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Assessment of Plasma Protein S Functional Activity and Antigen in Patients with Liver Cirrhosis

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Abstract: Protein S functional activity, free and total protein S antigen as well as C4b binding protein antigen were assayed in patients with liver cirrhosis and the amounts of free protein S and protein S bound to C4b binding protein in normal subjects and in patients with liver cirrhosis were also calculated from the area of two-dimensional immunoelectrophoresis. Cholinesterase activity correlated well with total protein S, C4b binding protein and protein S bound to C4b binding protein but not with the functional activity of protein S nor with free protein S antigen. The ratio of free protein S to protein S bound to C4b binding protein was 68±3% in normal subjects and 127±69% in patients with liver cirrhosis. C4b binding protein had an inverse correlation with the ratio of free protein S to protein S bound to C4b binding protein. From these data, we can conclude that protein S bound to C4b binding protein but not free protein S reflects the level of liver function of macromolecular synthesis in liver cirrhosis and that the binding condition between C4b binding protein and protein S in patients with liver cirrhosis is different from that in normal subjects.

Key words: Protein S functional activity, Total and free protein S antigen, Protein S bound to C4b binding protein, C4b binding protein, Liver cirrhosis

INTRODUCTION

The naturally occurring anticoagulant proteins, protein C and S, function to inhibit blood clotting. Protein S is a plasma protein produced vitamin K-dependently in the liver and inhibits the procoagulant cofactors Va and VIIIa by stimulating the proteolysis of factors V[1] and VIII[2], and by serving as a cofactor for activated protein C[1)-3)]. Persons with congenital deficiency of protein C are subject to severe recurrent venous thrombosis[4)-6)] because of their inability to regulate intravascular coagulation. Congenital protein S deficiency has also been known to be associated with recurrent thrombosis[7)-9)], because protein S is required for the expression of the anticoagulant activity of activated protein C. Protein S exists in two forms in human plasma, as free and bound form with C4b-binding protein (C4bp) which participates in the regulation of the classical pathway of complement activation[10)]. Free protein S has a cofactor activity for protein C, whereas C4bp bound-protein S has no functional activity[10),11)]. The two forms of protein S are balanced in human plasma by the level of C4bp [12),13)].

In liver disease, protein S and C4bp are reported to decrease because they are produced in liver cells[14)-17)] but there is no report regarding precise analysis of changes of protein S and C4bp until now. We have already

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reported a new technique to assay protein S functional activity[18]. In this study, using our new technique, we assayed changes of two forms of protein S and C4bp and evaluated their relationship to liver function of macromolecular synthesis in patients with liver cirrhosis precisely in order to elucidate the pathophysioloy of protein S in liver disease.

Materials and Methods

Materials

Purified venom activator "Protac", one vial contains 3 units of activity), goat IgG against human Protein S, and purified human protein C were obtained from American Diagnostica, Greenwich, CT. One unit of Protac activates all the protein C in 1 ml of normal plasma. Protein S-deficient plasma and antihuman C4b binding protein (C4bp) goat serum were obtained from Diagnostica Stago, Asnieres France. The concentration of protein S antigen in protein S-deficient plasma was less than 2% of that in normal plasma as determined by enzyme-linked immunosorbent assay (ELISA). Cephalin (Actin) and Owren's barbital buffer (50 mmol/*l*, pH 7.35) were purchased from American Dade, Miami, FL. Blood was collected into 38g/l trisodium citrate solution, in a 9:1 ratio of blood to antigoagulant. Platelet-poor plasma (PPP) was prepared by centrifugation at 4°C (2,000 xg for 20 min), and was then stored at −70°C until it was used. Normal plasma pooled from 20 ostensibly healthy normal subjects who had not been taking any drugs was used as standard plasma. Because there is not yet a generally recognized reference standard for protein S, the activity in normal plasma i.e., the amount of activity in 1 m*l* of average normal plasma is usually used to define the unit of biological and immunological activity of protein S. The protein S activity in normal plasma is stable for a month at −70°C, and the coefficient of variation (CV) for protein S activity under these conditions is less than 5%. We also used citrated plasma samples from 15 patients (one case was compensated and the others were decompensated) whose diagnosis of postnecrotic or postviral liver cirrhosis was based on liver biopsies and on anamnesis and clinical signs, such as splenomegaly, jaundice, esophageal varices, ascites, serum albumin, low serum level of non specific cholinesterase activity (ChE), high serum level of ALT and AST, alkaline phosphatase and virus antigen and antibody of hepatitis B or non-A, non-B hepatitis when needle biopsy was impossible[19]. In these cases, alcohol, biliary, cardiac, metabolic and inherited etiologies were excluded and complication of hepatocellular carcinoma was also excluded.

Methods

Protein S functional activity was measured as cofactor activity for activated protein C and was determined by the activated partial thromboplastin time method using an automated KC 10 coagulometer (Heinrich Amelung GmbH, Lieme, F.R.G.) which we previously reported[18]. We assayed total protein S antigen with an ELISA method (Asserachrom Protein S; Diagnostica Stago, Francoville, France). Protein S complexed with C4bp (protein S-C4bp) was precipitated with 3.75% polyethylene glycol 8,000 according to the method reported by Comp *et al.*[10] and free protein S antigen in the supernatant was also assayed by ELISA method. C4bp antigen was measured by the Laurell rocket technique[20]. Total and free protein S antigens were also assayed by two-dimensional immunoelectrophoresis according to the method reported by Comp *et al.*[10] ChE activity was measured by the method of Michel HO[20] and normotest (NT) was assayed by the method of Blombäck M using chromogenic substrate[22]. Linear regression analysis was carried out in accordance with F-test.

Mean values (mean±SD) and range of values of all assay systems in normal subjects (n=20) were shown in Table 1. The units of these parameters except ChE activity and protein S-C4bp were indicated by the percen-

Table 1. Mean values (mean±SD) and range of values of assay systems in normal subjects. ChE; non specific cholinesterase activity, NT; normotest, ATIII; antithrombin III activity, PS act; protein S functional activity, PS TAg; protein S total antigen, PS FAg; protein S free antigen, C4bp; C4b binding protein, PS-C4bp; protein S complexed with C4bp, PS-free/PS-C4bp; ratio of free protein S to protein S complexed with C4bp

Normal Subject (n=20)

	unit	mean±SD	range
ChE	ΔPH	1.00±0.22	0.6–1.45
NT	%	84±9	68–105
ATIII	%	100±15	70–134
PS act	%	102±21	68–143
PS TAg	%	106±25	60–115
PSFAg	%	106±20	74–145
C4bp	%	93±16	65–140
PS-C4bp	mm^2	0.86±0.16	0.67–1.08
PS-free/PS-C4bp	%	68±3	64–72

tile change of standard plasma which was prepared from mixed plasmas of 20 normal subjects.

Results

Normotest and antithrombin III activity in patients with liver cirrhosis were compared with ChE activity, which reflected the liver function of macromolecular synthesis. As shown in Fig. 1, normotest and ATIII activity correlated well with ChE activity (r=0.5826 and 0.8513, P<0.05 and 0.001, respectively). These results were the same as those previously reported[23)–25)]. Protein S functional activity, measured by our newly developed method, and free and total protein S antigens were compared (Fig 2). Protein S functional activity had a good correlation with free protein S antigen (r=0.8363, P<0.001) but had no correlation with total protein S antigen assayed by ELISA. C4bp which complexes with protein S and inhibits its function, was compared with free and total protein S antigens (Fig. 3). C4bp had no correlation with free protein S antigen but correlated well with total protein S antigen (r=0.9159, P<0.001). ChE activity and free and total protein S antigens were compared (Fig. 4). ChE activity had no correlation with free but correlated well with total protein S antigen (r=0.2994 and 0.6691, P<0.3 and 0.01, respectively). Protein S functional activity also had no correlation with ChE activity (data

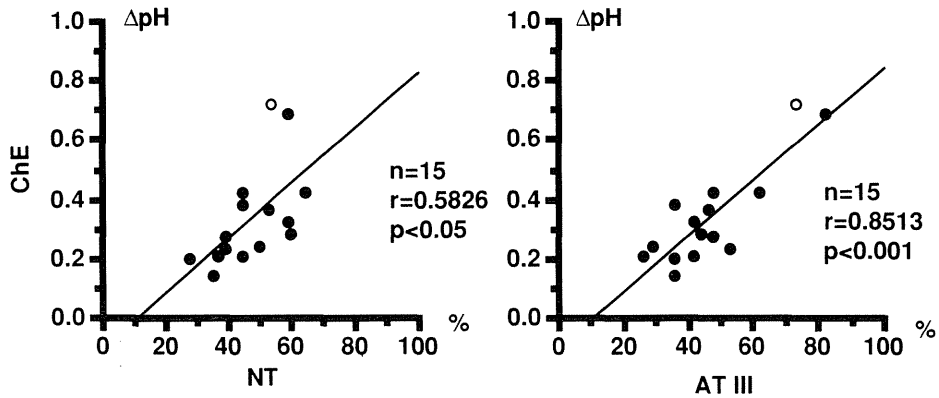

Fig. 1. Correlation between cholinesterase activity (ChE), normotest (NT), and antithrombin III activity (AT III) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

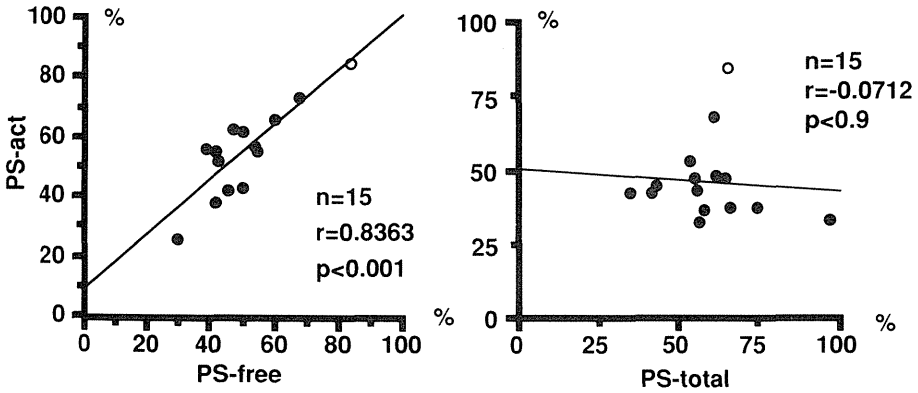

Fig. 2. Correlation between protein S functional activity (PS-act), free protein S (PS-free), and total protein S (PS-total) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

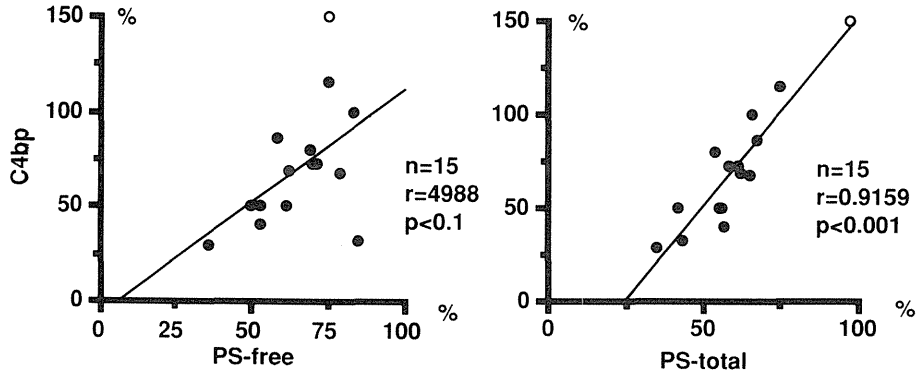

Fig. 3. Correlation between C4b binding protein (C4bp), free protein S (PS-free) and total protein S (PS-total) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

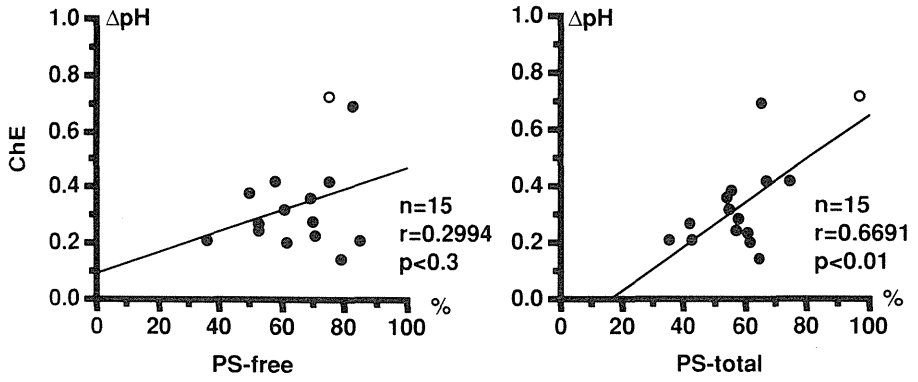

Fig. 4. Correlation between non specific cholinesterase activity (ChE), free protein S (PS-free), and total protein S (PS-total) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

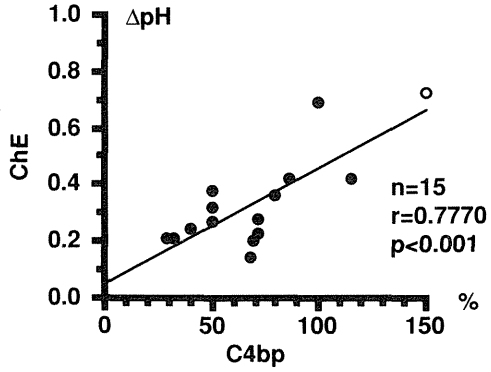

Fig. 5. Correlation between non specific cholinesterase activity (ChE) and C4b binding protein (C4bp) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

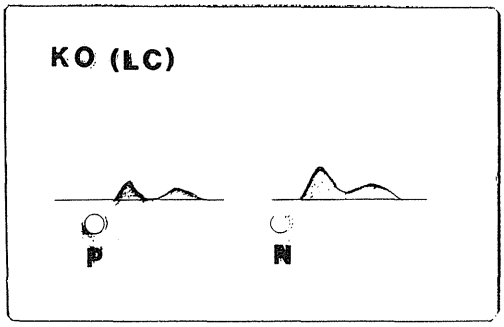

Fig. 6. Two-dimensional immunoelectrophoresis of protein S antigen. P: plasma of patients with liver cirrhosis, N: normal plasma

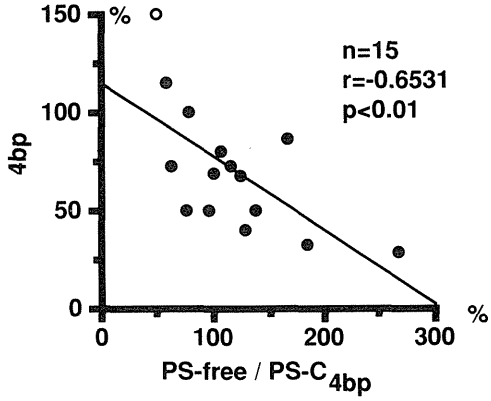

Fig. 7. Correlation between C4b binding protein (C4bp) and the ratio of free protein S to protein S complexed with C4bp (PS-free/PS-C4bp) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

Table 2. Mean values (mean±SD) and range of values of assay systems in patients with liver cirrhosis. ChE; non specific cholinesterase activity, NT; normotest, ATIII; antithrombin III activity, PS act; protein S functional activity, PS TAg; protein S total antigen, PS FAg; protein S free antigen, C4bp; C4b binding protein, PS-C4pb; protein S complexed with C4b binding protein, PS-free/PS-C4bp; ratio of free protein S to protein S complexed with C4b binding protein

		Liver Cirrhosis	
	unit	mean±SD	range
ChE	ΔPH	0.33±0.17	0.14–0.72
NT	%	48±14	30–86
ATIII	%	46±15	25–81
PS act	%	46±13	32–84
PS TAg	%	65±13	35–97
PS FAg	%	59±14	36–85
C4bp	%	70±32	29–150
PS-C4bp	mm^2	0.44±0.29	0.09–0.66
PS-free/PS-C4bp	%	127±69	49–266

not shown). C4bp had a good correlation with ChE activity ($r=0.7770$, $P<0.001$ Fig. 5). Mean values (mean±SD) and range of values of all assay systems in patients with liver cirrhosis were shown in Table 2. Normotest, ATIII activity, protein S functional activity and free form of protein S antigen were moderately decreased. To evaluate the relationship between free protein S and protein S-C4bp antigen in patients with liver cirrhosis, we analyzed these two proteins by two-dimensional immunoelectrophoresis as shown in Fig. 6. Liver cirrhosis and normal plasma were applied to separated well on the same gel plate for two-dimensional immunoelectrophoresis. After staining, two peaks of free protein S (fast moving) and protein S-C4bp (slow moving) were clearly detected. The areas of these two peaks were calculated with a planimeter and the percent ratio of free protein S to protein S-C4bp in normal subjects and patients with liver cirrhosis was determined. The mean value of the ratio of free

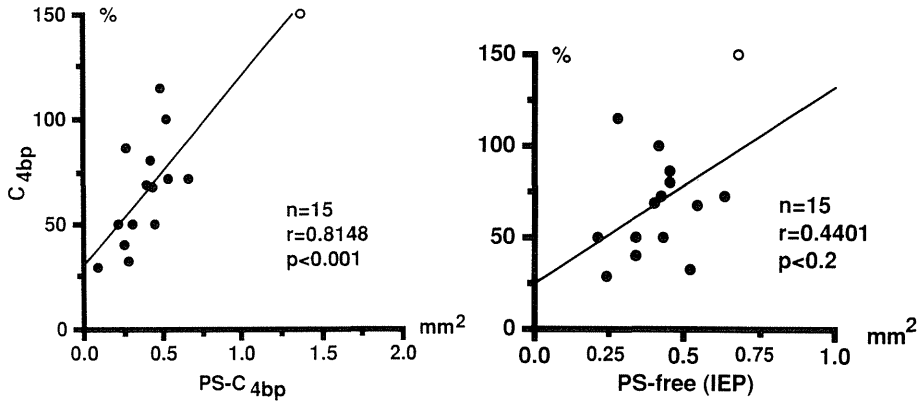

Fig. 8. Correlation between C4b binding protein (C4bp), protein S comlexed with C4bp (PS-C4bp), and free protein S in two-dimensional immunoelectrophoresis (PS-free, IEP) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

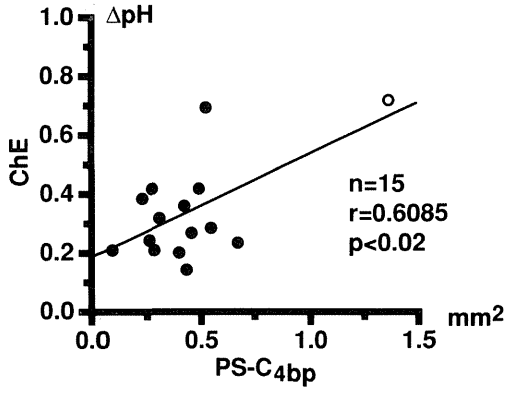

Fig. 9. Correlation between non-specific cholinesterase activity (ChE) and protein S complexed with C4bp (PS-C4bp) in patients with liver cirrhosis. Solid circles are decompensated cases and open circle is compensated case.

protein S to protein S-C4bp was 68±3% in normal subjects and 127±69% in patients with liver cirrhosis. The inverse correlation between C4bp and the ratio of free protein S to protein S-C4bp was statistically significant ($r=0.6531$, $P<0.01$, Fig. 7). In addition, C4bp correlated well with protein S-C4bp but not with free protein S as shown in Fig. 8 and protein S-C4bp also correlated well with ChE activity (Fig. 9). Total and free protein S measurd by ELISA correlated well with those measured by IEP ($r=0.7906$ and 0.6264, $P<0.001$ and 0.01 respectively).

Discussion

Protein S, a vitamin K-dependent protein synthesized in the liver[1], which complexes on the surface of membranes. This complex appears to be important in the expression of the anticoagulant activity of activated protein C[26]. Protein S has been observed to stimulate the proteolysis of factor V_a[1], factor $VIII_a$[2], and plasminogen activator inhibitor[3] by activated protein C, but is not the zymogen of a serine protease[27]. In plasma, protein S is present in two forms: free and bound to a high molecular weight protein known as C4bp[28]. When protein S complexes with this protein, it can not act as a cofactor for activated protein C[29]. In plasma, about 40% of protein S exists in the free form and the remainder exists as a C4bp complex[28]. Acquired protein S deficiency occurs during anticoagulation, in nephrotic syndrome and during pregnancy[14),30)–32]. There have been several reports of protein S reduction in liver disease[14)–17] but the precise relationship between liver function, protein S in free and C4bp complex form, and C4bp has not yet been identified. From our data, ChE

activity correlated well with ATIII activity and normotest, in addition to vitamin K-dependent coagulation factor and prothrombin time, and these factors have already been recognized as good parameters for the evaluation of liver function of macromolecular synthesis[23)–25)]. With regard to protein S, total protein S antigen had a better correlation with ChE activity than with free protein S antigen and activity and C4bp also correlated well positively with protein S-C4bp and total protein S but not with free protein S as well as correlating inversely with the ratio of free protein S to protein S-C4bp (pS free/pS-C4bp). Additionally, protein S-C4bp correlated well with ChE activity. Until now, free and total protein S, protein S-C4bp and C4bp levels have been reported to decrease in patients with liver disease[15),16),33)]. Our data were in agreement with these results, however ChE activity correlated well only with total protein S, C4bp and protein S-C4bp. In addition, ChE activity correlated well with protein S activity and antigen (free and total), C4bp and protein S-C4bp in normal subjects. Therefore, these data indicate that in liver cirrhosis, the major portion of protein S reduced according to the grade of liver dysfunction is not free protein S but protein S-C4bp and which may reflect the liver function of macromolecular synthesis. Because C4bp is produced only in the liver but protein S is a vitamin K-dependent plasma protein synthesized in the liver and the presence of protein S in platelets and the production of protein S in endothelial cells have also been reported[36),37)]. The ratio of free protein S to protein S-C4bp in plasma of patients with liver cirrhosis was also different from that in normal plasma. This implies some alterations in the relationship between protein S and C4bp. C4bp is present in two different molecular weight forms in plasma (570,000 and 510,000)[28)]. The high molecular weight form is composed of seven elongated subunits and a short subunit which binds to protein S[12)]. The β-chain of C4bp is protein S binding region[34)]. In liver cirrhosis, functional activity of protein S reduced to the same level as that of free protein S antigen. Therefore, there may be no abnormal protein S like PIVKA but some alteration of the domain in protein S which binds with C4bp, as opposed to the domain which contains r-carboxy-glutamic acid residues, since the two domains are present separately in protein S[35)]. In this study, we did not find any qualitative differences in protein S between normal subjects and patients with liver cirrhosis by two dimensional immunoelectrophoresis. Other methods are required to analyze the qualitative difference of connection between protein S and C4bp.

Thus, the plasma protein S level and its relationship to C4bp in various kinds of pathologial states may be complicated. A much more precise study of protein S is needed to clarify its pathophysiological role in various diseases.

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