MINIREVIEW

Protein S and Thrombotic Disease (43435)

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Protein S Structure and Function. Discovery of Protein S Function. The unraveling of the process of blood coagulation has slowly occurred throughout the 20th century. During the 1970s, the focus slowly began to change to questions dealing with regulatory mechanisms that could be important in thrombotic disease. A search for other inhibitors and/or regulatory factors began concurrent with studies on antithrombin III and its interaction with heparin. With the advent of improved chromatographic tools, it became possible to purify trace proteins and analyze their structures. In the early 1970s, Stenflo purified a new vitamin K-dependent protein that was designated protein C (1). It was observed that proteases like trypsin or thrombin could convert it to a serine protease (2). Kisiel and co-workers (3), closely followed by Walker and co-workers (4), recognized that this protease was unique in that it inhibited blood coagulation and that the mechanism appeared to be through the proteolysis of factor Va. Subsequently, it was observed that factor VIII was also a substrate (5, 6). Interestingly, it was found that the active form of factor Va was a better substrate than the circulating form of factor V (4), and the same was found to be true of factors VIII and VIIIa (6). Proteolysis of factors Va (4) and VIIIa (7) was observed to be dependent upon the presence of calcium ions and phospholipid vesicles. In 1980, Walker (8) proposed that activated protein C needed a cofactor protein for the expression of its anticoagulant activity and suggested that this protein was protein S. This discovery evolved from the earlier observations that since proteases derived from vitamin K-dependent proteins, factors Xa, IXa, and VIIa, all required cofactor proteins for the maximum expression of their activity, it was possible that activated protein C might also need a cofactor protein. Initial experiments showed that plasma could enhance the rate of the inactivation of factor Va by activated protein C. Fractionation of the plasma revealed protein S to be the factor involved in this enhancement. In addition to the effects on proteolysis, it was observed that the anticoagulant activity of activated protein C was dependent upon protein S. Therefore, protein S was observed to enhance the rate of inactivation of factor Va by activated protein C and to modulate the anticoagulant activity of activated protein C (8). This was the first demonstration of the relation between these two activities and the first demonstration of a function for protein S. This discovery became the basis for the opening of a new area into the investigation of the causes of thromboembolic disease.

Structural Properties of Protein S. Protein S is a vitamin K-dependent protein. It was first discovered by DiScipio and Davie (9) as a side product in the purification of other vitamin K-dependent proteins. Because no function had been associated with the protein, it was arbitrarily named protein S after the city in which it was discovered, Seattle. Protein S from human (10) and bovine (11) plasma has been sequenced using both recombinant techniques as well as standard sequencing strategies. The sequence of protein S has revealed that, like other vitamin K-dependent proteins, it consists of a series of well-defined domains. The Gla domain is found at the amino-terminal. Ten γ-carboxyglutamic acid (Gla) residues are found in this region, created by a posttranslational modification by a vitamin K-dependent carboxylase. This region has a high degree of homology with other vitamin K-dependent proteins (9). Following the Gla region is a disulfide-linked loop that contains a site that can be cleaved by thrombin (12). This is known as the thrombin-sensitive region. Cleavage of the thrombin-sensitive region converts single-chain protein S into a disulfide-linked, two-chain pro-

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tein. The thrombin-sensitive region is followed by four repeats of an epidermal growth factor (EGF)-like region. Characteristic of this type of domain is a high degree of disulfide cross-linking. Two unusual amino acids are found in this region. A β-hydroxyaspartic acid residue is found in the first EGF domain (13) and one β-hydroxyasparagine is found in each of the following three EGF regions (14). In other vitamin K-dependent proteins, the EGF region would be followed by the protease domain. Instead, protein S has a region that has some homology with steroid-binding proteins (15). This region makes up approximately two thirds of the mass of protein S and has only two disulfide bonds.

Like other vitamin K-dependent proteins, the interaction between calcium and protein S is important for its function. Protein S has several calcium-binding sites. The first is associated with the Gla region (16). Interaction of calcium ions with the Gla residues appears to be essential in the interaction between protein S and phospholipid-containing membranes. It has been shown that if as few as four of these residues are converted to γ-methylene glutamic acid, then the membrane-binding properties of the protein are lost (16). A second type of binding site is located in the EGF-like domains (17). Occupation of this site(s) can cause a conformational change in the protein. One of the sites is of extremely high affinity. The dissociation constant has not been estimated because of the extremely slow rate of exchange of bound calcium with calcium in the medium (17). Though the functional significance of these sites is unknown, they may be involved in the interaction between protein S and activated protein C.

The cDNA that codes for protein S has been cloned and expressed in mammalian cells allowing for the production of recombinant protein S (18). Recently, the gene for protein S and its pseudogene have also been characterized (19–21). It is located on chromosome 3 near the centromere (22, 23). It is at least 80 kb in length and contains 14 introns and 15 exons. Exons I and XV contain 112 and 1139 bp of noncoding sequence in addition to the amino- and carboxy-terminal region of the protein. Exons I through VIII encode protein segments that are homologous to other vitamin K-dependent proteins. Exons IX through XV encode sequences that are homologous to the sex hormone-binding globulin. The human genome also contains a second protein S gene closely linked to the α-gene on chromosome 3 (23). This 55-kb gene, known as the β- or pseudogene, does not code for an active protein, since there are several frame shifts and termination codons within the coding region (19).

Functional Properties of Protein S. The function of protein S is to enhance the proteolytic activity of activated protein C against factors V and VIII. No function has been described for the protein in the absence of activated protein C (8). The ability of protein S to enhance proteolysis by activated protein C has an absolute dependence on the presence of membranes (24). In addition, protein S has not been observed to form a soluble complex with activated protein C using physiological concentrations of both proteins (24). In the presence of membranes that contain phosphatidylserine, protein S has been observed to interact with activated protein C in a stoichiometric manner (24). Two experiments were carried out that indicated that the membrane-binding properties of protein S are essential for the expression of its cofactor activity. First, chemical conversion of the γ-carboxyglutamic acid residue to γ-methylene glutamic acid results in a protein that lacks cofactor activity and is unable to bind to phospholipid vesicles (16). Second, cleavage of protein S with thrombin results in a two-chain protein without cofactor activity (25, 26) that is also not able to bind to membranes (25) and has reduced calcium binding (25, 27). These observations suggest that the interaction with membranes may be important in the expression of its activity.

Studies of the protein S cofactor activity in the presence of membranes with varying lipid compositions have indicated that protein S has a greater effect on the enhancement of the inactivation of factor Va when phosphatidylserine is present in low concentrations in phospholipid vesicles containing both phosphatidylcholine and phosphatidylserine (28). The effects of protein S have also been observed to be sensitive to the composition of the fatty acid side chain, although the mechanism of this effect is unknown.

The effect of protein S on rates of bond cleavage by activated protein C has also been examined. Activated protein C has been observed to cleave three or four bonds in the factor VIII molecule. Upon the addition of protein S, the rate of factor VIII inactivation was enhanced approximately 5-fold and the rate of cleavage of each of the bonds was also enhanced to approximately the same extent (7). This suggests that the kinetic effect of protein S is not to cause the preferential cleavage of a specific bond, but, rather, to increase the rate of cleavage of all bonds catalyzed by activated protein C.

Solymoss *et al.* (29) have observed that human protein S has only a small effect upon the rate of inactivation of human factor Va by activated protein C, in contrast to what has been observed with bovine proteins. However, in human plasma, protein S has a significant effect on the anticoagulant activity of activated protein C. Solymoss *et al.* have observed, like others, that factor Xa can protect factor Va from inactivation by activated protein C, and that protein S can reverse this protection. They have proposed that the role of protein S is to modulate the protection of factor Va by activated protein C (29).

Marciniak (30) observed that the anticoagulant

activity of activated protein C was species specific. That is, activated protein C from one species was a poor anticoagulant of the blood of other species (30). The species specificity is not due to a specificity in the substrate rather than a specificity in the interaction with protein S. Protein S has been observed to enhance the anticoagulant activity of activated protein C in heterologous plasma (31).

It has also been observed that protein S also acts as a cofactor for the profibrinolytic activity of activated protein C. Immunodepletion of protein S from blood reduces the activated protein C effect upon fibrinolysis (32). In addition, it has been observed that the profibrinolytic effect of activated protein C, like the anticoagulant effect, is species specific. The species specificity can be corrected with protein S from the homologous species (33). In addition, it has been observed by most (33, 34), but not all, investigators (35) that the inactivation of plasminogen activator inhibitors by activated protein C is not a protein S-dependent reaction. This suggests that the mechanism by which activated protein C stimulates fibrinolysis is through some mechanism other than the inactivation of plasminogen-activator inhibitors.

Interaction of Protein S with Cells. Protein S has been found in platelets (36) and endothelial cells (37). Platelet protein S represents approximately 2.5% of the total protein S found in blood (36). It has been localized to the α-granules and has been observed to be released with platelets activated by thrombin. Some individuals with low levels of plasma protein S have also been observed to have low levels of platelet protein S (36).

In purified systems, native platelets do not support factor Va inactivation by activated protein C in the absence of protein S (38, 39). After platelet activation, however, they will support factor Va inactivation by activated protein C independent of added protein S. The latter effect can be inhibited with antibodies directed against protein S (38). These results have several possible implications. First, the platelet may have a protein S receptor on its surface and, second, protein S or a protein S-like protein from within the platelet can substitute for plasma protein S. Specific binding of protein S to unstimulated platelets has been observed (38, 40). The half-maximal concentration of binding occurred at 10 nM and there were approximately 1100 protein S binding sites/platelet. Platelet-bound protein S has been observed to be rapidly cleaved by a platelet-bound calcium-dependent protease (40).

Human umbilical vein (41) and bovine aortic endothelial cells (37) have been observed to synthesize protein S, but none of the other vitamin K-dependent proteins. Treatment of the cells with warfarin reduced the specific activity of the secreted protein, which suggests that the endothelial cells contain the vitamin K-dependent carboxylase (41). Treatment of endothelial cells with either interleukin 1 (42) or norepinephrine (43) suppressed the ability of endothelial cells to support the inactivation of factor Va by activated protein C. In the presence of protein S, like platelets, endothelial cells will promote activated protein C-catalyzed inactivation of factor Va (44).

Regulation of Protein S Activity

Interaction with C4b-Binding Protein. Dahlback and Stenflo (45) originally observed that C4b-binding protein (C4b-bp) co-purified with some vitamin K-dependent proteins, and that on gel filtration it co-migrated with protein S. This observation lead to studies on the importance of the complement system in the regulation of the protein C system.

In human (46) and rabbit plasma (47), protein S is found both free and bound to the complement component C4b-bp. Approximately 60% of the total protein S is found bound to C4b-bp. When bound to C4b-bp, protein S is unable to act as a cofactor for activated protein C (48), though it has been reported that activated protein C can interact with protein S that is in complex with C4b-bp. C4b-bp is a large (mol wt = 570,000) protein consisting of eight polypeptide chains (49). Seven of the chains, called α-chain, are identical and one, β-chain, is nonidentical. The nonidentical or β-chain is essential for the interaction between protein S and C4b-bp (49). In blood, several forms of C4b-bp exist. One form lacks the β-chain and contains seven α-chains. This form is unable to bind to protein S (50). A second, minor form has been observed that also has seven chains, but is missing one of the α-chain. This form is able to bind protein S. All forms that contain the β-chain have been observed to bind protein S (50).

Protein S and C4b-binding protein form a very stable complex. The complex is so stable that it can be easily purified (51). The affinity of the interaction has been estimated by several investigators. Dissociation constants have been reported between 10–7 M (45) and 10–10 M (52–54). The latter value appears closer to the consensus figure. However, if 10–10 M is correct, then it is unclear how any free protein S exists in plasma. It may be that there are other plasma components that regulate the interaction between these two proteins.

Studies have been carried out to identify the portion of protein S that interacts with C4b-binding protein. It has been observed by Nishioka and Suzuki (54) that activated protein C can interact with protein S bound to C4b-binding protein, which suggests that the binding sites on protein S for these two proteins are discrete. It has also been observed that protein S can modulate the binding of C4b-binding protein to membranes (55). Walker (56) observed that synthetic peptides from the carboxy-terminal disulfide-linked loop of protein S can inhibit the protein S interaction with C4b-binding protein. In particular, a peptide with the

sequence GVQLDLDEAI has been observed to inhibit the protein S interaction with C4b-binding protein, increase the amount of free protein S in plasma, and enhance the anticoagulant activity of activated protein C in human and rabbit plasma. In addition, the peptide has been infused into the rabbit and it has been observed to enhance the anticoagulant and profibrinolytic effects of activated protein C *ex vivo* (47).

Inactivation by Thrombin and Other Proteases. Protein S contains a structural domain that is known as the thrombin-sensitive region. This is a disulfide-linked loop that follows the Gla domain of the protein. Cleavage by thrombin converts protein S into a two-chain protein. The two-chain version has altered membrane and calcium-binding properties and is unable to function as a cofactor for activated protein C. The rate of inactivation by thrombin is sensitive to calcium ions which inhibits inactivation (25, 27). Platelets have also been observed to contain a protease that can cleave protein S that is bound to the platelet membrane (40). It has also been observed that neutrophil elastase can cleave protein S which results in its inactivation (57).

In the presence of calcium ions, protein S is insensitive to proteolysis (17). Because of this insensitivity, it is unclear whether any of the proteases that have been observed to cleave protein S are able to regulate protein S in a physiological setting.

Methods for the Detection of Protein S Defects

Antigenic Methods. Two types of immunological methods for the determination of proteins are commonly used and their uses have been extensively reviewed elsewhere (58, 59). Immunological assays for protein S are based on the observation that plasma contains two forms of protein S: free and bound to C4b-bp. Since only the free form is able to act as a cofactor for activated protein C, it has been rationalized that changes in the level of free protein S are related to susceptibility to thrombosis. Methods have then been developed for the separation of free and bound protein S. Separations have been made either electrophoretically (59), as with crossed immunoelectrophoresis, or by precipitation with polyethylene glycol (60). This is based on the observation that most of the protein S complex with C4b-binding protein can be precipitated with 3.8% (w/v) polyethylene glycol 8000 (60, 61). Detection and quantitation of the amount of free protein S can be determined either by enzyme-linked immunosorbent assay (62–64) or Laurell rockets (60), radioimmunoassay (65–67), and crossed immunoelectrophoresis (61). Commercial kits are available for each type of determination. Many investigators also determine C4b-binding protein either by enzyme-linked immunosorbent assay or Laurell rockets as a method for determining whether decreased free protein S could be due to elevated C4b-binding protein levels. As a cautionary note, my lab has observed that many of the polyclonal antibodies that are commercially available for C4b-binding protein will also react with protein S.

An alternative method for measuring protein S has been suggested by Schwarz and co-workers (68). In their method, called quantitative immunoblotting, samples are electrophoresed and then blotted onto nitrocellulose. Blots are then probed with radiolabeled anti-protein S. Quantitation occurs by developing the blot and measuring the intensity of the band by densitometry. This method of separating protein S from C4b-binding protein has the advantage of reduced variability due to the polyethylene glycol separation step, but has not been used widely because it is a more complex procedure.

Functional Methods. The function of protein S is to act as a cofactor for activated protein C in the inactivation of factors V and VIII. Hence, functional assays should show the ability either to enhance the rate of inactivation of these cofactors or to increase the anticoagulant activity of activated protein C in plasma that is deficient in protein S. Workers have concentrated on the development of a one-stage clotting assay that is sensitive to the concentration of protein S. Several of the assays have been developed to be sensitive to the ability of activated protein C to inhibit activated factor V. In these assays, either the factor X activator from Russell's viper venom (69) or factor Xa (70, 71) is used to initiate coagulation. It has been noted in assays that have been designated to modulate the activated partial thromboplastin time (APTT) that the source of the APTT reagent can have a great effect upon the activity of protein S (72–74). It appears that the lipid composition of the APTT reagent can alter the sensitivity of the assay to protein S. Plant sources appeared to work better than animal sources (72).

Several methods have been used to prepare protein S-deficient plasma. One method has been to immunodeplete plasma of protein S. Deficient plasmas have also been prepared by aluminum hydroxide adsorption of plasma and subsequent supplementation with purified prothrombin and activated protein C. Clotting was initiated with factor Xa, calcium chloride, and phospholipids (71). In addition, commercial kits for the functional assay as well as deficient plasmas are available.

Normal Levels of Protein S in Newborns and Adults. Normal levels of protein S have been determined in a number of studies to be approximately 24 μg/ml of plasma (67). This is the value that would correspond to 100% in most studies. On average, women have lower levels of protein S than men, and there is considerable variation. The normal range is approximately 67–136% of total in a mixture of men and women (71). Free protein S ranges between 81% and 133% for men and from 50% to 130% for women

(61). A value of 100% free refers to the amount of protein S present in a normal pool of plasma following polyethylene glycol precipitation. The median functional activity of 46 adults was 82%.

In comparison with adults, newborns have low levels of protein S when determined by immunological methods (75–77). In one study, newborns averaged 23% of the adult level (75). However, when assayed by functional assays, protein S levels in the newborn were 74% of the adult level (75). The reason for this discrepancy is that C4b-binding protein levels are very low or undetectable in the newborn (75). The result is that most of protein S is in the free form and functions as a cofactor. Protein S levels reach those of adults in full-term as well as premature infants by 6 months of age (78, 79).

Discrepancies between functional and antigenic levels of protein S can exist for a number of reasons. For example, near normal functional levels of protein S have been observed when antigenic levels are low due to decreased levels of C4b-bp. Normal total and free antigenic levels can be accompanied by low functional levels in cases in which the subject is being treated with oral anticoagulants or has a mutation that does not alter protein expression. In the former case, a disproportionately high level of antigen may reflect the presence of acarboxy-protein S in plasma. Hence, total reliance on either assay may result in missing several potential types of defects or abnormal conditions. The thorough laboratory evaluation of protein S should include the antigenic measurement of total and free protein along with a measure of the amount of functional activity.

Effect of Oral Anticoagulation Therapy and Liver Function on Protein S Levels

Because protein S is a vitamin K-dependent protein, its synthesis depends upon the presence of vitamin K for the carboxylation of 10 glutamic acid residues near the amino-terminal of the protein. Like other vitamin K-dependent proteins, functional and antigenic levels have been observed to be sensitive to warfarin. Fair *et al.* (41) have observed in tissue culture of the Hep G2 cell line that warfarin decreased the synthesis of protein S (41). Poort *et al.* (66) have observed a half-life of 42.5 hr in individuals given oral anticoagulant therapy. It has been observed that patients on long-term anticoagulant have only modestly reduced protein S levels. In one study, protein S levels were 71% in patients on long-term therapy compared with an assay mean of 82% for normal individuals using a functional assay (69). Several other studies present a different picture. van de Waart and co-workers (71) observed a functional level of protein S at 21% when by antigenic determination the level was 70%. Kobayashi *et al.* (73) observed functional activity to be 31% while free antigen was 51% in a similar patient group. The studies that indicate high antigenic levels of protein S in patients who are on oral anticoagulant therapy may be due to a significant plasma concentration of decarboxylated protein S.

Inherited Defects in Protein S Structure and Function

Protein S deficiency has been observed in a number of families. Most cases have been identified during a laboratory evaluation for thromboembolic disease. Protein S deficiency, as well as thrombosis, in these families was inherited in an autosomal-dominant fashion. Several types of inherited protein S deficiency have been observed. Type I is defined by total levels of protein S that are normal or near normal, while free levels are very low. Type II is characterized by both decreased levels of total and free protein S. A third type is defined by normal levels of free and total protein S, but the free protein S is not functional. Only one case of this latter type has been reported (80). Type I and Type II protein S deficiencies are not necessarily genetically distinct classes of defects. Several families have been observed that have inherited thrombosis associated with protein S deficiency, in which both type I and type II protein S deficiencies have been observed in the family. This suggests that other factors may influence plasma levels than have now been defined.

Engesser *et al.* (81) analyzed 136 members of 12 families with hereditary protein S deficiency. A total of 55% had venous thromboembolism, and in 77% it was recurrent. The mean age of the first event was 28 years and ranged from 15 to 68 years. At age 35, the probability of being free of thromboembolism was 32%. Though it may be generally held that protein S-associated thrombosis does not occur until the teenage years, several cases of thrombosis have been reported in children as young as 2 (82) or 3 years old (83, 84). In individuals with heterozygous protein S deficiency, there is no relationship between plasma levels of protein S and age of onset of thrombosis. There is a great deal of heterogeneity in the severity of onset. The most severe case reported was of a 21-year-old man who had a sudden myocardial infarction and died at home before the ambulance arrived (85). Protein S is associated with both venous and arterial thrombosis. In a small study of 34 patients with protein S deficiency, 25% had arterial thrombosis (70).

Only a small amount of information is available about the molecular basis of the defects in type I and II protein S deficiency. One element that can cause confusion in an analysis of the genome of affected individuals is that the human genome contains two closely linked protein S genes (23). Only one, the α-protein S gene, codes for an active protein. Though the β- or pseudogene do not code for an active protein, they may contain polymorphisms that map near to the

α-gene and segregate with thrombosis. Ploos van Amstel and co-workers (86) have observed an unusual *MspI* fragment in two families with inherited protein S deficiency. In one of the families, this polymorphism did not segregate with the phenotype; in the other, it did. The mutation was found to be in the protein S pseudogene (86). This suggests that polymorphisms in the pseudogene may be useful aids in mapping defects in the protein S gene. Schmidel and co-workers (87) studied a family with protein S deficiency. In this family, all of the members with protein S deficiency had type II protein S deficiency. Analysis of the genome indicated that there was a deletion in exon XIII. Another family with low protein S levels was observed to have a deletion in the middle of the coding region (88). Both deletion polymorphisms appeared to be the cause of the inherited thrombophilia. A protein S variant not linked to thrombosis designated PS-Heerlen, in which serine 460 was replaced with a proline, has been found. The variant protein has normal functional properties. The frequency of this allele was observed to be 0.52% in the general population (89). Schwarz and co-workers (90) have identified a family with a variant protein S molecule using immunoblotting after electrophoresis. The chemical defect was not identified; however, it may have been the PS-Heerlen variant that they were observing.

Acquired Defects in Protein S Structure and Function

Many cases of thromboembolic disease do not appear to be predisposed by some genetic factor that we understand at this time. Many of the cases involving low protein S levels in plasma appear to be acquired. Gladson *et al.* (91) have observed that the frequency of heterozygous protein S deficiency among young unrelated patients with venous thrombotic disease was approximately 5%. This compares with 4% for protein C and 3% for antithrombin III (91). Hence, among the identifiable anticoagulant proteins, protein S is the most common cause of thrombotic disease. Contrasting this, an Israeli study found antithrombin III to be low in 7.5% of 107 unrelated patients, and protein C deficiency in 5.6% and protein S deficiency in 2.8% of patients with recurrent thrombosis (92). The differences in these studies may be due to population differences in the expression of the disease.

Protein S and Cerebral Arterial Thrombosis. It has recently become apparent that the incidence of protein S deficiency in patients who have had strokes is greater than in the general population or populations of individuals with deep vein thrombosis. In one study of 103 patients with cerebrovascular disease, 20% had low free protein S levels (93). In a second, smaller study, 13 of 33 patients with cerebral arterial occlusions had either low total protein S or low free protein S (94). A number of case reports have appeared linking protein S deficiency with stroke (82, 94–97). In some of the cases, protein S deficiency was demonstrated to be inherited. However, in most cases, the other affected family members have had other forms of thrombosis.

Effect of Pregnancy and Oral Contraceptives on Protein S. Both pregnancy and the use of oral contraceptives have been observed to alter functional and immunological protein S levels. During pregnancy, total protein S has been observed to be reduced to 68% of normal levels (98). In 33% of the women studied, the concentration of protein S fell below the normal range (98, 99). C4b-Binding protein levels were either unchanged (98) or elevated (100), resulting in a disproportionate decrease in free protein S to approximately 40% of normal. In either case, it would appear that decreases in free protein S could enhance an existing thrombotic tendency due to a disproportionate decrease in free protein S levels. Low protein S level values tend to return to normal a few days after delivery (100). However, at least one patient has been reported in the second week postpartum with superior sagittal sinus thrombosis with dysfunctional protein C and decreased free protein S (101).

Women taking oral contraceptives also have significantly lower protein S levels (102, 103). In one study, total protein S levels decreased to 84% (104), whereas in a second study, total protein S decreased to 75% and free to 79% of normal (100). In a third study, women on oral contraceptives had total levels of protein S that were 85% of normal and free levels that were 86% of normal (104). Since a disproportionate decrease in free protein S was not observed, it is possible that the effect of oral contraceptives may be different from that of pregnancy. In a third study that examined highly trained female athletes, total protein S dropped from 111% to 85%, whereas no significant drop in free protein S was observed (105% to 94%). No examination of C4b-binding protein levels was carried out (102). Boerger and co-workers (104) suggested that a decrease in total protein S, with unchanged levels of C4b-binding protein, should result in a disproportionate decrease in free protein S. That this is not observed with women taking oral contraceptives suggests that either C4b-bp levels are diminished in this group, or that a different mechanism is working to change the levels of protein S.

Protein S Levels and Nephrotic Syndrome. Patients with nephrotic syndrome have a thrombotic tendency. A number of studies have been undertaken to determine whether protein S is involved in this tendency. Most studies tend to confirm that total levels of protein S are elevated in nephrotic syndrome (105–109). However, free levels have been observed to be elevated (105, 106), unchanged (107), and lowered (108). Some of the differences may be due to different patient populations that are being examined.

Lupus Anticoagulant and Protein S. Lupus anticoagulant, an antibody that is directed against negatively charged phospholipids, is associated with a high incidence of thrombosis (110). This has lead to a number of studies aimed at determining whether either protein C or protein S is altered in this condition. In general, total protein S levels have not been found to be altered in patients with Lupus anticoagulant (110–113). In addition, there has been no report of circulating antibodies against either protein C or protein S. Several studies have reported a reduction in free protein S levels in patients with systemic lupus erythromatosis. In one, the patient had elevated C4b-binding protein. Upon treatment with cyclophosphamide, C4b-binding protein and free protein S levels returned to normal, which suggests that the absence of free protein S was a consequence of the elevated C4b-binding protein levels (114). In one study, the reduced protein S levels were not associated with thrombosis (115). Parke and co-workers (personal communication) have observed that free protein S levels were depressed in eight of 11 women who had been chosen for study based on the presence of antiphospholipid antibodies and a history of habitual abortions. In this group, there was no relation between the level of C4b-binding protein and the free protein S level.

Disseminated Intravascular Coagulation and Liver Disease. A number of studies have examined the effects of liver disease on protein S levels. In liver disease, total protein S levels have been observed to be moderately reduced (73, 116, 117). This results in a significant reduction in free protein S levels (73, 116) and, in some cases, functional protein S levels (116). However, in one study in which free protein S levels were reduced to 64%, functional levels remained at 82%, which suggests that, at least in some cases, C4b-bp may also be reduced (73).

During disseminated intravascular coagulation (DIC), total protein S levels have been observed to vary from normal (117, 118) to significantly reduced (116, 119). One study (119) reports that much of protein S in the plasma from DIC patients is cleaved, though others report that low protein S activity is due to a redistribution onto C4b-binding protein (116). One study reports no changes in either total or free protein S levels during DIC (117). The confusion might suggest some degree of heterogeneity in the population of patients that have been identified as having DIC.

Protein S and Transplantation. Because free protein S levels may be regulated, in part, by C4b-binding protein, an acute phase reactant, and it has been reported that free protein S decreases during inflammation, several investigators have questioned whether protein S levels may be altered during transplantation. Protein S and protein C levels were followed in 21 patients after bone marrow transplantation (120). Twelve experienced significant drops in protein S and protein C levels. Only one patient developed venoocclusive disease of the liver, and no other thrombotic events were observed. Low protein S levels before the bone marrow transplant was predictive of the development of low levels after the transplant. Patients who have undergone kidney transplantation have been followed for changes in protein S levels (121). It has been observed that free protein S levels are decreased during transplant rejection. Total protein S levels were not measured, so it is not known whether this phenomenon was due to increased inflammatory response and increased C4b-binding protein levels or due to increased proteinuria as a result of kidney failure.

Future Trends

In comparison with other areas of medicine, the pathobiology of protein S is relatively poorly understood. It appears clear that individuals with low protein S levels have a relatively larger risk for the development of thrombosis than normal individuals. Yet, no large study has been carried out to identify the frequency of protein S deficiency in the population. This may be due, in part, to the difficulty in carrying out a protein S assay. However, with the availability of commercial kits for the determination of functional protein S levels, it should be possible to determine the frequency of the deficiency in the population. A second area that appears to be widely accepted but substantiated with little experimental data is the relationship between free protein S levels and the plasma concentration of C4b-bp. In addition, the mechanism that regulates the amount of protein S bound to C4b-bp is also unclear. In part, this may be due to the lack of good analytical tools for determining the capacity of C4b-bp in plasma to bind protein S. It appears that an understanding of the regulation of this interaction may be key to the understanding of many acquired protein S deficiencies, as well as type I protein S deficiency.

The effect of oral anticoagulation of protein S needs to be more thoroughly examined. There have been a number of discrepancies reported between the antigenic level of free protein S and functional protein S activity. This may be due to the presence of a substantial level of decarboxy protein S. Resolution of this issue may also result in a revised half-life for protein S.

The question of whether there are proteolytic mechanisms for the inactivation of protein S has yet to be resolved adequately. Though it appears that under most physiological conditions protein S is resistant to proteolysis, there are several reports that suggest that this may not be true *in vivo*. In particular, there is one report that reports degraded protein S in DIC.

The genetics of protein S deficiency are often confusing. This has been observed in cases in which type I and type II protein S deficiency exist in the same family

and they appear to be interchangeable. This suggests that better molecular descriptions of the deficiencies are needed.

Additional work is needed on the molecular description of the basis of action of protein S itself. Few studies have been carried out to determine the effect of protein S on the proteolytic mechanism catalyzed by activated protein C. In particular, it would be of interest to determine the effect of protein S on the interaction between activated protein C and the light chain of either factor V or factor VIII.

During the 11 years since the discovery of the function of protein S, much has been learned about its importance in thrombosis and hemostasis. Through this broad outline, it can be seen that much is left to be discovered about the biology of protein S that will increase our understanding of its relation to thrombotic disease.

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