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Characteristics of Fibrin Formation and Clot Stability in Individuals with Congenital Type IIb Protein C Deficiency

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Abstract

Many studies have shown that unregulated or excessive thrombin formation is potentially a cause of thrombosis; however, studies examining processes that contribute to fibrin stabilization in individuals predisposed to thrombosis are limited. In this study, we investigate a family with familial thrombosis via type IIb protein C (PC) deficiency. Using contact pathway inhibited whole blood, thrombin generation, fibrin clot formation and factor (f)XIII activation were monitored over time in control (n=5) and PC deficient (n=4, 34 – 69% PC by activity) subjects. The dynamics of thrombin generation varied significantly with the time required to reach the maximal rate of thrombin-antithrombin formation being much shorter in PC deficiency (5.8 ± 0.4 minutes) than in controls (9.7 ± 0.4 minutes; $p < 0.001$). PC deficient clots were significantly heavier than control clots ($p < 0.001$) and this difference could not be contributed exclusively to differences in fibrinogen levels between groups. FXIII was consumed faster in PC deficient subjects (23.7 ± 2.0 nM/minute) than in controls (5.1 ± 1.5 nM/minute; $p < 0.0001$) suggesting increased fXIII activation and incorporation of fXIIIa substrates into the clot. In plasma, the clot lysis time was increased in PC deficiency by both TAFIa dependent and independent mechanisms. Since PC deficient clots are both denser and show a greater degree of resistance to fibrinolysis, these clots would likely resist fibrinolysis and potentiate fibrin deposition observed in thrombosis.

Keywords

Carboxypeptidase U; fibrin; thrombosis; protein C deficiency; factor XIII; TAFI

Introduction

The processes of coagulation and fibrinolysis are inextricably linked through the activation of thrombin activatable fibrinolysis inhibitor (TAFI, also known as procarboxypeptidase U)¹ and factor (f) XIII² by thrombin. A blood clot is formed after thrombin cleaves fibrinogen to yield fibrin monomers that spontaneously assemble to form a mature fibrin clot. Activated fXIII (fXIIIa) is a transglutaminase that forms isopeptide bonds between fibrin monomers that increase the strength of the clot³. Additionally, fXIIIa cross-links α_2 -antiplasmin to fibrin which impairs clot lysis^{4, 5} by inhibiting plasmin, the enzyme responsible for digesting fibrin. Activated TAFI (TAFIa) attenuates fibrinolysis by removing

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lysine residues from plasmin-modified fibrin that act as cofactors for plasminogen activation⁶. While unregulated⁷ or excessive⁸ thrombin formation is presumed to be the primary cause of thrombosis, the indirect role of thrombin as an attenuator of fibrinolysis (through the activation of TAFI and/or fXIII) may also contribute to hemostasis. Recently, Meltzer *et al.* reported that TAFI levels are associated with venous thrombosis risk⁹ implying that TAFI contributes to fibrin deposition and a clinical event.

Protein C (PC) deficiency has been shown to be an independent risk factor for venous thrombosis¹⁰. PC deficiency is characterized as type I (consistent PC antigen and activity) or type II¹¹ (inconsistent PC antigen and activity). Since many afflicted individuals have no history of thrombosis, it has been suggested that another factor, together with a deficiency of PC, may be responsible for thrombosis¹⁰. Recently, cell adhesion molecule 1 was identified as a probable thrombophilia gene that increases the risk of venous thrombosis in PC deficiency¹² and other factors probably exist.

Previously, it was suggested that fibrinolytic activity may contribute to the variable pattern of thrombosis among PC deficient individuals¹⁰. In this small descriptive study, we have used a tissue factor (Tf)- initiated whole blood model of coagulation to evaluate elements of secondary hemostasis such as fXIII activation and consumption and fibrin formation that may contribute to fibrin deposition in individuals with type II, Vermont kindred IIb PC deficiency¹⁰. These individuals have a CAT+CCAT 3363 inserted (Ins) C mutation in exon 6 of the PC gene¹¹ which reduces the activity of activated PC to a greater extent than antigen levels.

Subjects, Materials and Methods

Subjects

Individuals with familial PC deficiency¹⁰ not on anticoagulation and healthy controls were recruited and advised according to a protocol approved by the Institutional Review Board at the University of Vermont Human Studies Committee. Informed written consent was obtained from 4 subjects with familial PC deficiency (one subject analyzed twice, S1) and 5 healthy controls, matched by age. The individuals with PC deficiency had PC levels ranging from 34 – 69% (mean $\pm$ SD, $49.8 \pm 12.6\%$ PC) at the time of the blood draw. The PC deficient patient characteristics are presented in Table 1.

Materials

HEPES, Tris-HCl, citrate, 1-palmitoyl-2-oleoyl-phosphatidyl serine, 1-palmitoyl-2-oleoyl-phosphatidyl choline were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant Tf was provided by Drs. Lundblad and Liu (Hyland division, Baxter Healthcare Corp, Duarte, CA) and was relipidated in phosphatidyl serine: phosphatidyl choline (PCPS) (75%:25%) vesicles by a previously described protocol¹³. Corn trypsin inhibitor (CTI) was prepared as described¹⁴. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPRck) and an α -thrombin antibody was provided by Dr. Richard Jenny (Haematologic Technologies, Essex Junction, VT). The burro α -antithrombin antibody was developed *in-house*. The rabbit polyclonal α -fXIII (D4679) was a gift from P. Bishop (ZymoGenetics, Seattle, WA, USA) and the mouse monoclonal α -fibrinogen 2E (α -chain; α -Fg2E) was produced *in house*. Potato tuber carboxypeptidase inhibitor (PTCI) was purchased from Calbiochem (San Diego, CA, USA) and PC-deficient plasma was purchased from Affinity Biologicals Inc. (Ancaster, Ontario). Soluble thrombomodulin (sTM) was purchased from American Diagnostica Inc. (Stamford, CT) and the human TM immunoassay was purchased from R&D Systems® (Minneapolis, MN).

Methods

Whole blood coagulation assay

Experiments were performed as previously described^{15, 16} at 37°C on a rocking platform. Briefly, venous blood was obtained by antecubital phlebotomy and added (1mL) to tubes preloaded with CTI (100µg/mL) and relipidated Tf (5pM, PCPS 1:2000 protein:lipid). Experiments were also conducted in the presence of sTM (10 nM). Over the course of 20 minutes, samples were quenched with 1 mL of a solution containing FPRck (50µM), benzamidine-HCl (20mM) and EDTA (50mM), pH 7.4. At the end of the 20 minute time course, samples were centrifuged at 1100 x *g* for 15 minutes to separate the serum from the cells and fibrin. The soluble fraction (serum) was stored at -80°C and later used to determine the time courses of thrombin generation, fXIII activation/consumption and fibrinogen consumption. The insoluble fraction was stored at -80°C and later used to determine the clot weights.

Thrombin generation and fibrinogen levels

Thrombin-antithrombin (TAT) complexes in serum were measured using an *in-house* immunoassay. Briefly, our *in-house* immunoassay follows standard ELISA procedures and makes use of a burro anti-human antithrombin capture antibody and a mouse monoclonal anti-human thrombin antibody. Data describing TAT formation were determined by fitting the TAT data to a sigmoid function. The best-fit was used to extract the maximal TAT level and the maximal rate for each time course. Fibrinogen levels were determined using the Clauss method at the clinical laboratory of Fletcher Allen Healthcare (Burlington, VT). The CVa for the assay is 6.1%.

Determination of clot weights

The insoluble fraction from the whole blood coagulation assay was analyzed as previously described¹⁷. Briefly, the insoluble clotted samples were washed repeatedly with 2 mL of a 150 mM NaCl solution, with a 12–24 hour interval between washes to allow the soluble material within the clots and the cells to diffuse into solution. The clots were repeatedly rinsed with H₂O to remove salt and subsequently lyophilized and weighed.

Fibrinogen consumption and fXIII activation and consumption

Serum samples were subjected to SDS-PAGE (4–12%) and transferred to nitrocellulose membranes (Bio-Rad) using a semi-dry transfer apparatus (250 mAmp for 1.5 hours). To detect fibrinogen in the serum, the primary antibody α-Fg2E was used at 5µg/mL and the secondary antibody (goat α-mouse IgG horseradish peroxidase) was used at a 1:5000 dilution. To detect fXIII(a) in the serum, the primary antibody was a polyclonal α-fXIII used at 5 µg/mL, the secondary antibody was goat α-rabbit HRP at 1:5000 dilution. Luminol (NEN Life Science Products Inc.) was used to detect the analyte via HRP conjugated IgG. Densitometry of each immunoblot was conducted using the Fujifilm chemiluminescent imager LAS4000 and Fujifilm MultiGauge imaging software (version 3.x). A relative fraction was calculated using the fibrinogen or fXIIIa present at each time point divided by total fibrinogen or total fXIII A-chain (fXIIIa + fXIIIaA) detectable.

Clot lysis assays

Citrated PC-deficient or normal plasma (40 µL) was added to a solution (40 µL) containing 10 pM Tf, 20 mM CaCl₂, 20 nM sTM, 0.2mg/mL CTI and 3 nM tPA in the presence or absence of 10 µM PTCl (final concentrations: 5 pM Tf, 5 mM excess CaCl₂, 10 nM sTM, 0.1mg/mL CTI and 1.5 nM tPA, ± 5 µM PTCl). Experiments were conducted at 37°C in a covered, 96-well plate and the turbidity was monitored at 400 nm over time using a

SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to determine the timing of coagulation and fibrinolysis. The lysis time was taken as the time at which the absorbance decreased by half during fibrin degradation.

Statistics

Between group comparisons were conducted using paired or unpaired *t* test for normally distributed data. All data are presented as mean $\pm$ standard error of the mean. For all statistical analysis $p < 0.05$ was considered statistically significant.

Results

Thrombin generation

Thrombin generation was assessed by measuring TAT complex formation during the course of Tf-initiated whole blood coagulation. Both the control and PC deficient groups had similar maximal TAT levels (298 ± 34 and 275 ± 44 nM, respectively; $p = 0.68$) and a similar rate of TAT formation (46 ± 6 and 61 ± 17 nM/minute, respectively; $p = 0.43$) (Figure 1 (panel A)). Experiments were also performed with sTM (10nM) to enhance the PC pathway. Addition of sTM had no effect on the rate of thrombin generation compared to in its absence ($p > 0.14$) (Figure 1, panel B). There was an obvious trend towards a reduction in the maximal TAT level in the presence of sTM compared to without, which was statistically significant in the PC deficient group ($p = 0.024$) and in the control group ($p = 0.01$). The most notable difference in thrombin generation when comparing controls to PC deficient individuals was the timing of TAT formation. The time required to reach the maximal rate of TAT formation was much shorter in PC deficiency (5.8 ± 0.3 minutes) compared to controls (9.7 ± 0.4 minutes, $p < 0.001$). STM shortened the time to reach the maximal rate of TAT formation in controls (6.2 ± 0.2 minutes, $p = 0.005$) but not in PC deficiency (5.7 ± 0.4 minutes, $p = 0.75$). In the presence of sTM, the rate of TAT formation increased 1.5-fold in the PC deficient group (90 ± 12 nM/minute) compared to the controls, however this was not statistically significant ($p = 0.14$).

Fibrin clots

The first time point containing a clot was 4 minutes in both control and PC deficient blood (Figure 2) which is consistent with the clot times of 3.7 ± 0.4 and 3.8 ± 0.2 minutes, respectively. By the 6 minute time point, the weight of the clots formed in control blood had reached a plateau of 1.45 ± 0.15 mg. In contrast, clots formed in PC deficient whole blood were significantly heavier ($p < 0.001$), with the maximal clot weight of 2.96 ± 0.35 mg occurring 8 minutes after Tf-initiated whole blood clotting. STM had no effect on the maximum clot weight among PC deficient subjects or among controls (data not shown). The difference between control and PC deficient clot weights could not be fully accounted for by differences in the plasma fibrinogen concentration (control: 3.06 ± 0.28 mg/mL, PC-def: 3.89 ± 0.34 mg/mL; $p = 0.087$) or incorporation of fibrin into the clot. In both control and PC deficient groups, all fibrinogen was consumed from the serum within 2 minutes of the clot time (data not shown).

Factor XIII activation and consumption

FXIII activation was quantified by western blot analysis of serum samples from the whole blood coagulation assay (Figure 3). The maximal rate of fXIII activation (panel A) was similar in controls and PC deficient samples (11.5 ± 1.0 nM/minute and 15.1 ± 4.7 nM/minute, $p = 0.13$) but the maximal level of fXIIIa detected in the serum differed significantly. The maximal fXIIIa concentration detected in the serum was greater in controls (59.6 ± 1.6 nM) compared to PC deficient subjects (39.2 ± 12.1 nM) ($p = 0.006$). To confirm this finding,

we also measured fXIII consumption from the serum by western blot analysis (Figure 3, panel B). Factor XIII was consumed from serum at a faster rate and to a greater extent in PC deficiency when compared to controls. The data from Figure 3, panels A and B, suggest that all fXIII is activated to fXIIIa in control samples, however, in PC deficiency there is a discrepancy between the amount of fXIIIa measured in serum (39.2 ± 12.1 nM, panel A) and amount of fXIII remaining in serum (≈ 5 nM, panel B) suggesting that ≈ 55 nM fXIII/fXIIIa is unaccounted for after the fibrin and cells are separated from the serum.

Clot lysis assays

Clot lysis experiments were performed to evaluate the TAFIa-dependent and independent effect of PC-deficiency on fibrinolysis. The clot lysis time was slightly elevated in PC-deficient plasmas (79.8 ± 9.8 minutes) compared to control plasma (60.6 ± 5.5 minutes). In the presence of the TAFIa inhibitor, PTCl, the clot lysis time was shortened significantly in both control and PC-deficient plasmas. The clot lysis time in PC-deficient plasmas (27.3 ± 4.9 minutes) was increased by 50% compared to the clot lysis time in control plasmas (18.4 ± 0.7 minutes). Differences between control and PC deficient groups did not reach the level of statistical significance in the presence of the TAFIa inhibitor or in its absence ($p=0.2$). There was a larger range, however, in the absolute lysis times among PC deficient subjects (+ PTCl: 17.6 – 37.1 minutes; no PTCl: 58.4 – 107.6 minutes) but not in controls (+ PTCl: 16.9 – 19.1 minutes; no PTCl: 49.8 – 67.1 minutes).

Discussion

In this study, we show that the dynamics of thrombin formation are affected in PC deficiency. PC deficient individuals show similar rates and maximal levels of thrombin generation to controls but the time required to reach the maximal rate of TAT formation was much shorter in PC deficiency compared to controls. STM shortened the time to reach the maximal rate of TAT formation in controls (6.2 ± 0.2 minutes, $p=0.005$) but not in PC deficiency (5.7 ± 0.4 minutes, $p=0.75$) and increased the rate of TAT formation in PC deficiency by 1.5-fold ($p=0.14$). We also show that the altered dynamics of thrombin generation in PC deficiency may contribute to thrombosis by increasing the clot density and consumption of fXIII (presumably through increased fXIIIa formation which becomes bound to the clot) and by increasing the clot lysis time by TAFIa-dependent and independent mechanisms.

Based upon plasma fibrinogen concentrations, plasma volume (hematocrit) and typical yield using this clot washing procedure, the expected clot weights in the control and PC deficient groups were 1.5 ± 0.1 and 1.8 ± 0.2 mg, respectively. The maximal clot weight of the control group was as expected (1.45 ± 0.15 mg), however, the maximal clot weight of the PC deficient group was significantly higher than expected (2.96 ± 0.35 mg, $p<0.0001$). The mechanism that contributes to the increased clot weight in PC deficiency is unknown at this time but due to the stringent clot washing procedure, the most likely explanation is that additional proteins are cross-linked to the fibrin clot. Previously, we have evaluated clot weights and fXIII activation in hemophilia A whole blood and determined that both the clot weight and extent of fXIII activation was decreased compared to controls and contributed to the formation of “discordant fibrin” in hemophilia A^{18,19}. By contrast, PC deficiency causes increased clot weight and a depletion of fXIII both of which leads to formation of a different type of discordant fibrin which has increased clot density. Whereas the discordant fibrin formed in hemophilia contributes to an overt bleeding diathesis, discordant fibrin in PC deficiency would likely contribute to thrombosis because the dense, highly cross-linked fibrin clots would be less permeable thus restricting blood flow and impeding fibrinolysis. Indeed, a number of studies have shown that fibrin is significantly altered in plasma from patients with thrombosis or individuals predisposed to thrombosis. Clots formed in plasma

from these subjects have significantly altered clot properties²⁰ compared to controls, which include stiffer and shorter fibrin fibers^{21, 22}, decreased fibrin permeability^{22–25} and compaction²⁴ and increased fiber diameter and fibrin density^{23, 24}. The architecture of fibrin in individuals with or predisposed to thrombosis are consistent with our finding that PC deficiency causes much heavier and therefore, denser clots in whole blood. Increased fXIII activation has also been shown to reduce fibrin permeability²⁶, the degree to which this occurs in PC deficiency should be explored in order to delineate the precise mechanism that causes altered clot structure in afflicted individuals. Therefore, future studies will focus on identifying proteins which are covalently attached to the fibrin clot.

Some PC-deficient subjects enrolled in this study had a relatively high level of PC (up to 69% PC) and it is expected that individuals with lower PC levels would have even more pronounced TAT formation than those studied here. Since the PC level is predicted to directly influence thrombin generation and as a result secondary haemostasis, the extent and rate of fXIII activation is also expected to be elevated in individuals with very low PC levels. The kinetics of PC activation is such that there would be little or no activation of PC in the absence of TM. In this study, we showed changes in the dynamics of thrombin generation in PC deficiency without adding exogenous TM. Since TM increases the rate of PC activation by thrombin by 1000-fold²⁷, the presence of low levels of TM would significantly increase activation of the PC pathway. In the PC group studied here, endogenous soluble TM was present in the plasma when measured suggesting some limited activation of the PC pathway in the absence of added TM. In another group of PC deficient individuals (n=10, unpublished observation) we found that the plasma concentration of soluble TM was 128 ± 7 pM, which is consistent with the endogenous soluble TM levels reported here.

Despite a major limitation of this study being the sample size, we did observe significantly altered dynamics with respect to thrombin generation and significantly increased clot weights in our small group of PC-deficient subjects compared to controls. The groups were also not matched equivalently by sex. Therefore, it is possible that some differences in clot weight are due to gender, however, a previous study conducted by our group¹⁸ shows a high degree of reproducibility in a gender-mixed group of controls (11 males and 9 females). Increasing the group size might also allow for a better determination of any associations between PC activity level and fXIII or clot weights.

From our data, fXIII levels were comparable between groups, however fXIII consumption and TAFIa dependent and independent clot lysis times were increased in PC deficiency. Individuals with PC deficiency also exhibited a wide range of lysis times which was not seen in the control group. This suggests that even in this small descriptive study, clots formed by some PC deficient individuals resist lysis more than clots formed by others. Further studies are needed to both confirm the findings of this descriptive study and to identify the specific mechanisms that contribute to increased clot weight and fXIII consumption in PC deficiency.

In conclusion, our data are consistent with the hypothesis that clot structure and stability contributes to thrombosis in PC deficiency. A relatively heavy clot formed in a fixed volume of blood is suggestive of a denser clot and using this reasoning, our data confirm in whole blood what others have shown in plasma – that dense, impermeable clots are formed by individuals with, or predisposed to thrombosis^{21–24}. An impermeable clot, in conjunction with attenuated fibrinolysis (described here) would likely lead to stabilization of an unwanted clot and potentially contribute to a clinically significant event.

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Figure 1.

Thrombin generation (TAT) during whole blood coagulation. The maximal level of TAT is similar in controls (●) and PC deficiency (○) in the absence of added sTM (panel A) and in its presence (panel B). STM slowed the rate of thrombin generation in control blood but not in PC deficiency and shortened the lag phase of TAT formation in both control blood and in PC deficiency compared to in the absence of sTM.

Figure 2.

Clot weights are increased in PC deficiency. In both control (●) and PC deficient (○) blood, clots were first observed at the 4 minute time point. By the 6 minute time point, the control clots had reached their maximal weight of 1.45 ± 0.15 mg. PC deficient clots achieved their maximal clot weight of 2.96 ± 0.35 mg by the 8 minute time point.

Figure 3.

Factor XIII activation and consumption from serum. The extent of fXIII activation as measured in the serum was 50% greater in control experiments (●, 59.6 ± 1.6 nM) compared to PC deficiency (○, 39.2 ± 12.6 nM) despite similar rates of activation (panel A). All fXIII can be accounted for in controls by adding fXIIIa in the serum (panel A) to fXIII remaining in the serum (panel B). PC deficiency showed that >90% of fXIII was consumed from serum (panel B) while only 35% could be accounted for as fXIIIa (panel A).

Figure 4.

PC-deficiency attenuates fibrinolysis slightly through TAFIa-dependent and independent mechanisms. In the presence of PTCl, a specific TAFIa inhibitor, PC-deficiency (grey) causes a 50% increase in the clot lysis time by TAFIa-independent mechanisms compared to controls (black). Experiments conducted in the absence of PTCl show that the clot lysis time is increased by just 35% in PC-deficiency compared to controls. Control and PC deficient groups did not have significantly different lysis times in the presence of the TAFIa inhibitor or in its absence ($p=0.2$), however, there was a much larger range in the absolute lysis times among PC deficient subjects (+ PTCl: 17.6 – 37.1 minutes; no PTCl: 58.4 – 107.6 minutes) than in controls (+ PTCl: 16.9 – 19.1 minutes; no PTCl: 49.8 – 67.1 minutes).

Table 1

Summary of the demographics and clinical histories of PC deficient subjects

Subject	Sex	Age	Thrombosis* (age)	PC (%)	D-dimer (%)	Soluble TM (pM)
S1	Female	36	DVT [‡] (22)	50	150	ND
S2	Female	33	None	46	170	115
S3	Female	67	None	69	130	ND
S4	Female	76	None	34	87	139

ND: Not determined

* DVTs were confirmed positive or negative by leg ultrasound

[‡] DVT occurred while taking oral contraceptives