Severe neonatal protein C deficiency: Prevalence and thrombotic risk

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Severe deficiencies of protein C, a pivotal coagulation-regulatory protein, have been reported in neonates as an apparently transient condition. In this prospective study, cord blood was collected at 193 deliveries and assays of protein C were correlated with clinical status, other coagulation results, and outcome. Protein C levels of <0.1 unit/ml were found most frequently in preterm infants with respiratory distress, infants of diabetic mothers, and infants of twin gestations. Levels of protein C correlated with levels of factor VIII activity but did not correlate with markers of consumptive coagulopathy. A protein C level <0.1 unit/ml was significantly correlated with the subsequent onset of thrombosis, even when the effects of gestational age and birth weight were excluded. Low cord blood levels of protein C may reflect delayed maturation or increased turnover in certain infants and appear to convey an independent risk of thrombosis, but the critical concentration of protein C necessary to maintain neonatal hemostasis is not known. (J PEDIATR 1991;119:793-8)

Protein C is a pivotal regulatory protein of the coagulation system that functions to limit coagulation and augment fibrinolysis. In addition to protein C, the system includes protein S, which is a cofactor of activated protein C, C4b binding protein, which is a complement component whose binding to a portion of protein S renders it inactive as a protein C cofactor, and protein C inhibitor, which neutralizes protein C. Levels of protein C activity and antigen have been reported to be low at birth (30% to 50% of adult values) and to rise very slowly after birth (Yoshioka K, Kinoshita S, Shinya O, et al. (National Hospital, Osaka, Japan): personal communication, 1985).[1-5] Although levels of total protein S are also very low in the fetus and neonate,

protein S function is significantly higher as a result of very low levels of C4b-bp.

We previously reported on 11 infants with transient neonatal levels of protein C similar to those reported in the homozygous-deficiency state.[6] Infants with homozygous protein C deficiency have disseminated intravascular coagulation, purpura fulminans, and large-vessel thrombosis within a few days of birth.[7-11] In older children and adults, heterozygous deficiencies of protein C have been associated with recurrent thromboses.[12-15] The sick preterm infant has a predisposition to DIC and thrombotic complications.[16-18]

C4b-bp	C4b binding protein
DIC	Disseminated intravascular coagulation
PIVKA-II	Protein induced by vitamin K absence–II

The level of protein C is very low in the preterm infant, but little is known regarding the physiologic function of the neonatal system. The role of severe neonatal protein C deficiency in the development of clinical complications is currently unknown. This article reports the frequency of

severe neonatal protein C deficiency and examines the relationship of the protein C level to neonatal DIC and thrombosis.

METHODS

Clinical protocol. This study was performed through the University of Colorado Health Sciences Center Neonatal Clinical Research Center. Cord blood samples were collected by the clinical research nurses, as described below. Clinical diagnosis and gestational age were assessed by review of the obstetrics record, Dubowitz score, and examination by the attending neonatologist. This study was approved by the University of Colorado Health Sciences Center Human Subjects Committee. Written, informed consent to obtain the ultrasonographic data was obtained from a parent of each infant with an indwelling umbilical artery catheter. The research nurses collected data regarding the clinical condition and follow-up of all study infants. No modifications of clinical care were made on the basis of the laboratory or ultrasound assessment of study infants.

Documentation of thromboses. All study infants who required placement of an umbilical artery catheter were examined with high-resolution real-time ultrasonography on day 3 of catheterization and within 12 hours of catheter removal to rule out the presence of aortic thrombosis. All infants were examined for other thromboses with appropriate studies on clinical indication.

Laboratory methods. Blood samples were obtained from the umbilical vein after separation from the infant at the time of delivery; a two-syringe technique was used. All samples were collected into citrate anticoagulant, immediately placed on wet ice, centrifuged at 1800g, and frozen at $-70°$ C until assay. Normal control values were derived from plasma obtained from a minimum of 30 healthy adults receiving no medications.

All samples were anticoagulated with 1 part anticoagulant to 9 parts whole blood. No attempt was made to correct anticoagulant volume for hematocrit values before sample collection, but hematocrit was determined on the sampled blood, and only specimens with a hematocrit value between 0.40 and 0.60 were retained for inclusion in the study.

To exclude vitamin K deficiency, we screened all plasma samples with a prothrombin time determination and PIVKA-II assay of noncarboxylated prothrombin as described previously.[19] Only samples with a negative PIVKA-II assay were included in this study.

Protein C components—protein C, protein S, C4b-bp, and protein C inhibitor—were assayed and compared with levels of the coagulation inhibitors antithrombin III and

heparin cofactor II. Protein C activity was measured chromogenically, and protein C antigen was measured by the Laurell rocket technique, as described previously.[6] The normal values for protein C activity were derived from a group of 38 subjects and had a mean of 0.99 unit/ml with a 2 SD range of 0.69 to 1.29 units/ml and an observed range of 0.67 to 1.40 units/ml. This assay has a lower limit of detection of 0.05 unit/ml. Protein C antigen was measured immunologically by the Laurell rocket technique, as described previously.[6] The lower limit of protein C antigen detection with this method is 0.10 unit/ml. The group of 38 normal subjects had a mean of 1.00 unit/ml with a 2 SD range of 0.70 to 1.30 units/ml and an observed range of 0.72 to 1.25 units/ml.

Total protein S was assayed by the Laurell technique with 0.75% monospecific polyclonal rabbit antihuman protein S antibody under conditions identical to those described for the protein C antigen assay. A control group of 56 normal subjects had a mean total protein S value of 1.02 units/ml with a 2 SD range of 0.70 to 1.34 units/ml and an observed range of 0.64 to 1.46 units/ml. Free protein S was quantitated by Laurell rocket immunoelectrophoresis after the protein S complexed to C4b-bp was precipitated by adding polyethylene glycol 8000 at 4° C. The electrophoresis was conducted at 10° C at 10 meter angle/slide for 18 to 24 hours with the same agarose and buffer system as that used for protein C antigen but with the addition of 2% polyethylene glycol 8000 to the sample and tank buffer. The control group of 38 normal subjects had a mean free protein S level of 0.97 unit/ml with a 2 SD range of 0.47 to 1.47 units/ml and an observed range of 0.58 to 1.80 units/ml. The lower limit of detection for the protein S assays is 0.10 unit/ml. Protein C inhibitor and C4b-bp were assayed by Laurell immunoelectrophoresis with polyclonal antibodies. Antithrombin III and heparin cofactor II were assayed chromogenically as described previously.[20, 21] Factor VIII activity was determined with a standard one-stage coagulant assay. Normal pooled plasma was diluted in hemophilic plasma to construct the standard curve.

In vivo activation of coagulation was assessed with measurements of *d*-dimer, prothrombin time, fibrinogen, and platelet count. The *d*-dimer assay of crossed-linked fibrin was performed with a latex agglutination using a monoclonal antibody purchased from Dade, Baxter-Hyland, Miami, Fla. A positive result indicates ≥200 ng/ml of circulating cross-linked fibrin. This assay has been reported to be sensitive to in vivo generation of thrombin and relatively insensitive to artifacts of sample collection.[22] Prothrombin time and fibrinogen and platelet count were obtained with standard techniques.

Cord blood samples were obtained from 193 infants.

Table I. Clinical and protein C results in study infants

Infant group	Gestational age (wk)	Weight (gm)	d-Dimer (%)	Protein C activity (units/ml)	Protein C antigens (units/ml)	Protein C <0.1 unit/ml		Thromboses	
						n	%	n	%
Term singleton, well (n = 65)	40 (37-42)	3310 (2350-4000)	10	0.40 (0.23-0.53)	0.39 (0.22-0.77)	0/36	0	0/36	0
Term singleton, distress (n = 21)	40 (37-40)	3200 (2400-3800)	35	0.33 (<0.05-0.63)	0.33 (0.17-0.55)	1/21	5	0/21	0
IDM (n = 17)	38.2 (34-42)	3275 (2520-3980)	42	0.36 (0.05-0.58)	0.31 (<0.10-0.58)	2/17	12	2/17	12
SGA (n = 6)	37.7 (34-40)	2000 (690-2640)	67	0.39 (0.29-0.51)	0.27 (0.20-0.35)	0/6	0	0/6	0
Term twin (n = 16)	38.3 (37-39)	2635 (1790-3580)	12	0.37 (<0.05-0.75)	0.24 (0.09-0.47)	4/16	25	1/16	6
Preterm singleton, well (n = 11)	32.1 (28-36)	1575 (1410-2710)	9	0.23 (0.11-0.33)	0.19 (0.15-0.30)	0/11	0	0/11	0
Preterm twin, well (n = 15)	31.2 (26-36)	1645 (900-2390)	0	0.13 (<0.05-0.38)	0.20 (<0.10-0.36)	4/15	27	2/15	13
Preterm singleton, RDS (n = 26)	30.9 (25-35)	1400 (690-2420)	24	0.13 (<0.05-0.29)	0.18 (<0.10-0.40)	9/26	35	5/26	19
Preterm twin, RDS (n = 16)	29.0 (25-34)	1260 (840-1860)	14	0.06 (<0.05-0.15)	0.13 (<0.10-0.36)	6/16	37	5/16	31

In columns 2, 3, 5, and 6, numbers in parentheses are ranges.
IDM, Infant of a diabetic mother; *SGA*, small for gestational age; *RDS*, respiratory distress syndrome.

Protein C activity, protein C antigen, total protein S, free protein S, C4b-bp, and *d*-dimer were assayed on all samples. Because of limitations in sample volume, other inhibitors could not be assayed on every sample. Antithrombin III assays were performed on 109 samples, heparin cofactor II was assayed on 47 samples, and protein C inhibitor was assayed on 10 samples. Fibrinogen concentration, prothrombin time, and platelet count were determined on 70 samples from high-risk infants. The high-risk group was defined as those neonates born with fetal distress, maternal diabetes, intrauterine growth retardation, or respiratory distress syndrome.[6]

Data analysis. Data were analyzed with statistical computer software (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill.) analysis of variance, multivariate analysis, and linear regression.

RESULTS

Imaging results. All infants were followed until discharge from the nursery. Fifty-five preterm infants required placement of an umbilical artery catheter; aortic thrombosis was documented in eight of these infants. Large-vessel thrombosis not associated with indwelling catheters was documented in three infants, including two infants of diabetic mothers (one renal vein thrombosis diagnosed with scintigraphy and one sagittal sinus thrombosis diagnosed with computed tomography) and one term twin (aortic thrombosis diagnosed with ultrasonography).

Coagulation results

Levels of protein C in normal and high-risk neonates. Levels of protein C activity and antigen are shown in Table I along with clinical data regarding the study infants. Sixty-five well term infants had a mean protein C activity of 0.40 unit/ml and a protein C antigen value of 0.39 unit/ml. The mean protein C activity of high-risk infants was not different from that of normal infants, although the range was wider, especially at the lower end. Eleven stable preterm singleton infants were studied and had mean protein C activity of 0.23 unit/ml and protein C antigen of 0.19 unit/ml. Preterm infants with respiratory distress syndrome, twin gestation, or both had decreased protein C activity that was consistently lower than the mean protein C antigen.

Frequency of severe protein C deficiency in newborn infants. Severe deficiency of protein C activity, defined as <0.1 unit/ml, was seen in no well term or preterm singleton infants. Overall, 10% of high-risk term infants had protein C levels <0.1 unit/ml. However, severe protein C deficiency was present in cord blood of 35% of all infants with respiratory distress syndrome. Protein C activity <0.1 unit/ml was significantly correlated with low gestational age (p <0.0001) and respiratory distress syndrome (p <0.0001). In all, 26 study infants were noted to have protein C activity <0.1 unit/ml. A coagulant assay may have revealed a greater deficit in the activity of protein C with its physiologic substrate than did the chromogenic assay used.

Table II. Neonatal coagulation inhibitor levels

Group	Protein C activity	Antithrombin III	Heparin cofactor II	Total protein S	Free protein S	C4b-bp	Protein C inhibitor
Term, well	0.40	0.59	0.51	0.25	0.42	0.12	0.79
Term, fetal distress	0.34	0.63	0.48	0.25	0.46	0.18	
Term, SGA	0.38	0.39	0.39	0.22	0.42	0.14	
Term, maternal diabetes	0.33	0.56	0.23	0.22	0.42	0.18	
Term, twin	0.34	0.52	N.D.	0.19	0.43	0.08	
Preterm, well	0.30	0.34	0.24	0.21	0.33	0.05	
Preterm, RDS	0.13	0.34	0.24	0.17	0.32	0.11	
Preterm, twin	0.13	0.33	0.25	0.17	0.31	0.12	

All results are in units/ml.
SGA, Small for gestational age; *RDS*, respiratory distress syndrome.

Table III. Relationship of cord blood protein C activity to catheter-related aortic thrombosis in infants with respiratory distress

	Protein C <0.1 unit/ml	Protein C ≥0.1 unit/ml	*p*
Aortic thromboses *(n)*	6/18 (33%)	2/37 (6%)	<0.01
Mean gestational age (wk)	31.4	30.5	NS
Mean birth weight (gm)	1540	1460	NS

NS, Not significant.

Therefore the magnitude of protein C deficiency in preterm infants with respiratory distress may be greater than these results suggest.

Cause of neonatal protein C deficiency. Acquired decreases in protein C and other regulatory proteins have been described in patients with DIC.[23] In this study the *d*-dimer test result was frequently positive in stressed term and preterm infants. However, there was no correlation between positive *d*-dimer test results and low protein C levels ($p = 0.5$). Results of the other screening tests of DIC performed in 70 high-risk infants showed a similar lack of correlation with low protein C values, although none of the study infants had evidence of severe DIC.

To determine whether severe deficiencies of protein C in cord blood may be related to increased activation and consumption, we compared levels of protein C in high-risk infants with levels of antithrombin III and factor VIII. Protein C antigen showed a strong correlation with antithrombin III antigen ($r = 0.71; p = 0.0001$). However, protein C activity showed a lesser correlation with antithrombin III activity ($r = 0.37; p = 0.0027$), primarily related to decreased protein C activity. Factor VIII activity was measured in cord blood of 21 high-risk infants. In these infants the mean factor VIII activity was 0.71 unit/ml, with a range of 0.06 to 2.40 units/ml. Factor VIII activity correlated with protein C activity ($r = 0.56; p = 0.0001$). These results are compatible with either increased turnover of both fac-

tor VIII and protein C in sick infants or a relationship in the expressed plasma levels of these two proteins. The cord blood results suggest that alteration in levels of protein C and factor VIII occurs before delivery of infants who subsequently have respiratory distress syndrome.

Table II lists the results of protein C and other coagulation-regulatory proteins in the study infants and shows a gestational age dependence. Severe deficiencies of the other regulatory proteins were not detected in any of the groups of study infants.

Very low levels of protein C activity and predisposition to thrombosis. Within the entire study group, the onset of thrombosis was inversely correlated with protein C activity, gestational age, and respiratory distress syndrome ($p <0.0001$ for each). Six term infants with maternal diabetes or twin gestation had severe protein C deficiency. The three cases of thrombosis that were not associated with umbilical artery catheters were identified in these two infant groups. To isolate the contribution of cord blood protein C level to thrombotic risk in preterm infants, we determined the relationship of protein C activity to aortic thrombosis for all preterm infants with respiratory distress syndrome and an indwelling umbilical artery catheter. Infants were segregated into groups with protein C level <0.1 unit/ml and ≥0.1 unit/ml. Between these two groups of preterm infants, there was no correlation of gestational age, birth weight, or severity of respiratory distress with protein C levels <0.1

unit/ml. However, there was a significant correlation of catheter-related thromboses in infants with severe protein C deficiency (p <0.01; Table III). When similar analyses were performed for antithrombin III, there was a less marked relationship between antithrombin III level and catheter-related thrombosis. However, only two sick preterm infants had an antithrombin III level <0.18 unit/ml; one of these two infants had an aortic thrombosis.

DISCUSSION

Genetic protein C deficiency is recognized as a cause of recurrent thrombosis in children and adults. The newborn infant has physiologically low levels of protein C that rise slowly after birth. The importance of protein C in the regulation of thrombus generation during fetal and neonatal life is currently unknown. We recently described the occurrence of severe protein C deficiency in newborn infants as a nonfamilial event; 5 of 11 infants with severe protein C deficiency had clinical signs of thrombosis. This prospective study was conducted to investigate the frequency of severe deficiency in newborn infants and to determine whether a neonatal risk for thrombosis is associated with a critical protein C level.

Disseminated intravascular coagulation and large-vessel thromboses occur with increased frequency in preterm infants with respiratory distress.[16-18] In this study, both severe protein C deficiency and neonatal thromboses were most frequent in preterm infants with respiratory distress. When protein C levels were analyzed in infants with prematurity, respiratory distress, and indwelling umbilical artery catheters, there appeared to be an independent risk of thrombosis contributed by protein C activity level.[24]

The cause of severe protein C deficiency in our patients cannot be determined with certainty. Individual differences in rates of maturation are possible; the near-term infants from diabetic or twin gestations could have delayed maturation relative to postconceptional age.

Disseminated intravascular coagulation results in the consumption of coagulation factors and can cause acquired protein C deficiency.[23] The discrepant protein C activity/antigen ratio noted in the high-risk preterm infants is compatible with DIC. The correlation of protein C activity with factor VIII activity is intriguing. However, the d-dimer test, as well as the coagulation screening tests, failed to support a diagnosis of DIC in infants with severe protein C deficiency. Levels of other coagulation-regulatory proteins in these infants similarly failed to document global consumption of coagulation factors. It is possible that the screening tests of coagulation activation used in this study were too insensitive to detect subtle consumption. Detection with more sensitive probes, such as an assay for the F 1 + 2

fragment of prothrombin[25] or an assay of circulating coagulation inhibitor complexes, would be necessary to rule out subtle protein C consumption.

We conclude that severe protein C deficiency of undertermined cause occurs in preterm infants with respiratory distress syndrome, infants of diabetic mothers, and some term gestations and appears to increase the risk of thrombosis in these infants. At this time there is no benefit to routine screening of protein C activity in high-risk infants. However, further study is warranted to determine whether treatment of high-risk infants who have protein C deficiency with anticoagulation or protein replacement is indicated to prevent or treat catheter-related thromboses.

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