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ORIGINAL ARTICLE

Association of increased fibrinogen concentration with impaired activation of anticoagulant protein C

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Summary. *Background:* Low levels of activated protein C (APC) are a risk factor for venous thrombosis. The mechanisms leading to interindividual differences in APC are not totally elucidated. Protein C is activated by the thrombin–thrombomodulin complex. As thrombin binds to fibrinogen and thrombomodulin through a common region, it is conceivable that fibrinogen influences the activation of protein C. This would help to explain the association between high levels of fibrinogen and an increased thrombotic risk. *Methods:* We analyzed the association between circulating APC levels and fibrinogen concentration in 382 healthy subjects. Subsequently, we studied the effect of increasing fibrinogen concentrations on the APC generation on cultured endothelial cells. *Results:* An independent inverse association between circulating APC levels and fibrinogen was found [β coefficient, −0.16; 95% confidence interval (95% CI) −0.26, −0.06; $P = 0.001$]. For each 100 mg dL^{-1} increase in fibrinogen, the independent risk of having low APC levels (< 0.7 ng mL^{-1}) was almost three times higher (OR 2.8; 95% CI 1.1, 7.2; $P = 0.04$). Accordingly, a notable association between increasing fibrinogen concentrations and the reduction in the thrombin–thrombomodulin dependent activation of protein C on endothelial cells was found ($r = -0.57$; $P = 0.002$). *Conclusions:* We present evidence of an inverse association between circulating APC and fibrinogen levels. According to this finding together with the results of our *in vitro* experiments, we propose that the impairment in the generation of APC on endothelial cells constitutes a new prothrombotic mechanism of fibrinogen.

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Introduction

Activated protein C (APC) is a major regulatory protein of the coagulation cascade. Protein C, the zymogen of APC, is activated by thrombin bound to thrombomodulin on cell surfaces. The activation is more efficient in the presence of the endothelial protein C/APC receptor (EPCR). APC, in concert with its cofactor protein S, exerts its anticoagulant function by cleaving activated factors V and VIII [1]. Low circulating levels of APC constitute a risk factor for venous thrombosis [2,3]. Although several reasons have been proposed to explain the origin of these low APC levels (i.e. low zymogen level, polymorphisms in the EPCR gene [4,5]), the underlying mechanisms by which circulating APC levels differ among individuals are not totally understood. In the search for new mechanisms explaining these differences, a direct involvement of fibrinogen is conceivable: it must be noted that thrombin binds to fibrinogen and thrombomodulin through a common region termed the anion-binding exosite-1 [6–8]. Therefore, fibrinogen could prevent the formation of the thrombin–thrombomodulin complexes, thus impairing the generation of APC. This mechanism could explain to some extent the association of high fibrinogen levels with thrombotic risk [9–12].

To test this hypothesis, we investigated the relationship between fibrinogen and APC levels in a group of healthy subjects and subsequently tested the ability of fibrinogen to influence the activation of protein C in cultured endothelial cells. We found an independent association between fibrinogen and circulating APC levels and accordingly, we found that increasing concentrations of fibrinogen were able to impair the generation of APC in endothelial cell cultures. We propose that fibrinogen plays a prothrombotic role by influencing the circulating APC levels.

Methods

Participants

Four-hundred and one subjects without thrombosis constituting the control group of a case–control study carried out to evaluate the role of APC in venous thrombosis were included in the study [5]. Plasma samples from 19 subjects were not available for fibrinogen determination, so finally 382 were studied (Table 1). The study was approved by the Ethics Committee. Informed consent was obtained from all participants.

Measurement of plasma fibrinogen levels

Fibrinogen concentration was determined in plasma using high-sensitivity standard reagent IL (Instrumentation Laboratory, Milan, Italy) on a ACL 7000 autoanalyzer (Instrumentation Laboratory).

Measurement of plasma APC levels

Levels of circulating APC were determined by a previously reported modification [2] of a method based on enzyme-linked immunosorbent assay (ELISA) detection of the APC complexed to its major plasma inhibitor, protein C inhibitor (PCI) [4]. 4.5 mL of fasting blood was drawn into two tubes containing 0.5 mL of 0.129 M trisodium citrate. Immediately after extraction 46 μL of 1000 U mL^{-1} heparin (Roger, Madrid, Spain) was added to one tube and the mixture was incubated at 37 °C for 30 min to force all circulating APC to form complexes with plasma PCI. More than 95% of the APC forms complexes with PCI under these conditions. Therefore, the concentration of APC:PCI in the tube with heparin is the sum of *in vivo* circulating APC:PCI complexes and the complexes formed *in vitro* from the circulating APC. To the second blood tube was added 46 μL of a mixture of 0.58 M benzamidine.HCl (Sigma, St Louis, MO, USA) and 0.5 mM PPACK (Calbiochem, La Jolla, CA, USA) to immediately inhibit circulating APC. By doing this, the formation of new APC:PCI complexes was prevented and the APC:PCI complexes in this tube would reflect the concentration of the *in vivo* circulating APC:PCI complexes. Hence, circulating APC concentration could be calculated from the difference in APC:PCI concentration in the two samples. The intra- and inter-assay variation coefficients were not higher than 5% and 8%, respectively. All samples were tested in duplicate for both conditions. APC determination was performed within 6 months after sampling.

Other laboratory measurements

C-reactive protein (CRP) was measured by a high-sensitivity nephelometric assay (Behringwerke AG, Marburg, Germany). Protein C, soluble endothelial protein C receptor (sEPCR) levels, and the polymorphism 4678G/C of the EPCR gene were measured as described in previous studies [2,5].

Effect of fibrinogen on APC generation

The experiment was based on previous procedures [13]: 50 000 EA.hy926 cells (endothelium-derived cell line expressing thrombomodulin and EPCR [14], kindly supplied by Dr C.J. Edgell, University of North Carolina, NC, USA) were incubated with 0.4 nM thrombin (ERL, Swansea, UK) in a 20 mM Tris buffer, pH 7.4, with 150 mM NaCl, 5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1% BSA, 0.01% Tween-20 and 0.02% NaN_3. After 15 min, the cells were washed once and 60 nM protein C (kindly supplied by Lilly, Indianapolis, IN, USA) was added in the presence of increasing concentrations of fibrinogen (ERL). After 30 min 0.2 μM lepirudin (Schering AG, Berlin, Germany) was added, together with 0.4 mM S-2366 (Chromogenix, Milan, Italy), whose proteolysis by APC was monitored in a

Table 1 Baseline characteristics of the healthy group, overall and stratified by tertiles according to fibrinogen levels

		Fibrinogen concentration		
	Overall ($n = 382$)	115–231 mg dL^{-1} ($n = 127$)	232–273 mg dL^{-1} ($n = 130$)	274–430 mg dL^{-1} ($n = 125$)
Age (years)	41.5 ± 13.1	37.6 ± 12.3	42.6 ± 12.5	44.4 ± 13.6
Sex, females (%)	191 (50)	65 (51)	59 (45)	67 (54)
Fibrinogen (mg dL^{-1})	252 ± 44	205 ± 21	253 ± 12	300 ± 26
APC (ng mL^{-1})	1.28 ± 0.47	1.44 ± 0.50	1.25 ± 0.44	1.15 ± 0.41
APC:PCI (ng mL^{-1})	0.55 ± 0.31	0.59 ± 0.34	0.53 ± 0.24	0.49 ± 0.32
Protein C (%)	102 ± 18	104 ± 19	102 ± 18	101 ± 14
PCI (%)	101 ± 22	99 ± 19	102 ± 23	101 ± 21
sEPCR (ng mL^{-1})	123 ± 73	127 ± 77	115 ± 64	127 ± 78
EPCR 4678G/C				
GG (%)	105 (27)	34 (27)	33 (25)	38 (30)
GC (%)	197 (52)	58 (46)	70 (54)	69 (55)
CC (%)	80 (21)	35 (27)	27 (21)	18 (15)
C-reactive protein (mg L^{-1})	1.38 ± 0.97	1.19 ± 0.99	1.35 ± 0.84	1.60 ± 1.04

Values given are mean ± SD.
APC, activated protein C; PCI, protein C inhibitor; EPCR, endothelial protein C/APC receptor.

iEMS Reader (Labsystems, Helsinki, Finland). The experiments were performed in triplicate. This method was also used to assess the effect of 495 mg dL^{-1} (15 μM) fibrinogen on the kinetics of the protein C activation. Under the conditions used, no formation of clots took place along the experiment. The data curve fitting the Michaelis–Menten equations was performed using Enzfitter software (Biosoft, Cambridge, UK).

Statistical methods

We compared APC levels between subjects at higher and lower fibrinogen concentrations with a Mann–Whitney test. A linear regression model was used to assess the relationship between different variables with APC levels. Fibrinogen, the main independent variable, was considered both as a continuous variable and as a categorical variable (tertiles). After running a univariate linear model, a multiple linear regression model was computed including the variables that could be confounding factors. The risk [expressed as odds ratios (OR)] of having low APC levels was modeled using a logistic regression analysis in which the outcome (dependent) variable was low vs. medium/high levels of APC, and the independent variables were the same as for the multivariate linear regression model.

The relationship between fibrinogen and APC *in vitro* was assessed using the Pearson correlation coefficient and a linear regression analysis.

Results

Relationship between fibrinogen and APC levels in healthy subjects

We analyzed the relationship between fibrinogen and circulating APC levels in 382 healthy subjects (Table 1). When a linear regression analysis adjusting for age, sex and factors which were shown to influence APC levels in the univariate analysis (CRP, protein C, and the EPCR 4678G/C polymorphism) was performed, an inverse, independent correlation was seen between fibrinogen and APC levels [β coefficient, −0.16; 95% confidence interval (95% CI) −0.26, −0.06; $P = 0.001$]. The APC–PCI complexes present in blood at the time of blood collection, which reflects APC generation *in vivo* already inhibited by PCI displayed a similar inverse correlation with fibrinogen levels (β coefficient, −0.13; 95% CI −0.16, −0.02; $P = 0.011$).

Subsequently, we categorized the population in tertiles according to fibrinogen values (Table 1), and performed a multivariate logistic regression analysis taking low APC levels (under 0.7 ng mL^{-1}, shown to be a risk factor for venous thrombosis [2]) as the dependent variable. Again, the subjects in the highest fibrinogen tertile were at higher risk of having low APC levels than the subjects of the low fibrinogen group (OR 3.0; 95% CI 1.0, 9.2; $P = 0.06$). This effect could be also seen when taking fibrinogen as a continuous variable: for each 100 mg dL^{-1} increase in fibrinogen, the independent risk of having low APC levels was almost threefold higher (OR 2.8; 95% CI 1.1, 7.2; $P = 0.04$). Hence, for the first time we present evidence that high levels of fibrinogen are independently related to low APC levels. Among the other factors studied, CRP, the genotype CC of the G4678C polymorphism of the EPCR gene and, more weakly, protein C were also independently associated with APC levels (not shown), although only protein C was independently associated with low APC levels [for each 1% increase in protein C, the risk of having low APC levels decreased (OR 0.9; 95% CI 0.8, 1.0; $P < 0.001$)]. PCI concentrations were essentially equal in the three groups and therefore did not explain the differences in the APC circulating levels.

Effect of fibrinogen on the activation of protein C

In the search for a molecular mechanism, which conforms with the inverse association between fibrinogen and APC levels observed, we studied the effect of fibrinogen on the thrombin-dependent APC generation on endothelial cells. Consistently with our findings, we found that increasing concentrations of fibrinogen dose-dependently reduced the activation of protein C on the surface of EA.hy926 cells (Fig. 1A): an inverse correlation was seen between fibrinogen and the initial rate of protein C activation ($r = -0.57$; $P = 0.002$). Interestingly, this finding was obtained using protein C at the physiological concentration and fibrinogen at a broad range of concentrations covering low and high levels resembling frequent clinical situations [9–12]. Therefore, these observations are coherent with the inverse association between circulating fibrinogen and APC levels observed in a group of healthy subjects. To better understand the nature of the effect of fibrinogen on the generation of APC, the effect of 495 mg dL^{-1} (15 μM) fibrinogen on the kinetics of protein C activation was analyzed (Fig. 1B). The apparent Michaelis–Menten constant of the protein C activation was similar in the presence and in the absence of fibrinogen while the V_{max} was lower when fibrinogen was present. This is coherent with a hampered binding of thrombin to thrombomodulin in the presence of fibrinogen.

Discussion

Low levels of circulating APC are considered a risk factor for venous thrombosis [2,3]. Although factors like the concentration of zymogen (protein C), the basal levels of thrombin (indirectly assessed by the measurement of F1 + 2 levels) or several polymorphisms in the EPCR gene can partly explain the different APC levels found among individuals [4,5], they are not enough to account for all the interindividual variability. In the search for new mechanisms contributing to influence APC levels, we wanted to explore if fibrinogen could play a role: high levels of fibrinogen have been associated with arterial and venous thrombosis [9–12]. However, they have been considered a disease marker rather than a causal agent [15–17]. Recently, some evidence linking them to a higher fibrin deposition in transgenic mice has been presented but the mechanism by

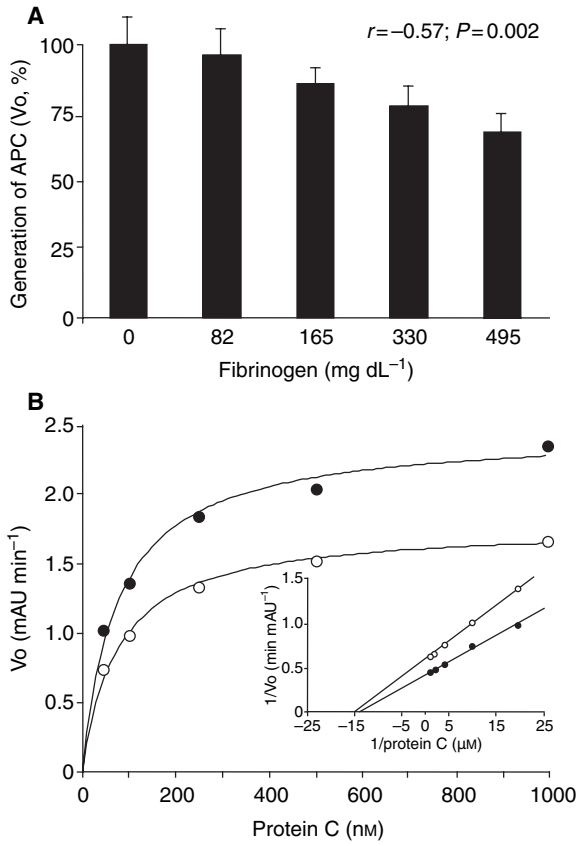

Fig. 1. Effect of fibrinogen on the activation of protein C by thrombin on the endothelial surface. (A) 0.4 nM thrombin and 60 nM protein C were added to EA.hy926 cells (50 000 well^{-1}) in the presence of increasing concentrations of fibrinogen, and the generation of activated protein C (APC) was monitored at 405 nm. For each condition, the mean ± standard deviation of three experiments is represented. The correlation between the fibrinogen concentration and the initial rate of protein C activation is shown. (B) The kinetic parameters of the activation of protein C by thrombin were calculated in the absence or the presence of 495 mg dL^{-1} (15 μM) fibrinogen under the same conditions described in panel A. The Lineweaver–Burk plot in the inset shows that the Michaelis–Menten constant (K_m) was similar in the presence (75.8 nM) or absence (76.9 nM) of fibrinogen while the V_{max} decreased from 2.45 mAU min^{-1} in its absence to 1.76 mAU min^{-1} in its presence. The experiment was repeated twice more with similar results. Solid circles, activation in the absence of fibrinogen; open circles, activation in the presence of 495 mg dL^{-1} (15 μM) fibrinogen.

which high fibrinogen levels are associated with higher thrombotic risk is not completely understood [18].

For the first time, we provide evidence that high fibrinogen levels are involved in impairing APC generation. First, we demonstrated that the variations in the concentration of fibrinogen in a group of healthy subjects are independently and inversely associated with the circulating APC levels. It is worth noting that this association was independent of the inflammatory status, estimated by the CRP level. Furthermore, we believe that by studying a group of healthy subjects we are underestimating the extent of the association, as it is not unusual to find patients exhibiting fibrinogen levels well above the highest concentration analyzed in our study (i.e. thrombosis, atherosclerosis, autoimmune diseases and sepsis) [9–12,19,20]. Secondly, to exclude the possibility that plasmatic fibrinogen does not influence the circulating APC levels but is an innocent bystander, we performed experiments of thrombin-dependent activation of protein C on endothelial cells. We could demonstrate that increasing levels of fibrinogen were able to reduce the initial rate of APC generation. The effect increased linearly with the fibrinogen concentration. These results, which conform with the findings obtained in the population studied, are also coherent with previous observations in which fibrinogen was shown to hamper protein C activation by competitively inhibiting the binding of thrombin to thrombomodulin in solution [21]. Nevertheless, the present study is the first one reporting this effect of fibrinogen using cell-anchored thrombomodulin. The fact that fibrinogen did not modify the apparent K_m but the V_{max} of the APC generation is consistent with the notion that fibrinogen, by binding thrombin, reduced the number of thrombin–thrombomodulin complexes available to activate protein C on the endothelial surface. This mechanism is plausible as thrombomodulin and fibrinogen share a common binding site on thrombin (i.e. anion binding exosite-1) [8], but could it account for the association between high fibrinogen concentrations and low APC levels in humans? The answer would be that it could. Huge amounts of fibrinogen are needed for it to play a competitive role, as the affinity of thrombomodulin for thrombin, in the nanomolar range, is much higher than the affinity of fibrinogen for thrombin (in the micromolar range). However, there is indeed a huge excess of fibrinogen as compared with thrombomodulin *in vivo*: assuming that the thrombomodulin concentration is around 0.15 nM in major vessels and 500 nM in the microcirculation [22], there is at least a 20 000-fold excess of fibrinogen over thrombomodulin in major vessels and a sixfold excess in the microcirculation. Under these conditions, variations in the plasmatic concentration of fibrinogen could be relevant for the formation of thrombin–thrombomodulin complexes and the subsequent rate of activation of protein C, especially in the major vasculature.

Thus we conclude that plasmatic fibrinogen and APC levels are inversely associated, and provide a mechanism which suggests that there is a cause–effect relationship. The fibrinogen concentration could be involved in the modulation of protein C activation. As low circulating APC levels are associated with an increased risk of thrombosis [2,3], we propose that elevated concentrations of fibrinogen may increase the thrombotic risk at least partly by decreasing APC generation.

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