparticles (PMPs) are generated. PMPs have previously been shown to form complexes with leukocytes. Here we examined the binding of complement components to PMPs. The complement components C1q, C4, and C3 were all detected on PMPs. Also, microparticles were shown by flow cytometry and Western blotting to expose C3(H_2O). As was seen for platelets, PMPs formed complexes with leukocytes, and this complex formation could be inhibited by ~50% by the addition of anti-CD11b mAb.

General Discussion and Future Perspectives

Several studies indicate that complement activation is associated with thrombotic processes such as myocardial infarction and stroke. In our work we have shown that clotting induced by either of the coagulation pathways induces complement activation. The activation was higher in PRP and blood as compared to PPP. This notion indicates that the activation is mediated by the blood cells and since PRP almost exclusively consists of platelets, it suggested that platelets are mostly responsible for the clotting induced complement activation.

In order to study platelet-induced complement activation, we have used a system in which platelets were activated by TRAP in PRP or whole blood that had been anticoagulated with lepirudin, thus allowing us to activate the platelets without causing any clotting reaction.

A significant increase in complement activation, demonstrated as generation of C3a and sC5b-9 was seen in the fluid phase when platelets where activated in PRP or whole blood by TRAP. This could be confirmed by using a specific complement inhibitor, Compstatin, which inhibited the generation of C3a and sC5b-9. Chelation of divalent cations by addition of EDTA totally abrogated this activation, and specific chelation of Ca^{2+} with EGTA-Mg^{2+} also totally inhibited the generation of C3a and sC5b-9, excluding activation by the alternative pathway. Furthermore, the activation cannot be the result of a lectin pathway activation, since PRP from an individual deficient in MBL induced complement activation after stimulation with TRAP to the same extent as in MBL sufficient individuals.

In the present study, we have demonstrated that CS-A is a strong activator of complement, making this substance a likely trigger of platelet-induced complement activation (Fig 11). This was confirmed by using purified CS-A and supernatants from activated platelets which activated complement to the same extent as was seen using TRAP-activated platelets. Pre-incubation of both CS-A and platelet supernatant with chondroitinase ABC abrogated the complement activation. By using CS-A, which had been immobilized on a polystyrene-surface, we were also able to show that C1q, both in purified form and in whole EDTA-plasma, can bind specifically to CS. The surface-bound CS did not activate complement in C1q-depleted serum, but when purified C1q was added, the ability of CS to activate complement was restored.

However, interaction between C1q and polyanions such as heparin and DNA are well established [249]. CS is also the sole GAG released from activated platelets [185]. Interaction of C1q and CS has also previously been described [191] and was suggested to occur in plasma as a specific C1q inhibitor [192, 195]. These observations were made by measuring the ability of CS to inhibit C1q-specific hemolytic activity. Here we show that this inhibition is accomplished by CP activation, thereby depleting the plasma of intact complement components, which subsequently leads to inhibition of the hemolytic activity of C1q.

We extended our study to include the platelet surface in order to reinvestigate the reported complement activation on the surface of activated platelets [91, 210]. Del Conde and co-workers have reported complement activation and deposition of C3b on activated platelets. This activation was shown to be dependent on P-selectin expression and to be mediated by the AP [210]. Peerschke et al. have also reported that complement is activated on the surface of activated platelets. Using an assay with platelets fixed to microtiter plates with polylysine and glutaraldehyde, they demonstrated that the activation involved CP components and is partially due to expression of gC1qR on the surface of activated platelets [91].

We have demonstrated that C1q, C4, C3 and C9 binds to the surface of activated platelets (Fig 11). These components represent the whole chain of components from the start of the CP to the end of the membrane attack complex. The presence of CS-A in platelets as the main GAG has been well established by both biochemical and histological techniques [185, 188]. CS is rapidly expressed on activated platelets. It is also released and has been reported to increase the plasma concentration of CS by up to 2 μg/mL within 3 min after activation [189], which is close to what we measured in Paper I. However, it is clear that this concentration is higher in the close vicinity of the platelets enhancing the activation of complement locally around the activated platelets.

Since CS-A also is expressed on TRAP-stimulated platelets, we hypothesized the binding of C1q to platelets is mediated by CS-A. By incubating purified C1q with soluble CS-A and low molecular weight heparin, the binding of C1q was substantially inhibited. The binding of C1q to CS-A expressed on activated platelets could be further supported by using an anti-CS-A mAb, which inhibited the binding of C1q to TRAP-activated platelets by approximately 50 %. The fact that large amounts of soluble CS-A or anti-CS-A mAb are needed to inhibit binding of C1q to activated platelets, indicates that the binding of C1q to CS-A is multivalent. C1q contains six globular heads, each harbors a binding site for CS-A. The affinity of a single globular head for CS-A is not known, but that for IgG is in the range of 10^{-3}M [25]. Assuming that the affinity for CS-A is similar, the avidity (i.e., the combined affinities of the each of the engaged globular heads in binding to CS-A) is very strong, explaining the extremely high binding capacity of C1q for CS-A.

C1q has earlier been shown to function as a receptor for IC on activated platelets. C1q and HAGG were allowed to bind to activated platelets and a two-fold increase in binding of HAGG to activated platelets was observed in the presence of C1q. By inhibiting binding of C1q with CS-56 mAb a clear inhibition of HAGG was obtained, indicating that C1q bound to activated platelets via CS-A may act as a receptor for IC. Previous studies have demonstrated that the binding of IC to platelets has the capacity to activate platelets. Thus, the binding of C1q to platelets is of potential pathological importance for IC diseases such as SLE and vasculitides. During exacerbations of these diseases, the levels of IC rise, resulting in binding of IC and activation of platelets.

In platelets, the CS-A chains are attached to a serine and glycine rich protein core forming the serglycin macromolecule. Similar serglycin molecules, but with different GAG composition are found in a variety of cells, e.g. mast cells, macrophages, lymphocytes, endothelial cells etc [173]. Serglycins of different compositions have been implicated as carriers of proteases but a study comparing serglycin-binding proteins in activated macrophages and platelets failed to identify any platelet derived proteins which have been linked to complement activation [177].

Considering the fact that we do not see any complement activation in C1q-depleted serum, and that the activity is restored upon addition of purified C1q, make us believe that it is unlikely that other non-complement proteases are responsible for the activation. The situation would have been different if we had used an analogous system of C1-depleted serum being reconstituted with intact C1, where it could be theoretically possible that activation of C1r and C1s could be accomplished by other proteases. Furthermore, the activation is dependent on Ca^{2+}-ions, which suggests C1q-target interaction.

Despite the observation that C1q binds to CS and activate the CP of complement in the fluid phase, C1q binding to activated platelets does not seem to activate complement. This was supported by the fact that activation of PRP in the presence of Compstatin or an inhibitory anti-C1q monoclonal antibody did not affect the binding of C3 or C9. Nor did addition of EDTA or EGTA to PRP prior to TRAP-activation affect the binding of C3, C4 or C9. By contrast, generation of both C3a and C5b-9 in the fluid phase of TRAP-activated PRP and blood was decreased in the presence of Compstatin, EDTA and EGTA. Western blot analysis of platelet surface-associated C3 and flow cytometry using monoclonal antibodies specific for different conformational forms of C3 showed that the bound C3 consisted of $C3(H_2O)$. This observation shows that the deposited C3 was activated by disruption of the thiol ester in C3 without proteolytic cleavage. How this is brought about is unclear. The mechanism for the $C3(H_2O)$ generation is at present under investigation. In order to test whether P-selectin was involved in binding of C3 in our experimental system, the blocking polyclonal antibo-

dy to P-selectin used in the original report by Del Conde [210] was employed. In our hands the polyclonal antibody did not affect the binding of C3.

We also investigated whether the reported expression of gC1qR on activated platelets was involved in the binding of complement components, and also its ability to induce complement activation. Two monoclonal antibodies, 60.11 and 74.5.2, were used for detection and blocking of this receptor. In our model, we could neither detect nor block the function of gC1qR and therefore concluded that these antibodies had no effect on the binding of complement proteins in our model.

Our results support the notion that complement is not activated on the platelet surface under physiological conditions. The lack of surface associated complement activation may be explained by the large number of membrane-bound (MCP, DAF, and CD59) and soluble (e.g. factor H, and C1INH) complement regulators found on the activated platelet surface, which most likely protects the platelet from complement attack. The binding of factor H to activated platelets has earlier been described through interactions with GPIIb/IIIa [169] or TSP [170]. However, it is well known that factor H possesses the ability of binding to heparin and glycoaminoglycans suggesting that it may bind to activated platelets through CS-A. We have shown that C4BP is present on the surface of activated platelets and binding of both C4BP and factor H to CS-A was confirmed using CS-A mAb CS-56, which substantially inhibited this interaction (Fig 11).

Supporting this, Ståhl et al have recently shown that aHUS patients with mutated factor H have a higher deposition of C3 and C9 and complement activation on the platelets compared to controls [220] but unlike the case in normal individuals this is true for both non-activated and activated platelets. Combining aHUS patient sera containing mutated factor H with normal platelets resulted in complement activation and activation and aggregation of platelets. The complement deposition and platelet activation was abrogated when platelets were preincubated with normal factor H or when normal serum was used [220]. Platelet activation and drop in platelet counts have also been reported in paroxysmal nocturnal hemoglobinuria (PNH). Insertion of C5b-9 complexes into the platelet membrane due to diminished surface expression of DAF and CD59 results in complement activation bringing procoagulant phospholipids to the platelet surface has been reported in PNH [218]. In both of these conditions, the absence of complement regulatory function on platelets has been suggested to contribute to deposition of complement activation products and to platelet aggregation and thrombocytopenia. In healthy individuals platelets are more resistant to activation due to the expression of a normal complement inhibitors repertoire.

Combined with our previous study, these findings show that complement activation occurs in the fluid-phase in the vicinity of the activated platelet but, under physiological conditions, there is no complement activation on the

surface of the platelets. This seems logical since such activation would damage the platelets and elicit thrombotic reactions.

The physiological relevance of the platelet induced complement activation in the fluid phase was demonstrated by the up-regulation of CD11b on leukocytes and the generation of leukocyte-platelet complexes mediated via generation of C5a (Fig 11). This was confirmed by the observations that Compstatin and C5aR antagonist (partially) inhibited these functions.

Formation of the platelet-leukocyte complexes has been demonstrated in various inflammatory and thrombotic conditions [227, 228]. This complex formation is believed to be due to initial interactions of P-selectin (CD62P) on the activated leukocytes and its ligand on leukocytes, PSGL-1. Firm attachment is subsequently mediated by the interaction of CD11b/CD18 and, so far, an unknown ligand on activated platelets. Our studies have, as discussed above, shown that activated platelets bound C3(H_2O)/iC3(H_2). This form of C3 acts as a ligand for soluble CR1 (Paper II). Further we found that platelet-leukocyte complexes were enhanced by the addition of native C3. Blockage by anti-CD11b monoclonal antibodies inhibited this C3-mediated complex formation by approximately 50 %, demonstrating further tethering by interactions between C3 on activated platelets and CD11b/CD18 on the leukocytes (Fig 11).

Upon activation of platelets, PMP are generated. Our studies have also shown that PMP bound complement components in a similar manner as activated platelets do. Western blot analysis showed that the majority of the C3 bound to PMPs consisted of C3(H_2O)/iC3(H_2O). By using anti-CD11b mAb we could counteract the binding of PMPs to activated leukocytes by approximately 50%, also suggesting that PMPs form complexes with leukocytes in a similar manner as activated platelets.

These complexes may be of pathological significance and the fact that PMPs and activated platelets contain tissue factor makes our finding more interesting, since it has been shown that leukocytes, mainly monocytes, acquire TF by binding platelets and PMPs [233, 246]. This means that complement activation may be a link between platelets/leukocytes and the coagulation system in autoimmune and cardiovascular disease.

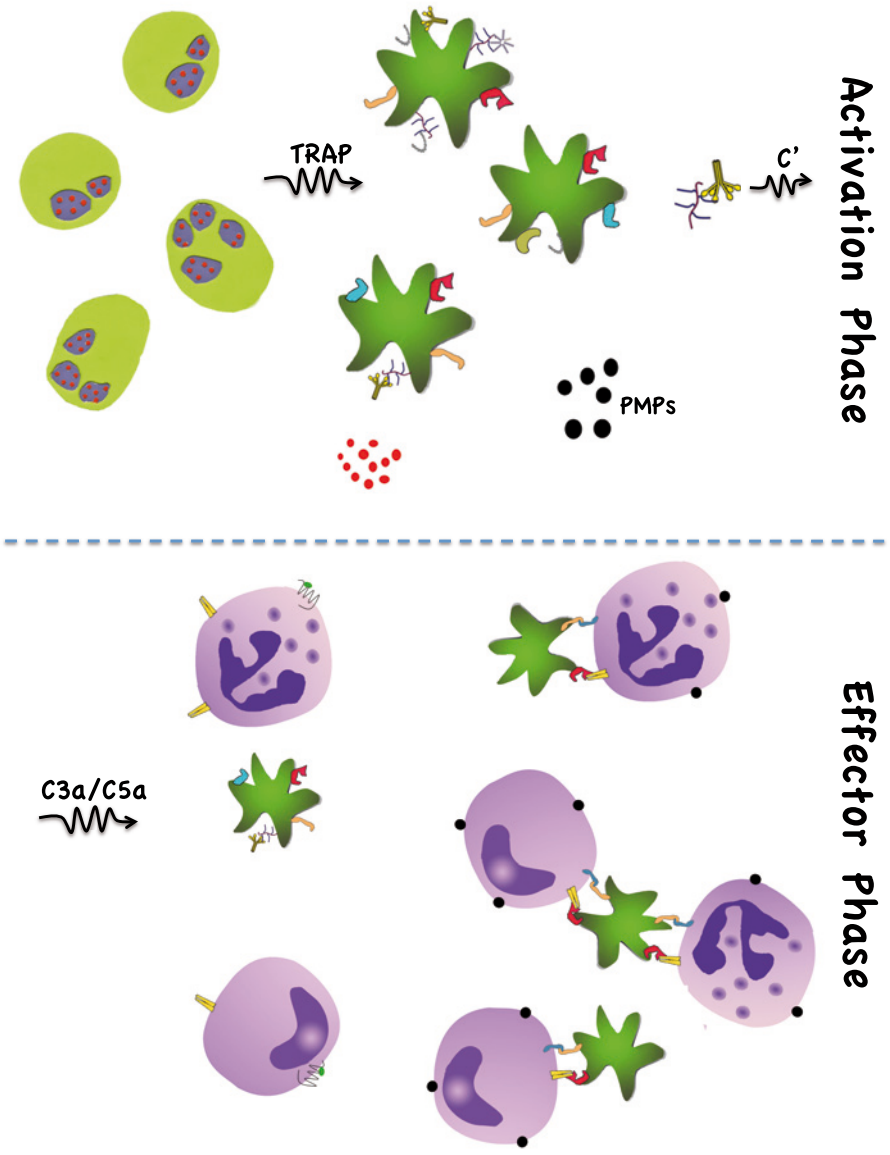

Figure 11: Schematic summary of the interaction between platelet activation, release of chondroitin sulfate, complement activation, and formation of platelet/PMP-leukocyte complexes. Further information regarding experimental setup and the interpretation of data, are found in the General Discussion section.

Complement activation is associated with several clinical conditions that involve platelet activation, e.g., SLE. Platelet activation is frequently seen in SLE patients during exacerbations. SLE patients also have increased levels of circulating ICs which has been associated with increased platelet activation through binding to Fc receptors present the on platelets. We have shown that activated platelets bind C1q, which increase the binding of ICs to activated platelets. This increased binding of ICs to platelets may have a role in the complex formation between platelets and leukocytes. Leukocytes express Fc receptors and increased binding of ICs to activated platelets may lead to further tethering of leukocytes.

We have also shown an increased formation of platelet-leukocyte complexes in the presence of C3. It will be interesting to investigate the association between TF expression on leukocytes and binding of C3 to activated platelets under pathological conditions. This may have clinical significance in SLE, since these patients often suffer from cardiovascular disease and other thrombotic complications.

Conclusions

Paper I

TRAP activated platelets activate the complement system via CP in the fluid phase by the release of CS-A, which binds C1q.

Paper II

Physiologically activated platelets bind complement proteins independently of complement activation. C3(H_2O) bound to activated platelets represent a non-proteolytically activated C3, which may act as a ligand for receptor interaction.

Paper III

The lack of complement activation on the surface of activated platelets may be associated to the binding of the complement inhibitors C1INH, C4BP and factor H.

CS-A expressed on activated platelets contributes to the binding of C1q, C4BP and factor H to the platelet surface.

Paper IV

The complex formation between platelets and leukocytes was enhanced in the presence of native C3. Platelet- and PMP-bound C3(H_2O)/iC3(H_2O) participate in this complex formation by acting as a ligand for CD11b/CD18.

Sammanfattning

Flera studier har visat att komplement-aktivering och trombotiska händelser, som är ett resultat av att koagulations-systemet aktiveras, hör ihop. Trombocyter (blodplättar) har länge misstänkts fungera som den viktigaste länken mellan de båda kaskad-systemen.

För att studera trombocyt-inducerad komplementaktivering har vi utvecklat ett system där trombocyter aktiveras med trombin receptor aktiverande peptid (TRAP) i helblod eller trombocytrik plasma (PRP), som är antikoagulerad med den specifika trombin-hämmaren, lepirudin.

Genom att frisätta chondroitin sulfat-A (CS-A) orsakar TRAP-aktiverade trombocyter en komplementaktivering i blodplasma, detekterat som bildat C3a och sC5b-9. CS-A interagerar med C1q och aktiverar komplementsystemet genom den klassiska aktiverings-vägen.

Våra studier visar även bindning av komplement-komponenterna C1q, C3, C4 och C9 till TRAP-aktiverade trombocyter. Dock beror denna bindning inte på någon proteolytisk komplementaktivering då blockering av komplement systemet på C1q- eller C3-nivå inte påverkar bindningen av komplement-proteinerna C3 eller C9. C3 som binder till aktiverade trombocyter består av C3(H_2O), vilket indikerar att C3 inte hade aktiverats genom proteolytisk klyvning. Bindningen av C1q är delvis beroende av CS-A exponering på aktiverade trombocyter.

Den uteblivna komplementaktiveringen på ytan av aktiverade trombocyter är sannolikt beroende av närvaron av flera komplement-hämmare, både naturligt cellbundna och sådana som binder in vid trombocytens aktivering. Vi kan bekräfta att C1INH och faktor H binder till ytan av aktiverade trombocyter. Vi har även sett att en annan potent komplement-hämmare, C4BP, också är associerad till ytan av aktiverade trombocyter. Bindning av faktor H och C4BP visades vara beroende på exponeringen av CS-A på ytan av aktiverade trombocyter.

Fysiologiska konsekvenser av dessa reaktioner som vi kunnat se är en ökning av uttrycket av CD11b på leukocyter samt ökad bildning av komplex mellan trombocyter och leukocyter. Trombocyter band till leukocyter genom minst två olika mekanismer: 1) aktivering av komplement-systemet vilket leder till generering av C5a som aktiverar leukocyterna; 2) genom att binda C3 (H_2O) / iC3 (H_2O), som kan fungera som ligand för det leukocytassocierade intergrinet CD11b/CD18.

De mekanismer vi har beskrivit bidrar till ökad förståelse av trombocyternas samspel med komplementsystemet. Dessa fynd kommer att öka kunskapen om komplementsystemets roll vid kardiovaskulär och trombotisk sjukdom.

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