Protein S deficiency

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In 1977, three years after the discovery of the γ-carboxy glutamic acid,[1] Richard DiScipio reported on the identification of a new vitamin K-dependent protein which was named protein S.[2] Three years later, Frederick Walker reported that bovine protein S functioned as a cofactor to activated protein C (APC) in the degradation of factor Va,[3] and it was later shown that APC-dependent degradation of factor VIIIa in the tenase complex requires the synergistic contribution of protein S and factor V.[4] In the early 1980s, Björn Dahlbäck and Johan Stenflo described the presence of two circulating forms of protein S, a free form and a complex of protein S with C4b-binding protein (C4b-BP), a regulatory protein of the complement cascade,[5] with only the free protein S form functioning as a cofactor to APC.[6] Association of familial protein S deficiency with recurrent thrombosis was reported in 1984[7] and it was soon shown that even a reduction of the free protein S form in plasma, in spite of total protein S levels within the normal range, could result in recurrent venous thromboembolism.[8] Since then, over a thousand papers have dealt with this protein clarifying its role within the framework of the natural anticoagulant systems.

Human protein S is a single-chain glycoprotein containing 635 amino acid residues.[9] 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).[9] Protein S is composed of multiple domains; the Ca^{2+}-binding Gla-domain followed from the NH_2-terminus 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.[9] The Gla-domain has high affinity for negatively charged phospholipid membranes, it also interacts directly with APC and is important for APC-cofactor activity.[9] Both thrombin and factor Xa can cleave Arg residues in the thrombin-sensitive region, leaving the Gla-domain attached via a disulphide bond. 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.[9] EGF1 and EGF2 are important for expression of APC-cofactor activity, EGF1 interacting directly with APC. The Hyn-containing EGF-like domains in protein S, in particular EGF4, contain very-high-affinity Ca^{2+}-binding sites, the Ca^{2+} binding being important for correct folding of the protein.[10] The two LamG domains of protein S comprise the COOH-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 factor Va and VIIIa. The C4b-BP binding site on protein S is fully contained in the LamG domains, both LamG1 and LamG2 contributing to the binding.[9]

Protein S and APC form a complex on negatively charged phospholipid membranes and, presumably *in vivo*, on endothelial cells, platelet and platelet microparticles Factor Va is inactivated by APC cleavage at 3 sites in the heavy chain: Arg 306, Arg 506, and Arg 679. However, the pattern of cleavage depends on whether protein S is absent or present. Only cleavage at Arg306, which bisects the A1 and A2 domain of factor Va, results in complete loss of factor Va activity. In the absence of protein S, rapid initial cleavage at Arg 506 occurs, resulting in a molecule with intermediate activity. This cleavage is apparently required for the optimum exposure of Arg 306. The presence of protein S increases the affinity of APC for the membrane surface, modulates factor Va protection from APC by factor Xa at Arg 506,[11] and relocates the active site of APC for preferential cleavage at Arg306.[12] This augments the rate of Arg 306 cleavage approximately 20-fold, and rapidly inactivates factor Va. Factor VIIIa is a heterotrimer consisting of A1 and A2 domains and the light chain. The A2 associates with the A1 domain via weak electrostatic interactions, with rapid dissociation at low concentrations. APC inactivates factor VIIIa by a similar mechanism to factor Va, although the APC cleavage sites are Arg 336 and Arg 562. Complete inactivation of factor VIIIa is correlated with cleavage at Arg 562. In the absence of protein S, Arg 336 is cleaved at a higher rate. In the presence of protein S and fragments of the B region of factor V (absent in factor Va), cleavage at Arg 562 is accelerated and factor VIIIa is inactivated more rapidly.[4]

Both free and C4b-BP-bound protein S[13] also express APC-independent anticoagulant activity, possibly due to direct interactions between protein S and factor Va, VIIIa and Xa. Although its physiological significance is still not clear this activity has also been shown in a flow system by perfusing plasma, anticoagulated with low-molecular-weight heparin, over tissue factor-containing matrices of stimulated endothelial cells, where protein S-depleted plasma gave a considerably higher and more prolonged prothrombin activation than plasma-containing protein S.[14] Recent observations if confirmed by other investigators may improve our understanding of the APC-independent protein S anticoagulant activity. According to Tilman Hackeng *et al.*, protein S specifically inhibits tissue factor activity by promoting the neu-

tralization of factor Xa by full-length tissue factor pathway inhibitor (TFPI), with a 10-fold reduction of the Ki of the factor Xa-TFPI complex .[15]

The protein C system plays a relevant role in modulating inflammation. In addition to the endothelial protein C receptor-dependent and independent PC/APC anti-inflammatory and cyto-protective activities,[16] the NH2-terminal lectin domain of thrombomodulin seems to dampen the mitogen activated kinase and nuclear factor kB responses in endothelium.[17] Protein S is also actively involved in the processes of apoptosis and inflammation. Cell death, either by necrosis or apoptosis, can greatly influence subsequent responses of the surrounding tissues. Exposure of the immune system to intracellular contents released during necrotic cell death can cause potentially harmful inflammatory responses and contribute to the development of autoimmune disease. Cell death by apoptosis is thought to limit these potential outcomes because the dying cells are removed by phagocytosis before cell lysis and exposure of the cell contents to the immune system take place. Under normal conditions, negatively charged phosphatidylserine is located in the inner leaflet of the cell membrane and the vitamin K-dependent proteins do not bind to the surfaces of normal cells. However, during apoptosis, phosphatidylserine is translocated to the outer leaflet of the cell membrane. This extracellular exposure of phosphatidylserine targets the intact apoptotic cell for rapid phagocytosis by macrophages, but it also leads to the Ca^{2+}-dependent binding, via the Gla-domain, of vitamin K-dependent proteins like protein S. Serum stimulates phagocytosis of intact apoptotic cells by macrophages apparently implicating a role for complement and integrins. By contrast, Anderson *et al.*[18] have identified protein S as the major factor responsible for serum-stimulated phagocytosis of apoptotic cells. In their experiments, purified protein S was equivalent to serum in its ability to stimulate macrophage phagocytosis of apoptotic lymphoma cells, and immunodepletion of protein S eliminated the prophagocytic activity of serum. An active role is also played by the protein S-C4b-BP complex. C4b-BP has a molecular weight of 570 kD and is composed of either six or seven identical α-chains (70 kDa) and either one (C4b-BPβ$^+$) or no (C4b-BPα) β-chains (45 kDa), resulting in a spider-like structure held together by disulphide bonds. 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.[9] In normal human plasma, at least 80% of the C4b-BP molecules contain the β-chain and bind protein S. The protein participates in the regulation of the classical complement pathway C3 convertase (C4bC2a complex), functioning as a cofactor to factor I in the degradation of C4b and as a decay-accelerating factor for the complex.[9] The non-covalent protein S-C4b-BPβ$^+$ complex in human plasma has a 1:1 stoichiometry and very high affinity. The binding of protein S to C4b-BPβ$^+$ abolishes the function of protein S as a cofactor for APC in factor Va degradation. In addition, although complex formation does not appear to inhibit the mild protein S enhancement of APC-dependent inactivation of factor VIIIa, it does inhibit the protein S/factor V synergistic effect. Although the C4b-BP concentration may increase to up to 400% of normal during inflammatory disorders, the synthesis of α-chains increases to a much greater extent than that of the β-chain. The large majority of C4b-BP molecules, therefore, lack the protein S binding site. Because protein S synthesis shows only a mild increase during inflammation, this ensures stable levels of free protein S during inflammatory states, even though the plasma C4b-BP level may be several times higher than normal. If the APC-cofactor activity of protein S is lost upon binding to C4b-BPβ$^+$, protein S can affect the C4b-BP-mediated regulation of the classical complement pathway by localizing C4b-BP to the surface of negatively charged phospholipids and controlling complement activation at sites where the coagulation system is activated. The binding of protein S-C4b-BPβ$^+$ to apoptotic cells can provide local regulation of the complement system and inhibition of inflammation in the vicinity of dying cells. Interestingly, while free protein S stimulates phagocytosis of apoptotic cells, addition of the C4b-BP-PS complex to serum deficient in both molecules abolishes the enhancing effect of serum on phagocytosis,[19] possibly because the bound C4b-BP inhibits the interaction between protein S and its receptors on the macrophages. Therefore, the presence of the C4b-BP-PS complex on apoptotic cells may lead to decreased phagocytosis. However, it may still be beneficial to the host, since it could prevent secondary necrosis by inhibiting further complement attack. By infusing protein S in a murine model of ischemic stroke, Liu *et al.*[20] observed decreased motor neurological deficit, infarction, and edema volumes. Although these findings may have been partly mediated by anticoagulant mechanisms, protein S also reduced the number of cortical neurons in which apoptosis was induced after hypoxia/reoxygenation injury by approximately 70%,[20] possibly by binding to these cells during apoptosis.

Acquired deficiency of protein S is observed in several pathological states which may be associated with an increased thrombotic risk. These include nephrotic syndrome,[21] disseminated intravascular coagulation, liver disease, and the use of drugs such as oral anticoagulants[22] and L-asparaginase.[23] Autoimmune protein S deficiency can also develop following an infection (most frequently chickenpox), leading to devastating thrombotic manifestations, especially in children, one week or later after the onset of the precipitating infection.[24,25] Most episodes of post-infectious purpura fulminans are probably associated with severe protein S deficiency due to specific autoantibodies of the G subclass. In the large majority of cases, this leads to an increased clearance of protein S from the circulation, although some may be

only neutralizing the APC-cofactor activity. Such autoantibodies are transient and their presence may be missed if not investigated early after the thrombotic episode. Interestingly, it has been suggested that acquired protein S deficiency may provoke a hyperinflammatory response.[26] Many authors have reported inhibition of APC anticoagulant activity in patients with lupus anticoagulants and thromboembolic manifestations. Optimal function of the APC-protein S complex requires the presence of phosphatidylethanolamine (PE), polyunsaturated fatty acids and phospholipid oxidation, a membrane composition which has little, if any, effect on the procoagulant reactions. PE and oxidation increase the binding of many antiphospholipid antibodies and the ability of at least some of them to inhibit the APC-protein S complex specifically. IgG purified from several antiphospholipid syndrome patients have been shown to inhibit only the lipid oxidation enhancement of APC activity in the presence, but not in the absence, of β_2-glycoprotein I. Oxidation of PE-containing membranes may induce specific interactions between APC and protein S which are disrupted by autoantibody-β_2-glycoprotein I complexes.[27]

Over the last 20 years, the increased venous thromboembolic risk associated with protein S deficiency has led to extensive investigation of potential carriers of the defect in families with thrombotic manifestations. There are two protein S genes (*PROS1* and *PROSP*) in the human genome but only *PROS1* is expressed, whereas *PROSP* is a pseudogene. Rare cases of homozygous or compound protein S deficiency relatively easy to diagnose have been reported in association with neonatal purpura fulminans. By contrast, the laboratory diagnosis of heterozygous protein S deficiency is still a major challenge. This deficiency increases the risk of thrombosis 3 to 10 fold, is uncommon in the general population ($\leq$0.2%) and is detected in 2-8% of patients with venous thromboembolism.[28] Commercially available tests include the measurement of total and free protein S antigens and of the APC-cofactor activity of protein S, but there is still no reliable diagnostic standard to evaluate their sensitivity and specificity. In addition, borderline-low protein S levels detected in some individuals appear to be disproportionately sensitive to the time, temperature, and dilutional conditions of the assays. Protein S levels are 30-40% lower than adult normal in neonates/infants until one year of age. They are higher in men compared with women and increase with age in women, but not in men. In women, protein S levels are lower before menopause, while taking oral contraceptives or undergoing hormone replacement therapy, and during pregnancy. Last, but not least, a series of comorbid disease states can influence protein S levels. The International Society for Thrombosis and Haemostasis Standardization Subcommittee has defined three types of hereditary protein S deficiency based on the plasma concentration of total protein S, free protein S, and APC-cofactor activity. Type I deficiency is identified by low levels of free and total antigen with decreased APC-cofactor activity. Type III deficiency is characterized by normal to low levels of total antigen, low free protein S, and an elevated fraction of protein S bound to C4BP. Approximately two thirds of protein S-deficient patients have type I deficiency, and one third have type III deficiency. The distinction between type I and type III protein S deficiency may be of clinical importance in a particular subject, but its biological basis and significance are controversial. Many cases of type III PS deficiency have the same molecular defects as type I deficiency and the age-related increase in C4BPβ^+, together with a limited ability to concomitantly increase PS levels, leads to the phenotype.[29] However, an apparently neutral polymorphism leading to protein S Heerlen (Ser460Pro) is the only abnormality detected in a much larger proportion of subjects with type III deficiency than expected from the population prevalence.[30] This mutation is in the SHBG-like domain, and it may lead to abnormal C4b-BPβ^+ binding. However, no evidence for this has been provided. Therefore, while type I protein S deficiency is essentially due to *PROS1* allelic heterogeneity, the molecular basis of type III protein S deficiency and its causative effect on thrombosis are still unclear. Type II protein S deficiency is characterized by normal levels of total and free antigen with low levels of APC cofactor activity. Its rarity is probably linked to inadequate methods of measuring the inability to effectively measure the APC-cofactor activity of protein S because commercial functional assays suffer a high rate of false positive results due to the presence of APC resistance,[31] lupus anticoagulants, and of high concentrations of prothrombin, factor VIIIa, and factor VIIa. The few mutations associated with type II protein S deficiency are located in the NH_2 terminus of the protein S molecule, including mutations of the Gla domain, causing impaired Ca^{2+}-induced phospholipid binding or mutations resulting in impaired interaction with APC, as found with an EGF1-lacking abnormal protein S or with mutations affecting the TSR.[32] Interestingly, a microdeletion reshaping the highest affinity Ca^{2+}-binding site in the EGF4 (protein S Basiglio)[33] results in an abnormal conformation of the Gla-domain, impairing phospholipid binding, without leading to a grossly misfolded protein.

Over 200 PROS1 defects segregating with protein S deficiency in family studies have been reported,[34] but the molecular mechanisms leading to reduced plasma protein S levels have only been investigated in a minority of cases, mainly involving missense or splice-site mutations. In this issue of the journal, Hurtado *et al.* report on the functional characterization at RNA and protein levels of 12 natural *PROS1* mutations associated with heterozygous type I protein S deficiency.[35] They observe defective synthesis, stability or secretion of the mutated protein S in association with missense mutations and, more interestingly, exclusion of the mutated

allele in association with the more frequent mutations generating a premature termination codon. Their work sheds light on an important mechanism which underlines quantitative protein S deficiency.

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