The role of coagulation factors Protein C and Protein S in diseases

Literature review

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Abstract

The coagulation cascade consists of several factors that all contribute to the balance between bleeding and haemostasis. Protein C (PC) and protein S (PS) are two of these factors, which both are known for their anticoagulant function. Activated PC (APC) is able to cleave activated factor V (FVa) and VIII (FVIIIa), whereas PS acts as a cofactor for both anticoagulant factors PC and tissue factor pathway inhibitor (TFPI). In addition to these well-known anticoagulant functions both PC and PS have several other functions. APC is shown to be able to act anti-inflammatory and anti-apoptotic, via activation of protease-activated receptor 1 (PAR1). Via activation of Tyro3, Axl and Mer (TAM) receptors, PS has anti-inflammatory functions and is involved in angiogenesis.
As anticoagulant factors, both PC and PS are known to be involved in the coagulation-related diseases thrombosis, purpura fulminans and atherosclerosis. In addition PC and PS play also a role in inflammation-related diseases, such as sepsis, systemic lupus erythematous (SLE), human immunodeficiency virus (HIV) and non-alcoholic fatty liver disease (NAFLD). The beneficial role of PC in sepsis even led to the development of (A)PC based treatments.
Via distinct signalling routes both PC and PS enhance the blood-brain barrier (BBB) integrity and thereby decrease the risk on neurological diseases. Besides, by crossing the BBB, APC is shown to play a role in the two neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) and in stroke. At last, APC is found to play a role in cancer, but for PS the role in this disease is still unclear. Associations between PC and PS deficiencies and coagulation-related and inflammation-related diseases confirm the role of PC and PS in these type of diseases.

Keywords: Protein C, Protein S, coagulation, diseases

Table of content

Introduction

Circulation of blood is essential for live in humans and many other organisms. In case of vascular injury, haemostasis, the process which causes bleeding to stop, is necessary to keep this undisturbed circulation intact. Haemostasis consists of two stages. During the first stage, called primary haemostasis, a platelet plug is formed via adherence, activation and aggregation of platelets.[1] Without any further processing this platelet plug dissolves within several hours, leading to re-bleeding.[2] The second stage of haemostasis, or secondary haemostasis is essential for countering this loss of the platelet plug. Secondary haemostasis eventually results in the formation of a fibrin network which stabilizes the platelet plug.[1] This fibrin network is formed via the coagulation cascade. This cascade comprises several serine protease zymogens and their cofactors (table 1). Together these blood clotting factors are responsible for a complex series of enzymatic reactions. The final outcome of these enzymatic reactions of the coagulation cascade is the formation of factor II, or thrombin. Thrombin can be considered as the major player in the coagulation cascade, because this protease is actual able to convert soluble fibrinogen into the covalently cross-linked fibrin fibers which stabilize the platelet plug.[1]

Factor	Name	Abbreviation
I	Fibrinogen	-
II	Thrombin	-
III	Thromboplastin*	TF
IV	Calcium	FIV
V	Ac-Globulin, proaccelerin, labile factor	FV
VII	Proconvertin, Spca	FVII
VIII	Antihemophilic factor	FVIII
IX	Plasma thromboplastin component	FIX
X	Stuart-Prower Factor	FX
XI	Plasma thromboplastin antecedent	FXI
XII	Hageman factor	FXII

Table 1 Nomenclature factors involved in coagulation cascade. Adapted from Wright (1962).[3]
* In this report referred to as Tissue Factor (TF)

The coagulation cascade needs to be initiated to activate thrombin. In case of vascular injury this occurs via the extrinsic pathway in which factor III, or tissue factor (TF) plays a major role. TF is a transmembrane protein that is expressed on perivascular cells.[4] To become an active coagulant factor TF requires binding of its cofactor VII (FVII) that circulates in the blood. Together, TF and activated FVII (FVIIa) form the extrinsic tenase complex. This complex is able to initiate the rest of the coagulation cascade via activation of another coagulant factor: factor X (FX).

Besides the extrinsic pathway, that is associated with vascular injury, also the intrinsic pathway is able to initiate the coagulation cascade. This intrinsic pathway is mainly an *in vitro* phenomenon which is activated upon enzymatic activity on negatively charged materials, like glass.[5] Factor XII (FXII) is the enzyme that becomes activated after binding to such negatively charged materials. Once enough active FXII is formed, the next coagulation factor, factor XI (FXI), is activated by FXII, leading to initiation of the coagulation cascade. Important to note is that initiation of coagulation is not the only result of the intrinsic pathway. Upon activation of FXII also the kallikrein-kinin system is activated leading to release of bradykinin.[5] Bradykinin is peptide hormone that is known to play a role in inflammation. In other words, the intrinsic pathway is not only important in the initiation of coagulation, but has also an important role in stimulating inflammation.

To come back to the set of enzymatic reactions that is required for the formation of thrombin, the extrinsic pathway is taken as starting point. As mentioned, the extrinsic pathway results in activation of FX. Activated FX (FXa) forms together with its cofactor, activated factor V (FVa), the prothombinase complex, that is able to convert prothrombin into thrombin (figure 1).[1] Additional to the formation of fibrin, this first small amount of thrombin is also used for amplifying the amount of thrombin via a positive feedback loop. Thrombin is in fact capable of activating factor XI (FXI).[1] So, activated FXI (FXIa) arises from both this positive feedback loop and the intrinsic pathway. FXIa is then able to activate factor IX (FIX), which is in co-operation with its cofactor VIII (FVIII) able to activate FX again. In this way even more thrombin is generated and a solid fibrin network is formed.

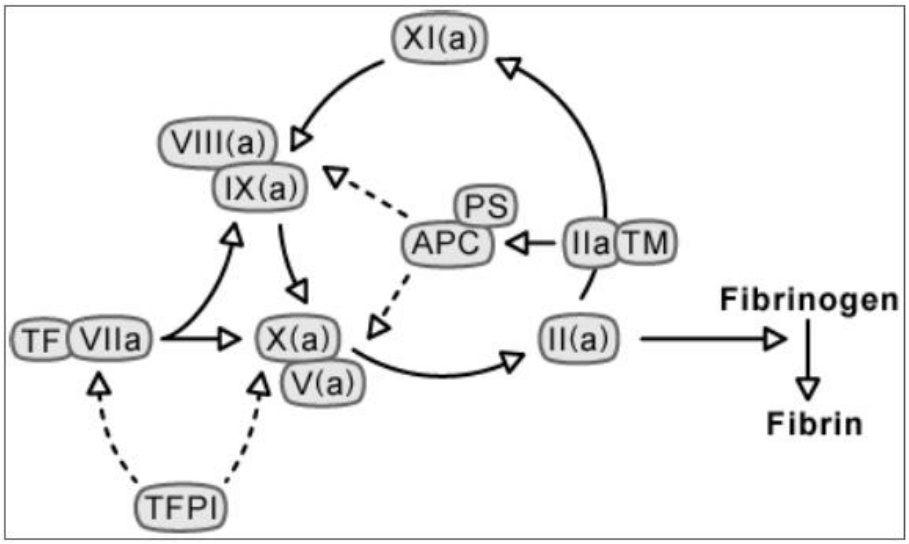

Figure 1 Schematic overview of coagulation cascade. Following the extrinsic pathway the coagulation cascade is initiated by TF. TF and activated factor VII (VIIa) form the extrinsic tenase complex, which is able to activate factor X (Xa). Next, Xa and its cofactor FVa form the prothrombinase complex, that converts prothrombin into thrombin (IIa). IIa cleaves fibrinogen into the fiber forming fibrin. Besides, IIa is also able to activate factor XI (XIa) initiating a positive feedback loop. XIa activates in turn, factor FIX (IXa), which is in co-operation with activated factor VIII (VIIIa) able to activate X again. TFPI acts as anticoagulant by inhibition of the extrinsic tenase complex and of Xa. Together with its cofactor PS, APC catalyses the inactivation of FVa and FVIIIa. **APC** active protein C; **PS** protein S; **TF** tissue factor; **TFPI** tissue factor pathway inhibitor; **TM** thrombomodulin. Adapted from C. Maas (2013) personal communication.

As described, thrombin is essential for coagulation, but excessive levels of this protease are dangerous in terms of increased thrombotic risk. Therefore strict regulation of the coagulation cascade is necessary. Not only the positive feedback loop as discussed above, but also inhibitory signals are part of this strict regulation. One example of such an inhibitory signal, or anticoagulant, is tissue factor pathway inhibitor (TFPI). Once coagulation is initiated TFPI is not only able to directly inhibit the extrinsic tenase complex, but also to inhibit FX (figure 1).[4] Two other important anticoagulant factors are protein C (PC) and protein S (PS). In presence of its cofactor PS, activated PC (APC) is able to catalyse the inactivation of FVa and activated FVIII (FVIIIa).[4] PS does not have an enzymatic anticoagulant function on its own, but acts as an anticoagulant by serving as a cofactor for both APC and TFPI.[4] Taken together, TFPI, APC and PS have an important role in the regulation of coagulation.

Besides their role in coagulation, APC and PS are shown to be involved in other processes, among which inflammation and apoptosis.[4,6,7] For this reason APC and PS might not only have a significant role in coagulation-related diseases, like thrombosis, but also in other diseases. Many studies have been performed to link specific functions of APC and PS to diseases in which these proteins seem to play a role. The goal of this thesis is therefore to give an overview of the role of both APC and PS in diseases. In the first two chapters both anticoagulant factors and their functions are described in detail. In the third chapter the role of APC and PS in several diseases is discussed.

Chapter 1 - Protein C

Activated protein C (APC) is known for its anticoagulant function, however several other, not coagulation-related functions are found for APC. In this chapter these functions will be discussed in detail, but first the structure and regulation of (A)PC are covered.

Structure of PC

The human PC gene is localized on the long arm of chromosome 2 (2q13-q14) and mainly expressed in the liver.[8,9] Around 1986, the nucleotide sequence and the presence of 9 exons and 8 introns was.[10,11] The PC gene encodes for the single-chain precursor of PC. Cleavage of the precursor leads to formation of the so-called prozymogen. Next, a second proteolytic cleavage is required to obtain the single-chain PC structure. PC however circulates mainly as a heterodimer, linked by a disulphide bond.[12] As shown in figure 2 the single-chain PC consists of a heavy and a light chain.[12,13] The heavy chain comprises the secondary structures that are typical for serine protease domains. The light chain comprises several epidermal growth factor (EGF)-like domains and a region with y-carboxyglutamic acid (Gla) residues. This Gla-rich domains are found to be highly conserved in vitamin K dependent proteins (VKDP).[12,14] Biological activity of these proteins depends on the posttranslational modification of the Gla domains. For this modification, called y-glutamylcarboxylation, vitamin K is an essential cofactor.[14]

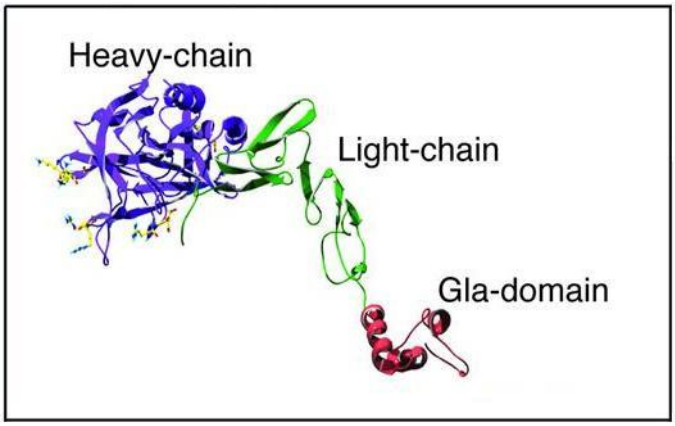

Figure 2. Crystal structure of activated protein C (APC).
APC heavy chain depicted in blue, the light-chain in green and the Gla-domain in red.
Adapted from Sarangi *et al.* (2010).[13]

Regulation of PC

PC is a serine protease zymogen, meaning that this protein has to activated before it is able to carry out its enzymatic functions. This activation of PC occurs once the coagulation cascade is initiated. One of the results of this initiated coagulation is thrombin circulating in the blood. This thrombin can be bound by the glycoprotein thrombomodulin (TM) present at the surface of endothelial cells.[15] This complex than is able to cleave the zymogen PC in the active protease APC. However activation of PC is actually found to be more complex. In 1996 endothelial cell protein C/APC receptor (EPCR) was identified as a type 1 transmembrane protein that is able to bind both PC and APC.[16] Both EPCR and TM are located at the surface of endothelial cells. So, binding of PC by EPCR enhances APC generation significantly by presenting PC towards the thombin/TM complex.[16]
Important to note is that EPCR not only enhances the anticoagulant functions of PC/APC. EPCR binds PC and APC with similar affinity meaning that APC is not per se immediately released.[16] This results in inhibition of its ability to act anticoagulant via inactivation of FVa and FVIIIa. However, this EPCR-related inhibition of APC is not considered to be the primarily way of inactivation of APC. Inhibition of

the proteolytic activity of APC is primarily achieved by serine protease inhibitors (SERPINs).[17] Especially the SERPINs protein C inhibitor, plasminogen activator inhibitor-1 and α1-antitrypsin are important for inactivation of APC.[18–20] The interaction between APC and these SERPINs is very slow. This is the reason that the circulation half live of APC is relatively long (approximately 20-25 min.) in humans.[18]

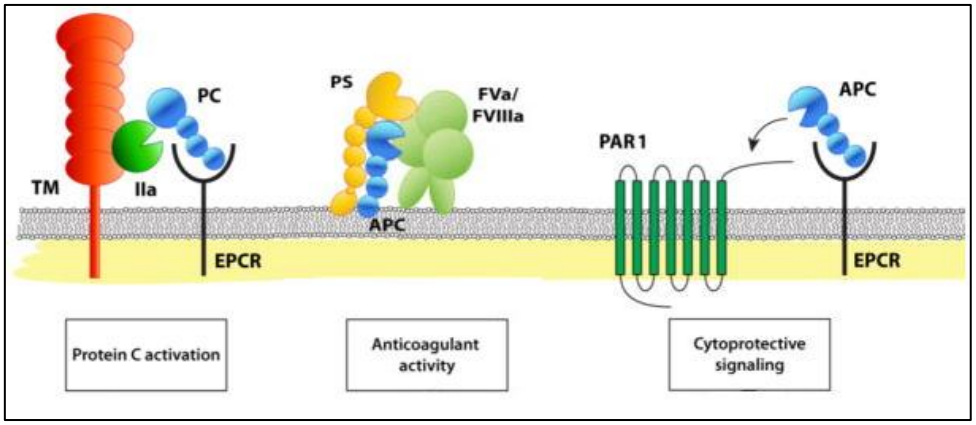

Figure 3 Schematic representation of the regulation of PC. PC activation occurs when thrombin (FIIa), bound to TM, cleaves PC, which is presented to FIIa by EPCR. Subsequently, APC performs its anticoagulant function via cleavage of activated factor V (FVa) and VIII (FVIIIa). In addition, by cleavage of PAR1 APC is able to stimulate cytoprotective signalling. **APC** activated protein C;**EPCR** endothelial cell protein C/APC receptor; **PAR1** protease-activated receptor 1; **PC** protein C;**TM** thrombomodulin. Adapted from Stavenuiter *et al.*[20]

Functions of APC

As mentioned before, APC is especially known for its anticoagulant function, which is performed via cleavage of both FVa and FVIIIa. Cleavage and thereby inactivation of FVa prevents the formation of the prothrombinase complex, resulting in low(er) levels of thrombin (figure 1). APC is able to cleave FVa at three sites: at Arg^{306}, Arg^{506} and Arg^{679}.[21] The actual place of cleavage determines whether FVa is inactivated partially (Arg^{506}) or completely (Arg^{306}). The cofactor of APC (PS) is not essential for cleavage of FV, but presence of PS does make the inactivation of FVa more efficient.[21] Then, the second anticoagulant function of APC. APC is able to cleave FVIIIa at Arg^{336} and Arg^{562} and both cleavages lead to inactivation of this coagulation factor.[22] Cleavage at Arg^{336} result in complete inactivation of FVIIIa and is therefore favoured in terms of anticoagulant functions of APC. Although PS accelerates cleavage at both sides, this cofactor has a greater effect on cleavage of Arg^{562}.

Protease-activated receptor 1

Besides this well-known anticoagulant function APC has several other, cytoprotective functions. Many of these functions are carried out via protease-activated receptor 1 (PAR1). PAR1 belongs to a family of G-protein coupled receptors that is known to transmit cellular signals that have been initiated by extracellular proteases.[23] This family of receptors becomes activated by proteases which cleave the extracellular N-terminus. PAR1 was initially known as 'thrombin receptor', because its major role in mediating thrombin signalling. However, not only thrombin, but also APC is able to cleave the N-terminus of PAR1.[20,24] Activation of PAR1 by thrombin and APC results in different functional outcome, what could be expected based on their opposite functional identity (promotion vs. inhibition of coagulation) (figure 4). PAR1 activation by thrombin, via fast cleavage at Arg^{41}, results in pro-inflammatory and endothelial barrier disruptive effects.[20,25] Whereas, APC-mediated cleavage of PAR1 at Arg^{46}, results in several cytoprotective effects.[25]

Interestingly to note is that Ludeman *et al.* showed that the cleavage of PAR1 by APC is 10^3-10^4 times less efficient compared to the thrombin-mediated PAR1 cleavage.[24] This group therefore concluded

that the probability of a physiological role of this APC-mediated cleavage is very low. About a year later, Bae and colleagues published a paper in which they provide evidence for a way in which APC is able to cleave PAR1 in presence of thrombin.[26] It is shown that all membrane bound proteins required for APC activation and APC-mediated PAR1 cleavage (TM, EPCR and PAR1) are gathered in caveolar microdomains.[26,27] APC-mediated, but not thrombin-mediated cleavage of PAR1 was shown to depend on these subtype op lipid rafts.[27] In 2009 Russo *et al.* hypothesize that these caveolae contain a subpopulation of PAR1, distinct from the non-caveolar PAR1 population that can be activated by APC. In this way APC might still be able to carry out its cytoprotective functions via PAR1 cleavage.

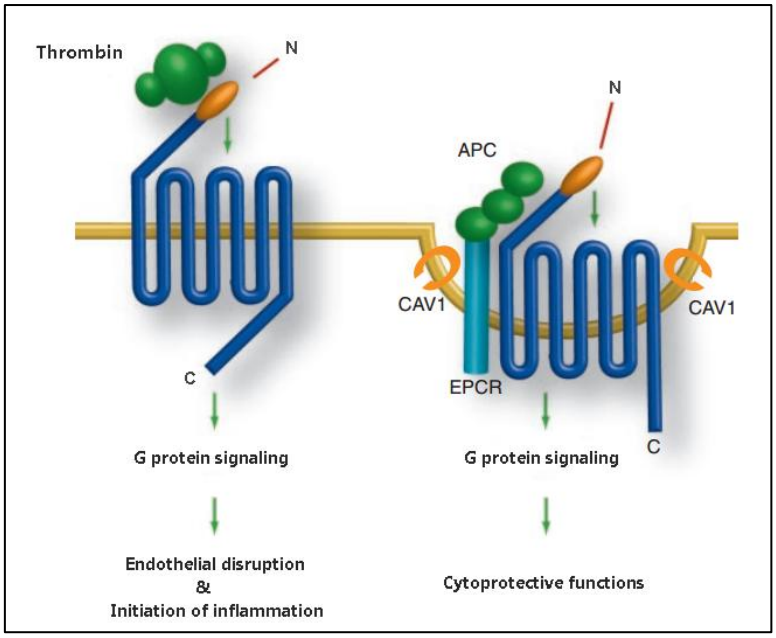

Figure 4. Schematic representation of PAR1 signalling by thrombin and APC.
Thrombin activates PAR1 signalling by cleavage at Arg[41] in the extracellular N-terminus. Thrombin-mediated PAR1 activation results, via specific G protein signalling, in endothelial disruption and initiation of inflammation. APC-mediated PAR1 signaling is initiated by cleavage at Arg[46] of the extracellular N-terminus and takes mainly place in caveolae. This PAR1 activation results via specific G protein signaling in several cytoprotective functions. **APC** activated protein C **CAV1** caveolin-1 **C** intracellulare C-terminus **EPCR** endothelial protein C receptor **N** extracellular N-terminus
Adapted from Russo *et al.* (2009)[23]

Anti-apoptotic function of APC

One of these APC-mediated cytoprotective functions is related to apoptosis. In 2003 two important studies concerning the anti-apoptotic effect of APC were published.[28,29] The first study, provided by Mosnier *et al*., showed that APC is able to inhibit staurosporine-induced apoptosis in an commercial available endothelial cell line.[29] Another important message of this paper was that both PAR1 and EPCR are required for this anti-apoptotic function of APC. The second study, published that same year also showed the importance of EPCR and PAR1 in the anti-apoptotic function of APC.[28] In this study Cheng *et al.* showed that APC inhibits apoptosis in endothelial cells that have been isolated from the brain of young adults that suffered from trauma. Moreover, Cheng and colleagues did not only show the essence, but also a possible mode of action of APC in inhibiting apoptosis in these hypoxic brain endothelial cells (BECs). In the presence of APC BECs show reduced p53 levels, and thereby a down-regulation of the pro-apoptotic factor Bax and an up-regulation of the anti-apoptotic factor Bcl-2.[28] Taken together APC is shown to inhibit (p53- mediated) apoptosis in endothelial cells both *in vitro* and *ex vivo*.

Anti-inflammatory function of APC

Besides this anti-apoptotic action of APC, this protein is also known to have several anti-inflammatory functions. One of those is affecting invasion of immune cells into tissues. To carry out their inflammatory function leukocytes need to invade into tissues, after adherence to endothelial cells. Integrins, such as intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1) are required for translocation across the endothelial barrier. In 2009, Elphick *et al.* published a study in which they investigated the effect of APC on neutrophil adherence and migration using a neutrophil adhesion assay.[30] This study showed that a recombinant form of human APC inhibited neutrophil adhesion and migration on proteins of the extracellular matrix. Moreover, Elphick and colleagues also showed the importance of the neutrophil integrins in this effect of APC. Both *in vitro* and *in vivo* experiments showed that the Arg-Gly-Asp (RGD) sequence of APC directly binds to β1 and β3 integrins present on neutrophil surface.[30] Four years later the molecular interaction between APC and the binding site of these integrins was visualised in a 3D structure, confirming the finding of Elphick *et al.*[31] Next to this protein-protein interaction of APC with integrins there is one study that claims that APC also has an effect on integrins at transcriptional level.[32] Authors of this study claim that treatment with APC leads to inhibition of VCAM1 in a human endothelial cell line. Other studies that validate these results, or found this transcriptional effect *in vivo* were not found.

A second anti-inflammatory function of APC is triggering endothelial permeability. During inflammation endothelial permeability increases in order to recruit immune cells out of the blood into the tissues. APC is found to enhances the endothelial barrier function. Finigan *et al.* showed that APC initiates rearrangements of the cytoskeleton of lung endothelial cells *in vitro*.[33] In that particular study not only this effect on vascular integrity, but also the involvement of Sphingosine 1-Phosphate1 ($S1P_1$) was shown. $S1P_1$ is a receptor for Sphingosine 1-Phosphate, which is a phosphorylated lipid growth factor that is among others known for its role in enhancing the integrity of the endothelial monolayer.[34] So, via transactivation of $S1P_1$ APC is able to reduce endothelial permeability and thereby indirectly act anti-inflammatory.[33]

A second indirect anti-inflammatory function of APC is the inhibition of thrombin-mediated cleavage of PAR1, via reduction of thrombin levels.[25] Interestingly, two separate studies published in 2000 showed that APC is also able inhibit proinflammatory signals on a thrombin independent manner. First, White *et al.* showed that treatment of APC reduces translocation of NFκB towards the nucleus in a human monocyte cell line.[35] One would expect that as a consequence of reduced translocation of this transcription factor proinflammatory effects are inhibited. Indeed, White and colleagues show that lipopolysaccharide (LPS) stimulated monocytes treated with APC excrete less tumor necrosis factor α (TNFα), an important proinflammatory cytokine, compared to LPS stimulated untreated monocytes.[35] This decrease in TNFα secretion by LPS stimulated monocytes was validated by the second paper. In that particular study both proinflammatory cytokines TNFα and macrophage migration inhibitory factor (MIF) were found to be down-regulated in LPS-stimulated monocytes upon treatment with APC.[36] The authors of this paper however mention that this effect was only achieved with APC concentrations far beyond physiological levels or in absence of serum. Presence of an 'endogenous serum-derived APC inhibitor' was suggested to be the explanation for this finding. Based on the facts that the efficiency of PAR1 cleavage is much higher and that its effects are shown to be opposite to that of APC, thrombin could be such an 'endogenous serum-derived APC inhibitor'.

Chapter 2 Protein S

As mentioned in the introduction Protein S (PS) has besides its well-known anticoagulant function other actions. Before these not coagulant-related functions are described the structure and regulation of PS are elaborated.

Structure of PS

Research on PS started around 1977, because that was the year in which PS was isolated for the first time.[37] PS is found to be mainly expressed by hepatocytes, but also many other cell types, among which endothelial cells, Leydig and Sertoli cells, osteoblasts and T cells, synthesize and secrete PS.[38] The gene encoding for PS contains 15 exons and 14 introns and is located at chromosome 3.[39,40] The first two exons code for a signal peptide and a pro-peptide which need to be cleaved to activate PS.[38] The other exons code for several domains of the protein (figure 5).

In short PS can be described as a 75 kDa single-chain glycoprotein which exists out of 653 amino acids.[38] The authors of the paper that described the isolation of PS in 1977 hypothesized that this 'newly purified human plasma protein' might be a VKDP.[37] As shown in figure 5, PS contains a N-terminal Gla-domain which indeed is a characteristic of VKDPs (see chapter 1).[41] Using this Gla-domain PS is able to bind negatively charged phospholipids present in cell membranes.[38] Directly next to this Gla-domain PS contains the so-called tenase sensitive region (TSR).[38] This region is formed by a disulphide-bridged thumb loop which is susceptible for cleavage. The third region comprises four EGF-like domains. Ca^{2+} induced conformational changes in these domains are thought to be responsible for improved anticoagulant function of PS.[38] The region closest to the C-terminal end of PS is a sex hormone binding globulin-like domain (SHBG) which consists out of two laminin G-like (LG) domains.[42] This SHBG region is known to mediate the biological activities of PS.

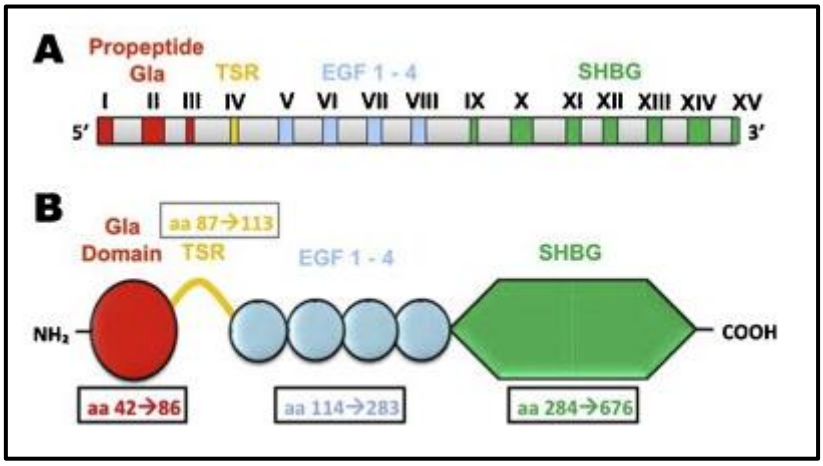

Figure 5. Schematic representation of Protein S (PS). The PS gene (A) exists of 15 exons and 14 introns. Together these exons code for in total 4 different regions of PS. Mature PS (B) contains a N-terminal Gla-domain which is characteristic for VKDPs. The second region comprises the TSR, formed by disulphide-bridged thumb loop. Then 4 EGF-like domains form the third region. The fourth region comprises a sex hormone-binding globulin-like domain (SHBG) consisting out of two laminin G-like domains. **EGF** epidermal growth factor-like domain; **Gla** γ-carboxyglutamic acid domain; **SHBG** sex hormone binding globulin-like domain **TSR** tenase sensitive region domain; **VKDP** vitamin K dependent protein
Adapted from Suleiman *et al.* (2013)[38]

Regulation of PS

PS expression is regulated at transcriptional level. Binding sites of several transcription factors, such as sp1 and FOXA2, were found in the promotor region of the PS gene.[43] Especially the effect of sp1 on the expression of PS has been investigated intensively. De Wolff *et al.* showed in 2006 that the PS promotor has four sp1 binding sites and that upon binding sp1 is able to trans-activate PS expression.[43] The finding of a mutation in the sp1 binding site in the PS promotor in a PS deficiency-related thrombotic patient, confirms the importance of this transcription factor in regulating PS expression.[44]

The second way to regulate PS is the cleavage of the TSR domain, which is known to inhibit PS function. For a long time it was thought that both thrombin and FXa are the factors responsible for this cleavage.[38] However a Dutch research group showed both *in vitro* and *in vivo* that not thrombin and FXa, but proteases derived from platelets are essential for TSR cleavage in PS.[45,46] This means that PS is not inhibited by stimulation of the coagulation cascade, but by increased presence of platelets i.e. haemostasis.

Besides these transcriptional and post-transcriptional levels of regulation PS function is also regulated at protein level. In human plasma only 40% of the total PS levels circulates in its free form.[47] The other 60% PS circulates bound to C4b-binding protein (C4BP). C4BP is a protein involved in the complement system, where its main function is to act as cofactor for the degradation of the classical complement factor C4b.[47] C4BP can have several phenotypes, consisting of 6-7 α-chains and one or none β-chains. Only the β-chains of C4BP contain the PS-binding domain. Therefore PS is only able to form a non-covalent complex with β chains positive C4BP.[38,47] The exact C4BP-binding site of PS is not known precise yet, but the LG domains in the SHBG domain are the main suspects.[47] It is plausible that binding of PS to C4BP with the domain that is important for its biological activity does affect its function.

Functions of PS

Just like APC, PS is mainly known as anticoagulant factor. PS however does not have direct anticoagulant actions on its own, but acts as a cofactor for both APC and TFPI. As cofactor for APC, PS assists in the cleavage and thereby inactivation of FVa and FVIIIa, leading to reduced thrombin levels. The role of PS in this process is visible in the changing rates of these cleavages in presence or absence of PS (see chapter 1).[21,22,47] In general PS increases the efficiency of the APC-mediated cleavage of FVa and FVIIIa.

The second way in which PS acts as an indirect anticoagulant is via TFPI. TFPI is among other able to inhibit FXa (see introduction).[4] This inhibition occurs via the formation of a reversible complex between TFPI and FXa. PS is shown to decrease the dissociation constant (K_i) of the binding between these TFPI and FXa, resulting in higher binding affinity and therefore more FXa inhibition.[48] Recently, the LG1 subunit in the SHBG domain of PS was found to be important for this function via interaction with TFPI.[49]

TAM receptor

Besides these two anticoagulant functions, PS is known to have several other functions and just like APC these functions are carried out via a specific type of receptors. In 1995 Stitt *et al.* showed that PS serves as ligand for the then still orphan TAM receptors.[50] These receptors are a family of receptors protein tyrosine kinases (PTK), including the members Tyro3, Axl and Mer.[51] Many cell types, among which macrophages, natural killer (NK) cells and Sertolli cells express TAM receptors. Upon binding of

their ligand these TAM receptors heterodimerize and become activated. In addition to PS, also growth-arrest-specific 6 (Gas6) functions as a ligand for TAM receptors. This Gas6 is a VKDP structurally closely related to PS.[7] Although Gas6 is thought to activate all three members of the TAM family, PS is found to only activate Tyro3 and Mer.[7,51] Activation of TAM receptors results in phosphorylation of the PTK domains. These phosphorylated tyrosine residues then initiate a whole signalling cascade resulting in several biological actions.

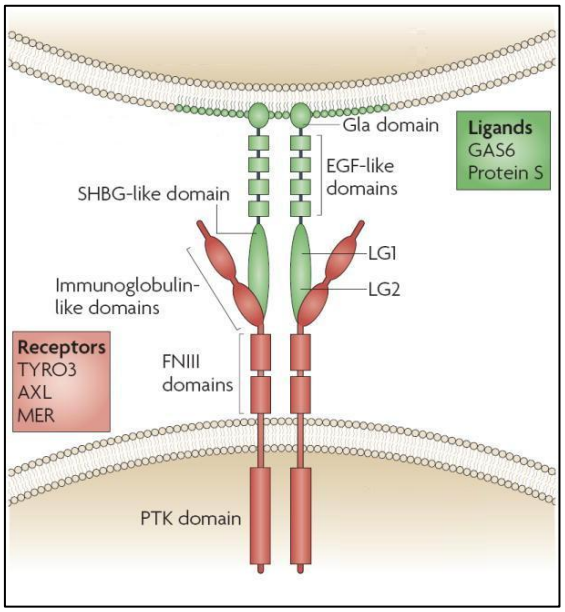

Figure 6. Schematic representation of TAM receptors and ligands. Tyro3, AXL and MER form the TAM receptor family which is identified as PTK. The immunoglobulin-like domain at the extra-cellular N-terminus is able to interact with the LG domains in the SHBG region of the ligands (GAS6 and PS). Upon ligand binding TAM receptor heterodimerize and auto-phosphorylation of PTK domains is stimulated. Both GAS6 and PS are bound to negatively charged phospholipids at the cell membrane via their N-terminal Gla domain. **EGF** epidermal growth factor; **FNIII** fibronectin type III; **GAS6** growth-arrest-specific 6; **Gla** y-carboxyglutamic acid; **LG** laminin G; **PS** protein S; **PTK** protein tyrosine kinase; **SHBG** sex hormone binding globulin Adapted from Lemke and Rothlin (2008)[51]

Anti-inflammatory function of PS

One of the biological actions PS-mediated TAM receptor activation results in, is inhibition of inflammation. In 2007 it was shown that activated TAM receptors initiate a signalling cascade that eventually inhibits toll-like receptor (TLR) –and cytokine driven immune responses.[52] A role for PS in this TAM-mediated anti-inflammatory effect was shown 6 years later. Dendritic cells (DCs) have a major pro-inflammatory role by presenting antigens and thereby activation of i.e. T cells. Carrera Silva *et al.* showed that such activated T cells express PS.[53] This T-cell derived PS is then able to activate TAM receptors present at the membrane of DCs.[53,54] Subsequently this PS-mediated TAM receptor activation in DCs results in inhibition of pro-inflammatory signals.[52,54]

Besides this inhibition of immune responses PS is also able to prevent the initiation of such responses. Remnants of death cells can initiate an immune response. These immune responses can be avoided by processing these remnants neatly. PS-mediated TAM receptor is shown to play an important role in this processing by initiating phagocytosis of apoptotic cells.[7,51] TAM receptors, activated by Gas6, were already known to play a role in clearance of apoptotic cells.[55] In 2003 Anderson *et al.* showed that purified PS was able to initiate macrophage phagocytosis of apoptotic cells, whereas PS-free plasma lacks this ability.[56] Together these findings suggest that apoptotic cells initiate macrophage phagocytosis by activation of TAM receptors on macrophage cell membrane via expression of PS on their cell membrane.

PS function in angiogenesis

At last PS might also have a role in angio –and vasculogenesis. Recently, Franineau *et al.* published a paper in which they show that PS is able to inhibit the formation of new blood vessels.[57] In this particular paper evidence for a inhibitory effect of PS-mediated Mer activation on vascular endothelial growth factor (VEGF-A) mediated angiogenesis in endothelial cells is provided. Although this evidence on the anti-angiogenic properties of PS appears to be quite convincing literature states that more research is required.[7] This statement is mainly based on former findings on angiogenic properties of the Axl receptor, as reviewed by Suleiman *et al.*[7] Noteworthy in the studies discussed in this review is that many of them are focused on the embryonic situation. Moreover, the majority of the reviewed studies are aimed at Axl and as mentioned before Axl is not the main TAM receptor target for PS. Taken together, in order to sort out what the actual effect of PS on blood vessel formation is, more research should be carried out.

Chapter 3 – Protein C and Protein S in diseases

In the previous two chapters the structure, regulation and functions of PC and PS have been discussed. Both proteins are shown to be involved several biological processes. For that reason both PC and PS play a role in several diseases. In this chapter the roles in those diseases are discussed.

Coagulation-related diseases

Thrombosis

For both PC and PS deficiencies are known (see latter paragraph).[58,59] Because both proteins have an important role in suppressing coagulation, a logic consequence of these PC and PS deficiencies is a pro-coagulant state and therefore an increased risk on coagulation-related disorders. Venous thromboembolism (VTE) is an example of such a state. VTE is a complex, multi-factorial disease that is characterized by the formation of a blood clot that obstructs veins.[60] The genes for PC and PS are shown to be related to VTE. In both genes several mutations that lead to deficient proteins are identified as cause for VTE.[61–63]

In contrast to the convincing role of PC and PS in VTE, the role of these proteins in arterial thrombosis and myocardial infarction is still a controversial subject. Several papers that describe PC deficient patients that suffer from arterial thrombosis and/or myocardial infarction are published[64,65], but research focused on the underlying mechanism remains to be done.

Purpura fulminans

Another coagulation-related disease that is associated with PC and/or PS deficiency is purpura fulminans (PF). PF is a fast developing thrombotic disorder characterized by necrosis of the skin.[66] In a later stage multiple-organ failure and severe VTE increase the mortality of this disease. Following severe acute infections, homozygous PC deficiency is the major cause of PF. In case of this homozygous PC deficiency, PF symptoms develop only a few hours to a couple of days after birth.[66,67] Interestingly, oral anticoagulant treatment, a tool to reduce the risk on thrombosis, can in combination with heterozygous PC deficiency also lead to PF-like symptoms.[68]

About the role of PS in PF is not much known yet, probably mainly due to the fact that homozygous PS deficiencies are rare.[66] In 1990 however the first PF case caused by PS deficiency was described.[69] Only 23 years later a second case was published.[70] In addition to this low prevalence, the high mortality of PF makes it difficult to become more knowledge about the role of PS, but also of PC in PF.

Atherosclerosis

The last coagulation-related disease that will be discussed in relation to PC and PS is atherosclerosis. In 2008 Thorp *et al.* showed that mice in which the Mer receptor was depleted, macrophages were less efficient in clearing apoptotic cells in atherosclerotic plaques.[70] Subsequently, the plaque progression and therefore the progression of atherosclerosis was accelerated. However, the authors of this paper only mention Gas6 as being a Mer ligand, meaning that PS is not per se related to these Mer-mediated actions in atherosclerosis.

On the other hand, the fact that PS levels are increased in human atherosclerotic arteries, implies a role of this protein in atherosclerosis.[71] In 2009 Liao *et al.* presented a study in which they present a role for PS in regulating macrophages.[71] Macrophages play an important role in the course of

atherosclerosis by taking up potential harmful lipids. At first this is a protective mechanism, but once macrophages can no longer handle (the amount of) these lipids, these cells actually start to accelerate the progression of atherosclerosis. Liao *et al*. showed that via activation of Mer, PS is able to down-regulate lipid uptake via and the transcription of scavenger receptor A (SR-A) *in vitro*. This indicates a beneficial role of PS in atherosclerosis.

The two previously described papers only include research on PS in atherosclerosis performed *in vitro* or in mice. In 2011 Hurtado *et al.* provided evidence for the role of PS in this disease in humans.[72] This group investigated RNA and protein expression of Gas6, PS and TAM-receptors in human carotid arteries with different degrees of atherosclerosis. The most important finding presented in this paper is that the protein expression levels of both PS and Mer were increased in atherosclerotic arteries compared to normal arteries.[72] In addition, they found that PS and Mer both were expressed in macrophages. Taken together, these results imply that the *in vitro* results found by Liao *et al*. might also apply to humans.

Inflammation-related diseases

Sepsis

Both PC and PS have an anti-inflammatory effect (see chapter 1 and 2). Therefore, a couple of the diseases in which these proteins are found to play a role in are linked to inflammation. The best-known inflammation-related disease in which at least PC is known to be involved in, is sepsis. A relation between PS and sepsis has not been described yet. Sepsis is described as a complex series of events which are the consequence of an excessive stimulation of the immune system after an infection.[73,74] In worst case scenarios sepsis can develop into severe sepsis, of which acute organ failure is a characteristic and which is lethal for 30-50% of the patients.[74] Already in 1984 the hypothesis that an thrombin-induced anticoagulant factor protects against an endotoxin infusion, which is a model for sepsis, was raised.[75] Three years later the same group of researchers identified this thrombin-induced anticoagulant factor to be PC.[76] In that particular paper they showed that in baboons injected with exogenous PC, symptoms of sepsis, induced by infusion of a lethal concentration of E. coli were prevented. Apparently PC has a protective role in sepsis. This beneficial role of PC in sepsis is confirmed by the fact that PC levels in septic patients are decreased and that low levels of PC are correlated with increased mortality.[74]

The finding of this beneficial effect of PC in sepsis led to the production of a human recombinant activated protein C called Drotrecogin alpha (DA). In 2001 Bernard *et al.* showed in a phase III clinical trial that treatment with DA leads to a 19,4% reduction of relative risk of death in patients suffering from severe sepsis.[74] Even though this group of researchers found that the risk of bleeding in the DA treated patients was increased, this human recombinant PC seemed to be a promising treatment to reduce mortality in severe sepsis. For the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) this latter finding was convincing enough to approve DA to treat severe sepsis in patients with high risk on death.[77] In 2008 a second clinical trial on the effects of DA in patients suffering from severe sepsis was started, because some doubts about the reliability of the results of the first trial existed.[78] In this second study Ranieri *et al.* were not able to reproduce the mortality reducing effects of DA in patients suffering from severe sepsis. Together with the high bleeding risk found in DA treated patients the result provided by Ranieri and colleagues led to worldwide withdrawal of DA from the market.[79]

However, after the withdrawal of DA, research after the beneficial role of PC in sepsis was not completely abolished. Recently, Piccin *et al.* showed that treatment with Ceprotin®, a human plasma-derived non-activated Protein C concentrate, in children suffering from severe sepsis is well tolerated.[80] In this particular study low mortality, no bleeding complications and reduced limb

amputation are shown after treatment with Ceprotin®. Promising results, but whether future studies will confirm these findings remains to be seen.

Systemic lupus erythematous

A second inflammation-related disease which is found to be related to PC and PS is systemic lupus erythematous (SLE). SLE is characterized by chronic auto-immune responses which affect multiple organs.[81] Interesting in respect to PC and PS is that patients suffering from SLE have a high risk on developing venous thromboembolism. Apparently, both inflammation and coagulation are involved in the development of SLE. For this reason, PC and PS, which both have anticoagulant as well as anti-inflammatory properties, might be involved in SLE. Evidence for this hypothesis was supplied by Meesters *et al.* In 2007 this research group presented a study in which they investigated several inflammation and coagulation markers in an age –and sex-matched cohort of SLE patients and controls.[81] An interesting finding of this study is that SLE patients showed a comparable level of thrombin generation in presence of increased levels of APC compared to controls. In addition, levels of VCAM1 were increased in SLE patients. These two findings suggest an increased APC resistance in SLE patients. As cause of this APC resistance the authors of this article come up with the lowered free PS levels that were found in SLE patients.[81] In 2010, the finding that PS might have an important role in SLE was reasserted. Circulatory levels of free PS did not differ significantly between SLE patients and controls, but a negative correlation between levels of free PS and disease activity was shown.[82] Reduced levels of free PS, possibly subsequent to inflammation induced up-regulation of C4BP, result in decreased activation of PC, TFPI and TAM receptors. Subsequently, this might lead to worsening of SLE by reduced suppression of haemostasis and inflammation.

Human immunodeficiency virus

Another illness in which the interplay between haemostasis and inflammation seems to play a role, is human immunodeficiency virus (HIV) infection. As the name states an HIV infection can be described as an immunodeficiency disease, but also many other phenomena are observed including haematological abnormalities.[83] One example of such a haematological abnormality is the altered presence and functionality of PS. In 1991 a link between HIV and PS was hypothesized, based on the fact that in a third of the HIV patients an acquired PS deficiency was found.[84] Around 1994 two papers concerning the cause of this acquired lack of PS in HIV patients were published.[84,85] Both papers came up with the presence of certain auto-antibodies directed against PS. Not only the anti-PS, but also the anti-cardiolipin auto-antibody, were shown to correlate with reduced PS levels in HIV patients. It is thought that PS deficiency in HIV patients is caused by binding and subsequently clearance of PS by these auto-antibodies.[83] As mentioned in the previous section also inflammation induced changes in C4BP levels might be an explanation for the altered PS levels in HIV patients. However, studies focused on this potential cause of the PS deficiency in HIV patients failed to show a relation between PS and C4BP levels.[86,87]

Besides the clear role of PS in HIV infections, also some studies describe HIV patients with an acquired PC deficiency.[88,89] On the other hand, also data that shows unchanged levels of PC in HIV patients is published.[90] Therefore, the role of PC in HIV infections is uncertain. Neglecting this uncertainty, Baker *et al.* proposed an interesting perspective on the involvement of PC and other coagulant factors in HIV.[91] Liver failure is common amongst HIV infected patients, partially due to the prolonged use of potential toxic drugs.[92] PC is mainly produced in hepatocytes (see chapter 1). Baker *et al.* suggest that due to the HIV-induced liver dysfunction PC (and other coagulant factors) levels are reduced.[91] Reduced levels of PC might subsequently contribute to the inflammatory and thrombotic state as observed in HIV.

Non-alcoholic fatty liver disease

The last inflammation-related disease that will be discussed in the light of PC and PS is non-alcoholic fatty liver disease (NAFLD). NAFLD actually consists of several stages, including steatosis, steatohepatitis (NASH), fibrosis and cirrhosis.[93] Besides these liver-related disorders, NAFLD is associated with several other metabolic disorders, among which insulin-resistance.

In 2005 Assy *et al.* showed that PC levels in NAFLD patients in the steatosis and in the NASH stage are increased compared to healthy controls.[94] The two latest stages of NAFLD, fibrosis and cirrhosis, were not taken along in this study. In that same year the same group of researchers published another paper in which the primary results were validated.[95] In addition, a correlation between levels of PC and amount of infiltrated fat in the liver was shown. In 2010, a third study concerning PC levels in NAFLD patients was published. Kargili *et al.* studied several coagulant factors in NAFLD patients.[96] In this study no significant changes in PC and PS levels were found. The fact that this research group did not further distinguish between the different stages of NAFLD might be an explanation for this finding. Recently a new paper concerning coagulation in NAFLD was published. In this paper Tripodi *et al.* showed a significant decrease in PC level in NAFLD patients at the cirrhosis stage compared to controls.[93] No difference in the PC level was found in the steatosis or NASH stage. Taken together these four papers implicate that the level of PC changes along the progression of NAFLD. It appears that PC levels increase during the first two stages and decrease in the last two stages. Assy *et al.* came up with a possible explanation for increased PC levels in the first two stages.[94,95] As mentioned before, NAFLD is associated with insulin-resistance.[93] A consequence of this insulin-resistance is that overall liver synthesis increases, including PC synthesis.[94,95] Studies to confirm this hypothesis, and to gain more knowledge on the mechanism behind the decreased PC levels in the latter two stages of NAFLD and the effect of these changing PC levels are required.

As shortly mentioned in the previous paragraph also PS has been studied in relation to NAFLD. Just like the result for PC in NAFLD, Kargili *et al.* were not able to show a significant difference in the PS level between (non-specified) NAFLD patients and controls.[96] On the other hand Assy *et al.* showed a significant increase in the level of PS in both steatosis and NASH stage compared to healthy controls.[95] Another interesting finding of this latter study was the relation between PS levels and fibrosis. It appeared that PS levels were decreased in NAFLD patients with severe fibrosis compared to NAFLD patients with normal fibrosis. Assy *et al.* mention advanced fibrosis-induced decreased liver synthesis as a potential mechanism behind this finding. However, they also mention that more research is required to sort out whether the decreased PS level is a cause or an effect of severe fibrosis.

Neurological diseases

The human central nervous system (CNS) is separated of the rest of the body by the blood-brain barrier (BBB). Only substances that are able to cross this BBB are able to interact or to affect the CNS. Deane *et al.* showed that APC, assisted by EPCR, is able to cross the BBB.[97] Interestingly, this study showed that the transport PC across the BBB was significantly lower in affinity. The fact that brain do not express thrombin and TM and therefore lacks the ability to activate PC, the transport of already activated PC (APC) across the BBB is preferable.

Whether PS is able to cross the BBB has not yet been described in literature. However, an important role for PS on the integrity of this BBB is shown by Zhu *et al.*[98] This group of researchers showed that after PS-mediated Tyro3 activation, $S1P_1$ is activated. As discussed in chapter 1, $S1P_1$ is a receptor involved in regulating the integrity of endothelial monolayers. In respect to the BBB, activation of $S1P_1$ results in cytoskeletal rearrangements, which protect the BBB integrity.[98] By protecting the

integrity of the BBB, PS protects the CNS against potential harmful peripheral influences and might thereby decrease the risk on neurological diseases.

Neurodegenerative diseases

To come back to the role of APC in neurological diseases, neurodegenerative diseases are interesting to start with. Amyotrophic lateral sclerosis (ALS) is an example of such neurodegenerative diseases. A common used murine model for ALS is one in which mice have a mutated form of superoxide dismutase-1 (SOD1).[99] In 2009, Zhong *et al.* showed that in this ALS mouse model treatment with APC slows disease progression and extends survival.[99] This beneficial effect on ALS arises from PAR1 (and PAR3)-mediated translocation of the transcription factor SP1, resulting in down-regulation of SOD1. Based on this SOD1 silencing function of APC treatment Zhong *et al.* propose APC as a promising treatment for ALS patients.

Another neurodegenerative disease that is interesting in relation to APC is multiple sclerosis (MS). MS is a disease in which both inflammation and neurodegeneration play a role.[100] In 2008 a proteomic analysis of MS lesions identified APC as factor involved in this disease.[100] The same paper also showed that in mice with experimental autoimmune encephalomyelitis (EAE), a model for MS, treatment with APC significantly reduced disease severity and pro-inflammatory cytokine production. Five years later Alabanza *et al.* studied the effect of APC on EAE mice in more detail by injecting these mice with a blocking antibody that inhibited the endogenous PC and APC.[101] Surprisingly, this (A)PC level reducing therapy also resulted in attenuated disease severity. In addition to this finding, Alabanza and colleagues discovered two other interesting results of the anti-(A)PC treatment. First of all, they found an increased infiltration of leukocytes in the brain.[101] Based on the inhibiting effect of APC on invasion of immune cells (see chapter 1) that is blocked by the anti-(A)PC antibodies this finding is not that surprising. Secondly, Alabanza *et al.* found that myeloid-derived suppressor cells (MDSCs), a subset of $CD11b^+$ cells, were increased in the periphery of anti-(A)PC treated EAE mice. MDSCs are known as potent T cell suppressors and indeed $CD4^+$ T cell levels were reduced in anti-(A)PC treated EAE mice.[101] Based on these results it seems that this anti-inflammatory effect of MDSCs only occurs in absence of APC. Interesting to note is that the latter effect only could be found when mice were treated with anti-(A)PC antibodies prior to the EAE induction, but not when the anti-(A)PC antibody was given once mice already suffered from EAE. This implicates that APC has specific effects at different stages of the disease. Altogether, targeting APC in MS led to conflicting results concerning its effect on inflammation. Therefore more research concerning the exact mechanisms of APC in the different stages of MS is required to see whether APC might function as a target to treat MS.

Stroke

Besides neurodegenerative diseases, stroke is another neurological disorder in which PC is found to play a role in. Stroke can be described as an event that occurs as consequence of interruption of the blood supply to the brain.[102] This interruption of blood supply is often caused by occlusion of blood vessels by a blood clot. For this reason strokes can also be described as a coagulation-related disease. However, Shibata *et al.* provided evidence for the fact that the cytoprotective functions of APC (see chapter 1) are involved in strokes as well.[103] This research group showed that treatment with APC reduced the infarcted area in a murine model of stroke. Not only APC its anticoagulant function, but also its anti-inflammatory appear to be the underlying mechanism for this beneficial effect in stroke. Compared to non-treated ischemic mice, APC-treated ischemic mice showed a reduced infiltration of neutrophils, what is probably due to the reduced expression of ICAM1 that was also shown.[103] In addition to this anti-inflammatory role, also APC its anti-apoptotic role might play a role in stroke. In chapter 1 the study of Cheng *et al.* was already mentioned. In this study hypoxic BECs were used to study the anti-apoptotic role of APC.[28] BECs can become hypoxic as a consequence of stroke,

implying that the anti-apoptotic effect of APC found in these cells might also contribute to the smaller infarcted area found in APC treated stroke subjects.

The previously discussed papers only concern *in vitro* or murine studies. That PC also plays an important role in strokes in human can be supported by the finding of a PC (and PS) deficient patient that suffered form an ischemic stroke.[102] Except for papers that describe PS deficient patients that suffered form (ischemic) stroke[102,104], no literature concerning the role of PS in strokes is available. Based on the anticoagulant and cytoprotective properties of PS (see chapter 2), one might imagine that PS, just as PC has a beneficial role in stroke, but more research is required to confirm this hypothesis.

Cancer

Already since 1823 haemostatic abnormalities were found to be present in cancer patients.[105] Many years later anticoagulant treatment in cancer patients, even without these haemostatic abnormalities, showed to improve the survival of these patients.[106] The mechanism behind this beneficial effect of anticoagulant therapy in cancer patients was initially found by studies focused on thrombin. In 1992 Nierodzik *et al*. showed that thrombin is able to enhance the binding of tumour cells to platelets *in vitro*.[107] In addition, in that same paper thrombin is shown to stimulate metastasis in several tumour cell lines. Furthermore, thrombin is also shown to induce angiogenesis via PAR1-mediated up-regulation of VEGF.[108] Taken together, a pro-coagulant state, represented by relatively high thrombin levels, is disadvantageous in relation to cancer development. Counteracting this pro-coagulant state with anticoagulant treatment, such as APC therapy, appears therefore to be beneficial for cancer patients.

In addition to countering the detrimental effects of a pro-coagulant state, APC is found to play another beneficial role in cancer. As discussed in chapter 1, APC enhances the integrity of the endothelial monolayer via $S1P_1$. Van Sluis *et al.* showed that this function of APC is important in reducing metastasis.[109] In this study Van Sluis and colleagues showed that in mice treated with an anti-APC antibody cancer cell extravasation was reduced compared to non-treated mice. In addition, this group of researchers showed that an $S1P_1$-mediated up-regulation of the vascular endothelial cadherin underlies this beneficial effect of APC on cancer progression.[109]

Based on the function of PS (see chapter 2) one might expect that PS also plays a role in cancer. The fact that PS is found to be expressed in many cell types confirms this hypothesis.[38] However, confirming literature on the underlying mechanisms of PS in cancer is not available.

PC and PS deficiencies

As mentioned before, deficiencies of both PC and PS are known. Because these deficiencies have several pathological consequences, a description of these deficiencies and their consequences is essential in this overview of the role of PC and PS in diseases.

Inherited PC deficiencies are most often caused by mutations that affect the structure of PC.[18] Nowadays, already over hundred mutations in the PC gene are known.[58] In addition, acquired PC deficiencies can arise as a consequence of anticoagulant therapy such as Coumadin.[110] Described symptoms of PC deficiency are mainly coagulation-related. Especially severe PF and VTE in new-borns are common symptoms. However, Shaw *et al.* showed that also inflammation markers (IL-6 and IL-8 levels) are significantly increased in severe PC deficient patients (PC activity ≤ 40%) compared to non-severe PC deficient patients (PC activity >40%).[111] Implicating that PC deficiencies might result in inflammation-related diseases.

Also for PS many mutations that affect the structure and/or the function of the protein are found. PS deficiencies are divided into three groups, based on quantity of the free –and C4BP bound levels of PS and the PS activity.[112] In addition to these relatively rare inherited types of PS deficiencies, PS deficiency can also be acquired by many circumstances among which anticoagulant therapy. Consequences of a PS deficiency are coagulation-related. Homozygous PS deficiency is generally incompatible with life due to severe PF during the first days of life.[112] Heterozygous or acquired PS deficiency is associated with increased thrombotic risk.

For both PC and PS an association between acquired deficiency and inflammation-related diseases have been proposed based on case studies.[88,113] A difficulty in proving evidence for this association is the uncertainty about the acquired deficiency as being the cause or a consequence of the disease. The PC or PS deficiency might be acquired by non-disease related circumstances, such as diet[112] and subsequently lead to development of an inflammation-related disease. On the other hand inflammation-related diseases, or the treatments for these diseases might result in PC or PS deficiency. Although the origin of the acquired PC or PS deficiency differs, both cases imply a role for PC and PS in inflammation-related diseases.

Conclusion

Besides the well-known anticoagulant function of both APC and PS, this literature review describes several of their not coagulant-related functions. APC was shown to be able to act anti-inflammatory and anti-apoptotic via activation of the PAR1 receptor. PS was found to play a role in suppressing inflammation and in angiogenesis via activation of TAM receptors. Due to this broad range of functions, both APC and PS could play an important role in many diseases. Because both APC and PS are originally known as coagulant factors, their involvement in coagulant-related diseases, such as thrombosis, PF and atherosclerosis seems to be plausible. Moreover, the fact that PC and PS deficiencies result in PF and thrombosis-related diseases confirms that both (A)PC and PS play an significant role in coagulation-related diseases.

Subsequently, the inflammation-related diseases sepsis, SLE, HIV and NAFLD in relation to APC and PS were outlined. The production of human recombinant (A)PC treatments against sepsis, confirms the important beneficial role of APC in this disease. But whether APC and PS play a significant (causative) role in other inflammation-related diseases is not fully substantiated. Based on the lack of inflammation-related symptoms in PC and PS deficiencies, the probability that a PC or PS deficiency on its own is causative for an inflammation-related disease is low. However, from the roles in the previous mentioned diseases and the association between acquired PC and PS deficiencies and those diseases can be concluded that both (A)PC and PS do play an important role in the progression of inflammation-related diseases.

Next, the role of APC and PS on neurological diseases was discussed. Because PS is unable to cross the BBB, PS is only able to have an indirect role in neurological diseases by increasing the BBB integrity. In contrast, APC is able to cross the BBB and is therefore able to directly interact with the CNS. However, studies focusing on the relation between APC and ALS and MS do not show a convincing role for this protein in these neurodegenerative diseases. An explanation for this finding might be that APC is only one of the many factors that influence these diseases. That APC plays an important beneficial role in stroke, another neurological disease, is well supported by *in vitro* and murine studies and by the fact that PC deficient patients have an increased risk for stroke. Not only the anticoagulant function, but also cytoprotective functions of APC are shown to contribute to this beneficial effect in stroke. Another disease in which APC seems to have a beneficial effect is cancer. By preventing a pro-coagulant state and extravasation of tumour cells via enhancement of the endothelial barrier, APC is thought to reduce the progression of cancer. Effective anticoagulant treatments in cancer patients indeed indicate an important role of APC in caner, but whether this beneficial effect in humans is acquired via reduction of extravasation has not been proven yet. No literature about the role of PS in cancer was found.

Taken together, (A)PC and PS do not only play a significant role in coagulation-related, but also in inflammation-related diseases. Furthermore, both coagulation factors are shown to have a role in affecting the progression of several other diseases.

List of abbreviations

ALS	Amyotrophic lateral sclerosis
APC	Activated protein C
BBB	Blood-brain barrier
BEC	Brain endothelial cell
C4BP	C4b-binding protein
CNS	Central nervous system
DA	Drotrecogin alpha
DC	Dendritic cell
EMA	European medicines agency
EPCR	Endothelial protein C receptor
FDA	Food and drug administration
FNIII	Fibronectin type III
Gas6	Growth-arrest-specific 6
Gla	y-carboxyglutamic acid
HIV	Human immunodeficiency virus
ICAM	Intercellular adhesion molecule
Ki	Dissociation constant
LG	Laminin G-like
LPS	Lipopolysacharide
MDSC	Myeloid-derived suppressor cell
MIF	Macrophage migration inhibitory factor
MS	Multiple sclerosis
PAR1	Protease-activated receptor 1
PC	Protein C
PF	Purpura fulminans
PS	Protein S
PTK	Protein tyrosine kinase
RGD	Arg-Gly-Asp sequence
S1P1	Sphingosine 1 - phoshpate receptor 1
SERPIN	Serine protease inhibitor
SLE	Systemic lupus erythematous
SOD1	Superoxide dismutase-1
TAM	Tyro3, Axl and Mer
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TSR	Tenase sensitive region
VCAM	Vascular adhesion molecule
VEGF-A	vascular endothelial growth factor
VKDP	Vitamin K dependent protein
VTE	Venous thromboembolism

Nederlandse samenvatting

De bloedsomloop is essentieel voor het leven van de mens en vele andere organismen. Coagulatie, ofwel bloedstolling, is één van de stadia van hemostase, het proces dat de bloedsomloop intact houdt. De coagulatie cascade bestaat uit verschillende stollingsfactoren, welke bijdragen aan het stoppen van een bloeding. Anticoagulantia, ofwel eiwitten die coagulatie remmen, waaronder Protein C (PC) en Protein S (PS), zijn belangrijk om trombose te voorkomen. PC en PS staan voornamelijk bekend om deze anticoagulante werking, maar beide eiwitten blijken ook andere functies te hebben. Geactiveerd PC (APC) blijkt, via activatie van de protease activated receptor 1 (PAR1) een belangrijke rol te spelen in het onderdrukken van ontsteking en geprogrammeerde celdood (apoptose). Van PS is bekend dat het, via activatie van Tyro3, Axl and Mer (TAM) receptoren, een rol heeft in het onderdrukken van het ontstekingsproces. Daarnaast lijkt PS een rol te spelen in de vorming van nieuwe bloedvaten (angiogenese).

Van zowel APC als PS is bekend dat ze een rol spelen in stolling gerelateerde ziektes, waaronder trombose, purpura fulminans (een trombose geïnitieerde ziekte welke word gekarakteriseerd door afsterving van de huid) en arteriosclerose (aderverkalking). Beide eiwitten lijken daarnaast ook een rol te spelen in ontsteking gerelateerde ziektes, zoals sepsis, systemische lupus erythematodes (SLE), humaan immunodeficiëntievirus (HIV) en non-alcoholic fatty liver disease (NAFLD). Het effect van APC op de progressie van sepsis is erg positief gebleken. Dit heeft dan ook geleid tot het op de markt brengen van de humaan recombinant (geactiveerd) PC medicijnen tegen sepsis.

APC en PS blijken ook allebei een positief effect te hebben op de integriteit van de endotheel laag die de hersenen van de periferie scheidt (BBB). Door de hersenen op deze manier beter te beschermen tegen potentiële gevaren vanuit de periferie, verlagen APC en PS de kans op neurologische ziektes. Daarnaast is gebleken dat APC in staat is om de BBB te passeren en om zo op nog een andere manier een rol te spelen in neurologische ziektes. Zo is APC gebleken betrokken te zijn bij de twee neurondegeneratieve ziektes amyotrofische laterale sclerose (ALS) en multiple sclerose (MS) en bij beroertes.

APC lijkt ook een rol te spelen in kanker. Door het tegen gaan van stolling en het verstevigen van de endothele barrière is APC therapie effectief gebleken in het verminderen van uitzaaiingen. Ook PS wordt tot expressie gebracht door veel tumor cellen, maar de exacte rol van dit eiwit in kanker is nog niet onderzocht.

PC en PS deficiënties kunnen veroorzaakt worden door genetische afwijkingen of worden verkregen door omgevingsfactoren, waaronder behandeling met anticoagulantia. Een associatie tussen deficiëntie van zowel PC als PS en stolling gerelateerde ziektes is gevonden, maar of deze deficiënties ook een link met ontsteking gerelateerde ziektes hebben is nog onduidelijk.

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