Anniversary Issue Contribution

The tale of protein S and C4b-binding protein, a story of affection

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For *Thrombosis and Haemostasis*' 50th birthday I have been invited to write about a favorite topic, focusing not only on the scientific aspects but also including some historical and personal aspects. I have chosen to write about vitamin K-dependent protein S describing not only how 30 years ago I got involved in studying the molecule but also report what the present knowledge is. Vitamin K-dependent protein S is indeed a fascinating molecule with multiple functions in different biological systems. The protein has been of special interest to me during most of my scientific career, and the following review is a personal tribute to the molecule. Protein S has lead me on my scientific journey for many years, and I hope I will have a chance to continue exploring its many secrets. So, how did it all begin?

Discovery of complex in human plasma between vitamin K-dependent protein S and the complement regulator C4b-binding protein

After finishing medical school and after a few years of clinical work, I joined the Clinical Chemistry department at the Malmö General Hospital in 1977. The hospital was linked to Lund University as teaching hospital, and the head of the department was the legendary professor Carl-Bertil Laurell, who among many other things had invented the crossed immunoelectrophoresis and the Laurell rockets (electroimmunoassays) – techniques that would greatly affect my research on protein S. Another eminent scientist in the laboratory was Dr. Johan Stenflo who in 1974 had reported on the discovery of the γ-carboxy glutamic acid (Gla) (1). Gla is the result of a vitamin K-dependent posttranslational modification of Glu and uniquely present in the Gla domains of vitamin K-dependent proteins. The Gla residues are important for calcium binding to the Gla domain, for the folding of the domain and for the ability of the domains to bind negatively charged phospholipids membranes (2). The discovery of Gla was a great scientific achievement and lead the way to many other discoveries including the anticoagulant protein C and protein S (3). Carl-Bertil Laurell decided that Johan Stenflo should be my supervisor, which I am very grateful for.

The identification of Gla resulted in development of methods to measure the content of Gla in proteins, a tool that was instrumental for the discovery of new vitamin K-dependent proteins. The first to come was bovine protein C in 1976 by Johan Stenflo, the name protein C was given as the protein eluted in the third peak – the C peak – on a chromatography (4). Just a year later, Richard DiScipio, who was a PhD student in Earl Davie's laboratory in Seattle, reported on the identification of yet another new vitamin K-dependent protein, a protein they named protein S (5).

One of the projects during my thesis work was to devise a purification procedure for human protein C, which proved to be quite difficult and many different techniques were tried. During one of these trials, I got a highly pure protein, but it was not protein C, it was protein S. An antiserum was raised in rabbits, and I set out to devise a method to measure protein S in plasma with the intent to investigate protein S plasma concentrations in thrombosis patients. Being in the laboratory of Carl-Bertil Laurell, rocket electrophoresis was a natural choice. The initial trials were not encouraging, as I did not get nice rocket-shaped immune-precipitates but rather balloon-shaped ones. The inventor of the rocket technique was close by, and I consulted him on the matter. "This does not look like an immune-precipitate – try a crossed immunoelectrophoresis" was the advice of Carl-Bertil Laurell, and so I did. In this technique, the plasma proteins were first separated in an agarose gel electrophoresis and then run in 90° into an antibody-containing gel. Two immune-precipitates were observed, a sharp one in the α1 region and a fuzzy second one migrating like fibrinogen in the β2-γ-region. The immune-precipitate in the α1 region corresponded to the position of isolated protein S when it was run in the same analysis. The comment of Carl-Bertil Laurell on the β2-γ-precipitate was "it looks like a complex with fibrinogen". This stimulated me to search for the nature of this precipitate. After a series of experiments, I was convinced that it was not fibrinogen to which protein S was complexed. I tried many other known plasma proteins migrating in the β2-γ-region but had little success.

As so often in science, serendipity provided the needed help and guided me to the identity of the protein that interacted with protein S. At the time, we routinely purified vitamin K-depend-

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ent proteins using barium-citrate absorption of plasma, which was followed by fractionated ammonium-sulphate precipitation and some additional chromatographic steps. The vitamin K-dependent proteins were recovered in the 40–60% ammonium sulphate precipitate, whereas the 40% precipitate (mainly consistent of barium-sulphate) was always discarded – nobody had to my knowledge checked if any proteins were in this fraction. One day, a failed ammonium sulphate precipitation procedure made me realize that the complexed form of protein S was actually present in the 40% precipitate – in almost pure form. Now it was possible to characterize the protein to which protein S was bound. The protein was of very high molecular weight being composed of multiple 70 kDa subunits and on agarose gel electrophoresis it migrated like fibrinogen. N-terminal sequencing gave a sequence which had not been reported previously, so the identity of the protein was still unknown to me. Again, I was lucky, and the identity of the protein was soon to be revealed. I attended a lecture on the complement system given by Dr. Anders Sjöholm, who was a collaborator of Prof. Anna-Britta Laurell in Lund. Both were experts on the complement system. After the lecture, I approached Dr. Sjöholm and described my new findings and asked him if the complement system contained a protein that matched the description of the one that I had found. "It might be C4b-binding protein (C4BP)" was the comment of Dr. Sjöholm – and he continued to tell me that C4BP had just been described as a complement regulatory protein functioning as a cofactor to factor I in the degradation of C4b. Dr Sjöholm had obtained an antiserum against C4BP and generously provided me with an aliquot to test. Imagine my excitement when the C4BP antiserum reacted with my protein. It was exciting that the vitamin K-dependent protein S formed a complex with the complement regulatory protein C4BP(6). In the summer of 1980, Frederick Walker reported that bovine protein S functioned as a cofactor to activated protein C (APC) in the degradation of factor (F) Va (7). This was indeed interesting, and the results taken together suggested that protein S played regulatory roles in both the coagulation and complement systems.

Unusual structure of protein S-C4BP complex revealed by electron microscopy

In May 1981, I presented my thesis on the activation of prothrombin on the platelet surface, which included studies on the binding of factor Xa to platelet, the resulting activation of prothrombin, the inhibitory effect of activated protein C, and the purification and characterization of human factor V (8–11). The protein S-C4BP studies were not part of my thesis, but the discovery of the protein S-C4BP complex (6) made me eager to learn more about the complement system. I was lucky to receive a Fogarthy postdoctoral fellowship and go to Scripps Clinic and Research Foundation in La Jolla to the laboratory of Hans J. Müller-Eberhard, a truly leading scientist in the field of complement research. Scripps was a fascinating research institute and provided fantastic opportunities to learn novel techniques. One of the other fellows in the laboratory, Craig Smith, was an expert on high resolution of electron microscopy of proteins and had devised a special way to prepare samples for negative staining, the so called "pleated sheet" technique. C4BP was obviously an interesting candidate to examine with this technique because it was of high molecular weight, contained multiple subunits and had distinct binding sites for protein S and C4b. Craig taught me the tricks of the pleated sheet technique, and with a couple of prepared golden grids I went to the electron microscopy laboratory not knowing what to expect. It was a fantastic moment when the C4BP presented its spider- or octopus-like structure to me and I took a number of photos of C4BP and of mixtures of C4BP and protein S and mixtures of C4BP and C4b (Fig.1). Based on these photos, I could draw a schematic model of the C4BP molecule localizing the binding site for protein S to a unique previously unidentified subunit (the β-chain that was later purified and cloned by my PhD student Andreas Hillarp) and C4b to each of the extended tentacles (the α-chains) (12, 13). The model still is

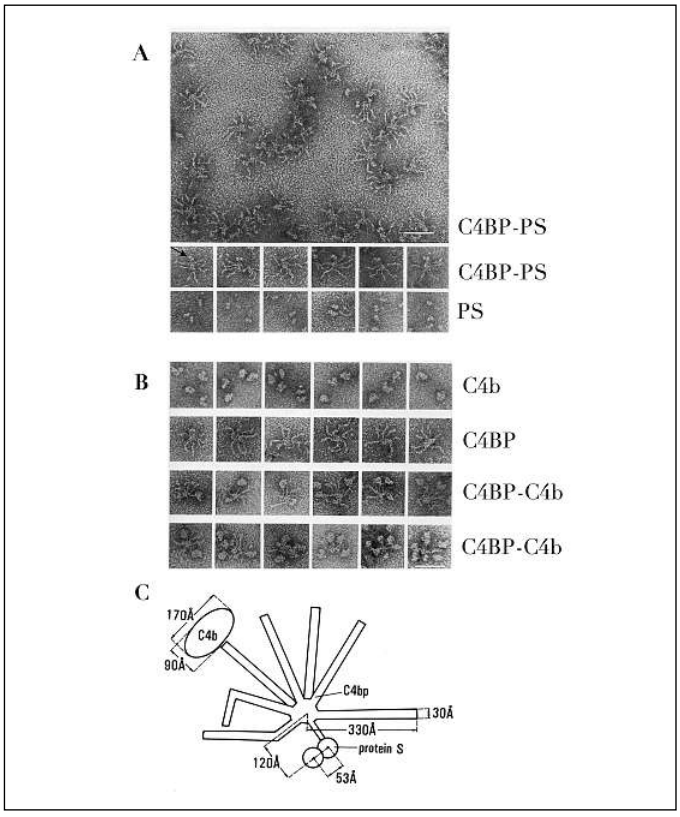

Figure 1: Unusual structure of C4b-binding protein and its complexes with protein S and C4b. The purified C4BP-protein S complexes were examined by high resolution negative staining using the "pleated sheet" technique and examined in the electron microscope. A) Top: a field with multiple C4BP-protein S complexes; Bottom: selected images of complexes or images of purified protein S (PS), as indicated. The arrow points at the protein S that is attached to a unique subunit close to the central core of C4BP. B) C4b was purified and subjected to examination by electron microscopy, and selected images are seen in the top row. The panel below shows C4BP molecules and the two lower panels demonstrate C4BP-C4b complexes with increasing number of C4b bound per C4BP from upper left to lower right. The images clearly visualize that the binding sites for C4b on C4BP are located at the peripheral end of each tentacle. C) A schematic model based on the observations made in the electron microscope detailing the dimensions of the participating molecules. The figure is a modification of figures published back in 1983 (12; printed with permission by PNAS).

valid even though we have a more detailed understanding of the involved molecular interactions.

This is the story of how I got involved in studies on protein S and C4BP 30 years ago. I am still intrigued by protein S and C4BP and in particular fascinated by the many functions of protein S. In recent years it has been revealed that protein S probably has important roles also outside the coagulation and complement pathways, and in my present research I follow the path where protein S leads me. In the following description I will leave the personal approach and focus on what is currently known about protein S.

Protein S gene and synthesis

Protein S circulating in plasma is mainly derived from liver synthesis, but in addition, endothelial cells, testicular Leydig cells and osteoblasts do synthesize protein S (reviewed in [14]). Platelets contain protein S, but whether this is derived from megakaryocytic synthesis or from uptake of plasma protein S is not known. A megakaryocytic cell line has been demonstrated to synthesize protein S suggesting that megacaryocytes have the capacity to make protein S. In the human genome there are two protein S genes (PROS1 and PROSP), but only PROS1 is expressed, whereas PROSP is a pseudogene. Both genes are located on chromosome 3 close to but on different sites of the centromer, PROS1 at q11.2 and PROSP at p21-cen. There is a high degree of sequence identity between the exons (97%) of the two genes. PROS1 is approximately 80 kb long and contains 15 exons and 14 introns. Chimpanzee and gorilla also have two genes, whereas the genome of orangutan, rhesus monkey and African green monkey only contain one protein S gene, suggesting that the gene duplication event occurred after the branching of the orangutan from the African apes (references in [14]).

Structure of protein S

Human protein S is a single-chain glycoprotein containing 635 amino acid residues, the calculated molecular weight of the apoprotein being 70,690 (14–16). The mature protein S is extensively post-translationally modified, containing three N-linked carbohydrate side chains and the modified amino acid residues γ-carboxy glutamic acids (Gla), β-hydroxy aspartic acid (Hya), and β-hydroxy asparagines (Hyn) (2, 14, 17). The primary structures of protein S from many other species have been determined, e.g. monkey, bovine, porcine, rabbit, rat and mouse protein S.

Protein S is composed of multiple domains (Fig. 2); from the NH_2-terminus the Ca^{2+}-binding Gla-domain is followed by a small thrombin-sensitive region (TSR), four epidermal growth factor (EGF)-like domains, and the sex-hormone-binding globulin (SHBG)-like region comprising two laminin G (LamG)-type domains) (14–16). The intron/exon organization of the gene correlates with the domain structure of protein S, suggesting that it has evolved through a combination of exon shuffling and gene duplication events.

The Gla-domain has high affinity for negatively charged phospholipid membranes, it also interacts directly with APC and is important for the APC-cofactor activity (14, 18–20). In human protein S, thrombin can cleave at Arg49 and Arg70 and FXa at Arg60 in the thrombin-sensitive region, leaving the Gla domain attached via the disulfide bridge. The TSR is also involved in the interaction between protein S, and APC and after cleavage the APC-cofactor function of protein S is lost (14–16). EGF1 and EGF2 are important for expression of APC-cofactor activity, EGF1 interacting directly with APC. The EGF1 contains a Hya and the three following Hyn in the molecule (2). The importance of the hydroxylation of Asp/Asn in protein S is not understood, and human protein S synthesized under conditions that inhibit the hydroxylation still expresses full cofactor function and C4BP binding. The Hyn-containing EGF-like domains in protein S, in particular EGF4, contain very-high-affinity Ca^{2+}-binding sites (K_d down to nM), the Ca^{2+} binding being important for correct folding of the protein (21, 22). The two LamG domains of protein S comprise the carboxy-terminal SHBG-like region. It contains three N-linked carbohydrate side chains of unknown function. The second LamG domain has been shown to play a role in the APC-mediated cleavages of both FVa and FVIIIa. The C4BP-

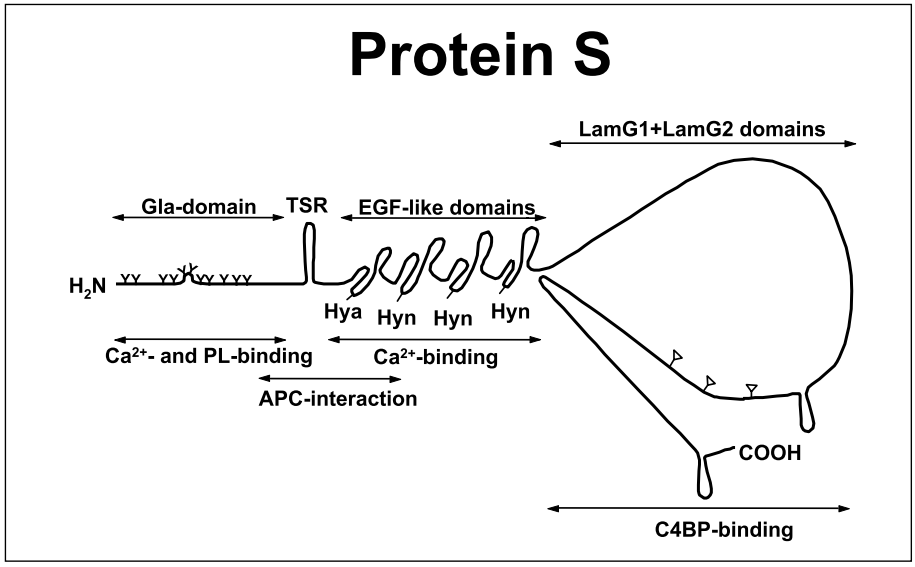

Figure 2: Schematic model of human protein S. Vitamin K-dependent protein S is a single chain molecule with multiple domains. The Gla-domain, which contains eleven Gla residues (Y) binds Ca^{2+} and negatively charged phospholipids and interacts with APC. The thrombin sensitive region (TSR) interacts with APC, cleavages by thrombin or FXa resulting in loss of the APC-cofactor activity. EGF1-EGF4 contain the modified amino acids β-hydroxy aspartic acid (Hya) or β-hydroxy asparagin (Hyn) as indicated. EGF1 and EGF2 are important for the APC-cofactor activity of protein S. LamG1 and LamG2 constituting the C-terminal half of protein S contain the binding site for C4BP. This region is also known as the SHBG region. The three N-linked carbohydrate side chains are indicated (s).

binding site on protein S is fully contained in the LamG domains, both LamG1 and LamG2 contributing to the binding. Several regions have been shown to be important for the binding, but the details of the binding site are still not known (reviewed in [14–16]).

Anticoagulant APC-dependent activity of protein S

Protein S works as an APC cofactor in the cleavage of FVa and FVIIIa (16, 23, 24). Protein S and APC form a complex on negatively charged phospholipid membranes, protein S increasing the affinity of APC for the membrane approximately 10-fold (Fig. 3). The interaction between APC and protein S affects the location of the active site of APC in relation to FVa and the membrane (25). The interaction between APC and protein S has mainly been characterized in in-vitro systems using phospholipid vesicle preparations. *In vivo*, presumably endothelial cells, platelets and platelet microparticles provide the phospholipid surfaces for the protein S-APC interaction (26, 27).

The APC-mediated cleavages at Arg506 and Arg306 in FVa have different dependence of protein S (Fig. 3). Thus, even though protein S provides stimulation to both cleavages, the APC-mediated cleavage at Arg506 in FVa is less dependent on the presence of protein S than the cleavage at Arg306 (23, 24, 28). A positively charged exosite on the surface of the serine protease domain of APC interacts specifically with a negatively charged area around Arg506. The cleavage at Arg506 results in partial loss of FVa activity, whereas the cleavage at Arg306 results in full inactivation. FXa in the assembled prothrombinase complex protects the Arg506 site from degradation by APC, presumably through sterical hindrance, i.e. FXa when bound covers the Arg506 area (29).

Protein S is not the only APC cofactor of importance in APC-mediated degradation of FVIIIa, because FV has been found to enhance the APC-mediated FVIIIa degradation (30). The APC-cofactor activities of FV and protein S are synergistic and result in efficient control of the activity of the tenase complex.

APC-independent anticoagulant activity of protein S

Several laboratories have reported that protein S also expresses APC-independent anticoagulant activity (31–33). The physiological importance of this activity is unclear and its molecular mechanism has been the topic of debate. The APC-independent anticoagulant activity has been suggested to be due to direct interactions between protein S and FVa/FVIIIa and/or FXa. It has been shown also in a flow system using endothelial cells and plasma, where protein S-depleted plasma gave considerably higher prothrombin activation than plasma containing protein S (34). It has been suggested that the direct anticoagulant activity of protein S is due to competition for phospholipid (35). However, such phospholipid dependence is only observed when the protein S preparations contain multimers of protein S (36). Whether protein S multimers are present in plasma or generated during purification is also a matter of discussion (37, 38). Furthermore, it has recently been suggested that protein S specifically inhibits tissue factor (TF) TF activity by promoting the interaction between full-length TF pathway inhibitor (TFPI) and FXa (39). This is an interesting observation that potentially will result in a better understanding of the direct anticoagulant activity of protein S.

Interaction between protein S and C4b-binding protein

This review started with a personal description of how I got involved in studies of protein S and of its complexes with C4BP 30 years ago. Since then, we have learnt a lot about the complex, its structure-function relationships, and about the potential physiological role of protein S in the complex. The protein S-C4BP complex in human plasma has a 1:1 stoichiometry, the interaction being non-covalent and of high affinity (K_d approximately 10^{-10} M) (6, 14, 15, 40). C4BP is important for the regulation of the classical complement pathway C3 convertase (C4bC2a complex), functioning as a cofactor to the serine protease factor I in the degradation of C4b and as a decay accelerating factor for the complex (14, 40). The high-molecular-weight (570 kDa) C4BP is composed of seven identical α-chains (70 kDa) and a single β-chain (45 kDa), disulphide bridges linking the chains. As mentioned in the introduction, C4BP demonstrated a spider- or octopus-shape in the electron microscope, the different subunits radiating from a central core (Figs. 1 and 4) (12). A binding site for C4b is located on each of the α-chains, whereas the single binding site for protein S is located on the β-chain. The binding sites for C4b and protein S have been elucidated in detail and molecular models of the α- and β-chains have been created (40, 41).

In normal human plasma, at least 80% of the C4BP molecules contain the β-chain (C4BPβ+) and bind protein S. The molar concentration of C4BPβ+ is approximately 30–40% lower than that of protein S, and because the protein S-C4BP inter-

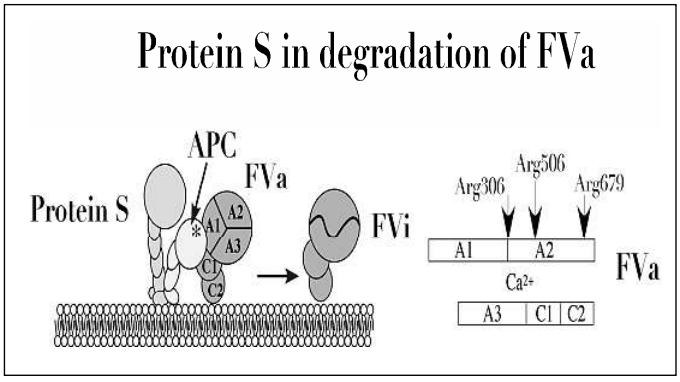

Figure 3: Protein S stimulates degradation of FVa by APC. APC cleaves the membrane-bound FVa at three sites, at Arg306, Arg506 and Arg679. The presence of protein S increases the efficiency of APC, the two molecules forming a membrane-bound complex. The Arg306 cleavage site is more dependent on the presence of protein S than the Arg506 site, but both cleavages are stimulated by protein S. The Arg679 cleavage seems to be less important than the other two, but its physiological significance is not fully understood.

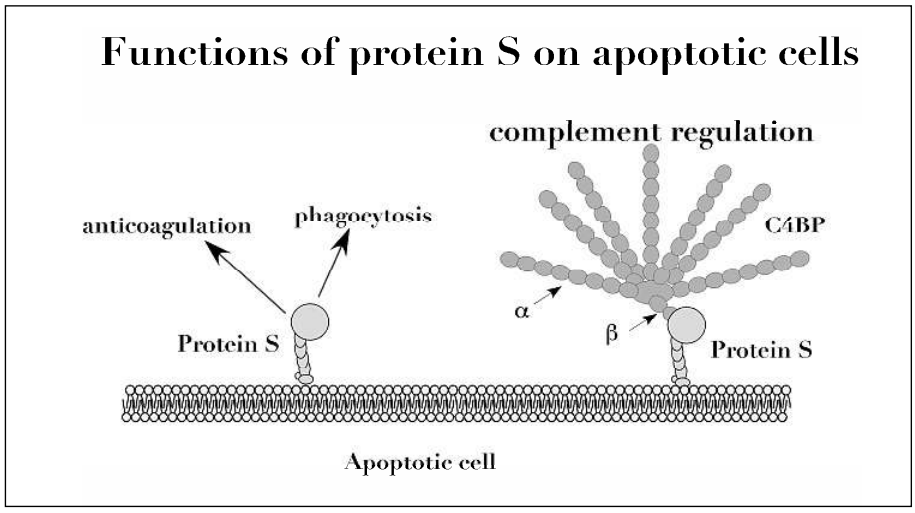

Figure 4: Protein S and the protein S-C4BP complex bind to apoptotic cells. The dying apoptotic cells expose negatively charged phosphatidylserine on their surfaces, which is an "eat me" signal to neighboring cells and professional phagocytes. The Gla-domain of protein S mediates binding of both free protein S and the protein S-C4BP complex to the apoptotic cells, providing the potential for regulation of both coagulation and the complement systems on the surface of apoptotic cells. In addition, protein S stimulates phagocytosis of the apoptotic cells, whereas the protein S-C4BP complexes seem to have the opposite effects. The octopus-like shape of C4BP is shown, the arrows indicating the α- and β-chains.

action is of very high affinity, free protein S is equivalent to the molar surplus of protein S over the C4BPβ+ (14, 15). The concentration of C4BP increases up to 400% of the normal level during inflammatory disorders, the synthesis of α-chains increasing more than that of the β-chain. This is due to differential regulation of the α- and β-chain gene expression by cytokines. As C4BP molecules synthesized during inflammation contain only α-chains they are unable to bind protein S. The protein S synthesis does not increase much during inflammation, which ensures stable levels of free protein S during inflammatory states even though the plasma C4BP level may be several times higher than normal.

The protein S-C4BP interaction provides a link between the regulation of blood coagulation and the complement system. C4BP modulates the protein S anticoagulant activity, because the APC-cofactor activity of protein S is lost upon binding to C4BP (42). Protein S on the other hand can affect the C4BP-mediated regulation of the classical complement pathway by localizing C4BP to the surface of negatively charged phospholipid, e.g. on apoptotic cells (see below). The binding of protein S-C4BP to apoptotic cells can provide local regulation of the complement system and inhibition of inflammation in the vicinity of dying cells.

Protein S binds to apoptotic cells and stimulates phagocytosis

The negatively charged phosphatidylserine is under normal conditions not exposed on the surface of cells but located in the inner leaflet of the cell membrane. As a consequence, the vitamin K-dependent proteins including protein S do not bind to the surfaces of normal cells. Phosphatidylserine is exposed on the cell surface under certain situations, e.g. during apoptosis or platelet activation, which explains why protein S can bind to apoptotic cells and on platelet microparticles (43, 44). The binding of protein S is calcium dependent and mediated by the Gla domain. Protein S binding stimulates phagocytosis of the apoptotic cells through mechanisms yet to be defined (45). In contrast, binding of the protein S-C4BP complex to the apoptotic cells counteracts the phagocytic process, suggesting that the bound C4BP inhibits the interaction between protein S and its receptors on the macrophages (46). Protein S and the protein S-C4BP complex may also have additional functions on the apoptotic cell surface, e.g. protein S may be anticoagulant, whereas the protein S-C4BP complex may control the complement system.

In the early 1990s, Gas6, a new member of the vitamin K-dependent protein family, was discovered and found to be homologous to protein S. Gas6 is the product of the growth arrest specific gene 6 and, as the name implies, that Gas6 synthesis was found to be induced by growth arrest, e.g. by serum starvation of cultured cells (47–49). Gas6 is reported to inhibit apoptosis, stimulation mitogenesis and growth. Gas6, unlike protein S, is not expressed in the liver, and the concentration of Gas6 in plasma is 1,000-fold lower than that of protein S. Gas6 is widely expressed, e.g. by endothelium, fibroblasts and smooth muscle cells, just to mention a few. Gas6 binds and activates the TAM receptor tyrosine kinase Tyro3(Sky), Axl, and Mer, which results in induction of tyrosine phosphorylation and intracellular signaling (50, 51). The Gla-domain of Gas6 binds to apoptotic cells and stimulates their phagocytosis. Protein S has been reported to work as ligand for the Tyro3(Sky) receptor, but its physiological significance was questioned because only protein S from certain species could stimulate the receptor (52). Thus, bovine protein S stimulates the human receptor, whereas human protein S did not. The structural difference between human and bovine protein S accounting for this species specificity was located to the LamG1 domain of protein S (53). The three-dimensional structures of the two LamG domains of Gas6 and of an Axl-Gas6 complex have been determined (54, 55).

Protein S deficiency as risk factor of venous thrombosis

Familial protein S deficiency is an inherited risk factor for venous thrombosis present in 2–4% of thrombosis patients and 0.03–0.13% of the general population (15, 24, 56). Heterozygous protein S deficiency increases the risk of thrombosis 5- to 10-fold. Homozygous or compound heterozygous protein S

deficiency is extremely rare and without treatment incompatible with life due to extensive microvascular thrombotization. The risk of thrombosis in protein S deficiency is much higher if combined with other genetic or acquired conditions predisposing for thrombosis, e.g. APC resistance due to factor V Leiden, explaining the varying penetrance of thrombotic symptoms in patients with protein S deficiency (57).

Inherited protein S deficiency has been categorized into three different types (I-III), type I patients having decreased free and total protein S, type II denoting functional deficiency with normal protein level, and type III being characterized by low free protein S but normal concentration of total protein S. In several large family pedigrees, types I and III were found to coexist suggesting the two types to be phenotypic variants of the same genetic disease (14, 15, 58). The plasma levels of protein S and C4BPβ+ are approximately equimolar in protein S deficiency, which explains the low plasma levels of free protein S in type I patients, because the affinity of the protein S-C4BP interaction is so high. This clarifies why analysis of free protein S has higher predictive value for protein S deficiency than analysis of total protein S (59). Very few type II deficiencies have been described. Although many mutations were identified in cases with protein S deficiency, a consistent observation reported from many laboratories is that mutations are only found in around 50% of the patients. The explanation for this has been unclear, but recently it was shown that in pedigrees where no mutation can be found in exons, the protein S deficiency is still linked to the PROS1 gene, suggesting the defect to be in or close to the PROS1 gene (60). In several such families, large deletions were found raising the possibility that the PROS1 gene may be more prone than other genes to suffere large deletions (61). In type I, most of the identified gene defects are missense or nonsense mutations (15). In type II, mutations have been found in the Gla-domain resulting in defective γ-carboxylation, and presumably provoke folding problems and instability as well as poor Ca^{2+} and phospholipid binding. A Thr103 to Asn mutation in EGF1 causes type II deficiency presumably due to poor interaction with APC (62). In EGF2, a Lys155 to Glu mutation results in a functional defect in protein S (protein S Tokushima). Protein S Tokushima has poor APC cofactor activity and interacts poorly with APC, suggesting that EGF2 is important for the APC-cofactor activity of protein S (63). Individuals with inherited protein S deficiency have similar clinical manifestations as those with other thrombophilic conditions, i.e. mainly venous thrombosis in lower extremities (14–16, 57).

Several factors influence the concentration of protein S in plasma, e.g. pregnancy and oral contraceptives decrease the plasma protein S levels. In pregnancy, the levels of both total and free protein S may reach levels similar to those found in patients with inherited deficiency (14, 15, 59). The levels of protein S decrease during treatment with oral anticoagulants, since protein S is a vitamin-K-dependent protein. Acquired protein S deficiency is found associated with nephrotic syndrome, the high molecular weight of C4BP not allowing its glomerular filtration, whereas free protein S is lost in the urine resulting in decreased levels of functionally active protein S. This may contribute to the thrombotic risk associated with nephrotic syndrome. Acquired protein S deficiency in patients with autoimmune disease or HIV infection may be the result of immunological mechanisms yet to be defined. Acquired protein S deficiency with autoantibodies against protein S and severe thrombosis is occasionally observed after varicella infections in children (14, 15).

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