Functional protein C and anti-cardiolipin antibody in children with portal vein thrombosis

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Background: Portal vein thrombosis (PVT) is a common cause of portal hypertension in children from developing countries. Deficiencies of proteins C and S and elevated anticardiolipin antibody (aCL) levels have been shown to predispose to venous thrombosis. We studied these factors in children with idiopathic PVT. Methods: 19 children with PVT (mean [SD] age 5.7 [2.1] y; 15 boys) were studied; all had variceal bleeding, and had PVT on ultrasonography. Functional protein C activity was measured using a clotting assay; if it was normal, a clotting assay for functional protein S activity was performed. IgG aCL levels were measured in all sera using an in-house standardized solid-phase ELISA.

Results: Protein C functional activity ranged from 4% to 109%. Eight children had activity below 70%, the lower cut-off of the normal range. Protein S assay, done in 10 of the 11 children with normal protein C activity levels, was normal (above the cut-off level of 65% of the normal range). IgG aCL levels were abnormally elevated (>mean ± 2SD of 16 healthy control children) in nine children; of these, three had associated protein C deficiency. Thus, of the 19 children with idiopathic PVT, 14 had abnormality in one or more tests. Conclusion: A majority of children with PVT of unknown etiology have functional protein C deficiency or abnormally elevated levels of aCL antibodies. [Indian J Gastroenterol 2001;20:47-49]

Key words: Coagulopathy, portal hypertension, protein S

Portal vein thrombosis (PVT) is responsible for 70%-80% of portal hypertension among children in the developing world.\(^1\) In a large majority of children with this disease, no underlying cause can be found on conventional investigations; such cases are labelled as idiopathic PVT.\(^4\)

Protein C (PC) is a naturally occurring anticoagulant that gets activated by thrombin and thrombomodulin. It inhibits activated coagulation factors Va and VIIIa.\(^5\) It also promotes lysis of blood clot by increasing the levels of circulating plasminogen activator. Deficiency of PC has been shown to be associated with recurrent venous thrombosis at various sites.\(^6\)\(^7\) Protein S (PS) is a vitamin K-dependent protein synthesized in the liver as an inactive precursor, which is activated by vitamin K-mediated post-translational carboxylation. PS acts as a cofactor of activated PC, with which it appears to form a complex that binds to phospholipid surfaces in the presence of calcium and inhibits the activity of factors V and VIII. Deficiency of PS has been associated with spontaneous thrombosis.

Similarly, presence of anticardiolipin (aCL) antibodies is believed to play a role in the pathogenesis of spontaneous thrombosis in venous channels.\(^7\) Several hypotheses have been forwarded to explain this: binding of aCL to vascular endothelium initiating the coagulation cascade, decreased release of prostacyclin from endothelium, interference with activation of proteins C and S, activation of platelet membrane phospholipids.\(^9\)\(^10\)

To elicit the role of these factors in the pathogenesis of PVT, we studied functional PC and PS levels and aCL in children with PVT of unknown etiology.

Methods

Nineteen consecutive children with PVT, aged below ten years (mean 5.7 [SD 2.1] y; range 1.5-9; 15 boys), were studied; they had presented with gastrointestinal bleeding from endoscopically-documented esophageal varices. The age at onset of symptoms was 3.7 (SD 1.1) y (range 1.5-5). All patients had an ultrasonographically demonstrable extrahepatic portal vein block and/or cavernoma. No patient had features suggestive of venous thrombosis at any other site or family history of thromboembolic manifestations. Patients with clinical or biochemical evidence to suggest liver dysfunction (n=2), those with malnutrition and those who had received anticoagulants or fibrinolytic drugs were excluded. All children had normal prothrombin time index (90%, range 76% to 100%); mean platelet count was 125,000 (SD 50,000)/cm\(^3\) (range 75,000 to 180,000). The study was approved by the Ethics Committee of our institution and informed consent was obtained from one parent of each child. PC estimation was also done in four healthy controls (mean age 5.8 [3.0] y; range 2.5-9 y; 3 boys). PS estimation was done in three healthy control children (2 boys). For aCL assay, 16 healthy control children (aged 5.9 [2.3] y; range 2.5-11; 10 boys) were studied.

Protein C activity

Functional PC activity was assayed using a commer-
cially-available clotting assay system (Staclot; Diagnostica Stago, France). This assay is based on the prolongation of activated partial thromboplastin time by PC. In brief, venous blood was collected in trisodium citrate anticoagulant (9:1), centrifuged at 2500xg for 15 min, and plasma was stored at -20°C till analysis, which was done within 30 days of sample collection. To 100 μL of 1:10 dilution of test sample in a plastic test tube, 0.1 mL of lyophilized human plasma free of PC and 0.1 mL of purified extract of Agkistrodon C, a PC activator, were added and the mixture was incubated at 37°C for 180 s. Thereafter, 0.1 mL of 0.25M CaCl₂ prewarmed at 37°C, was added to the tube, which was kept in a 37°C water bath with gentle shaking. Time taken for the earliest clot to appear after addition of CaCl₂ was measured using a stop-watch. Calibration was done using three dilutions of a thrombocalibrator (Diagnostica Stago, France), which is a lyophilized pooled standard serum. On plotting the clotting time values of various concentrations of calibrators, a straight line was obtained, from which protein C activity of each test specimen was read against its respective clotting time. As specified by the manufacturer, PC level below 70% of that of the thrombocalibrator was considered as abnormally low.

**Protein S activity**

Functional PS activity was assayed only in children who had normal PC activity. It was done using a commercially available clotting assay system (Staclot; Diagnostica Stago, France); this assay is based on inhibition of activated clotting factor Va by protein S. Sample collection and storage were similar to those used for PC estimation. As specified by the manufacturer, protein S level below 65% of that of the thrombocalibrator was considered abnormally low.

**ELISA for anticardiolipin antibody**

IgG aCL antibody level was measured using the method of Loizou et al. Briefly, wells of 96-well plates were coated with 50 μL of cardiolipin in alcohol (50 mg/mL; Sigma, USA) overnight at 4°C. Wells were blocked with 150 μL of phosphate buffered saline (PBS; 0.15M, pH 7.2) containing 10% fetal calf serum (FCS) for 2 h at 4°C. Following washing with PBS, 50 μL of diluted sera and positive control (1:50) was added in triplicate and plates were incubated for 2 h. After washing six times with PBS, 50 μL of anti-human IgG conjugated with horse radish peroxidase (1:1000; Dakopatts, Germany) was added; after 2 h of incubation and washing, 50 μL of substrate (ortho-phenylenediamine 0.4 mg/mL in citrate-phosphate buffer and 0.04% hydrogen peroxide) was added and incubation was done for 30 minutes at 37°C. Reaction was stopped with 2.5N sulfuric acid and the plates were read at 492 nm.

All samples were run in triplicate. Interassay and intra-assay variabilities were less than 10%. The concentration of aCL (GPL units) in the test serum was read from a curve generated by running doubling dilution of a standard serum. Anticardiolipin antibody levels exceeding mean ± 2 SD of those in normals were considered as abnormal.

**Results**

Protein C functional activity in children with PVT ranged from 4% to 109%. Eight of 19 children had PC activity below 70%, the cut-off of normal range (Fig 1). All four healthy children had normal PC activity. There was no difference in clinical features of patients with low and normal PC activity.

Protein S assay was done in 10 of 11 children with normal PC levels (one child did not come for testing). Protein S functional activity was normal in all these children (range 84%-130%) and in the three healthy children.

Anticardiolipin antibody levels in normal children and those with PVT are shown in Fig 2. The level in
control children was 4.3 (2.9). Thus, aCL levels exceeding 10.1 were taken as abnormal. The aCL levels in children with PVT were significantly higher (10.9 [6.3]; p<0.001). Nine of 19 children had abnormal aCL levels; three of these children also had low PC activity.

Thus, of the 19 children with PVT, 14 had one or more abnormalities; these included five with PC deficiency alone, six with elevated aCL levels and three with both PC deficiency and abnormal aCL levels.

Discussion

Eight of 19 children with PVT in our study had abnormally low PC activity. In France low PC activity was reported in 9 of 20 children with PVT. In Mexico, an area where PC is common, 56% of adult patients with PVT had low PC activity. In a small study from India, one of four adult patients with PVT had low PC activity.

Family studies of PC activity in children with PVT do not support a genetic origin of this defect. The fact that levels of PC do not improve after successful shunt surgery excludes the possibility of excessive consumption of PC in the spleen and/or in congestive gut. Increased clearance of PC on altered endothelial cells of the cavernoma or portosystemic collaterals may explain low levels of PC activity. PS deficiency has also been associated with PVT in a few reports, but we have not found this in any of our patients.

The role of aCL antibodies in the causation of PVT has not been well studied. In a follow-up study of children undergoing liver transplantation, one of five children who developed PVT showed presence of aCL antibodies. A 5-year-old girl with idiopathic PVT has been reported to have aCL antibodies. Our data suggest that presence of these antibodies may have pathogenic importance in patients with PVT.

Three of our patients who had both PC deficiency and abnormally elevated aCL levels need mention. Anti-cardiolipin antibody is known to inhibit activation of PC, leading to decrease in functional activity of the latter. Since we performed a clotting assay that measures only the functional activity of PC and not total PC concentration, it is not possible to determine if the PC deficiency in these patients was a primary event or was secondary to elevated levels of aCL antibodies.

In conclusion, a majority of children with 'idiopathic' PVT have functional PC deficiency or abnormally elevated aCL antibodies.

References


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