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The Release of Tissue Factor Pathway Inhibitor and Platelet Factor 4 After Heparin Injection in Patients with Thrombocytosis

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Platelet factor 4 (PF4) and tissue factor pathway inhibitor (TFPI) are two proteins with high affinity for heparin. They are each stored in platelets, as well as on endothelial cell surfaces, from where both are displaced or released following an injection of heparin with a rapid and marked increase in serum levels. Prior work has demonstrated that the platelet count is one of the factors affecting the levels of heparin-releasable PF4. We therefore characterized the response to a dose of intravenous heparin previously demonstrated to completely displace PF4 from the non-platelet pool in subjects with normal or increased platelet counts. Seventeen patients with essential thrombocytosis (ET), 10 patients with polycythemia vera and high platelet counts (PV-H), 7 patients with polycythemia vera and normal platelet counts (PV-N) and 10 controls received an initial bolus of 40 I.U./kg of unfractionated heparin, followed 2 hours later by a 2nd bolus of a fixed dose of 1000 I.U. TFPI activity did not show any variation among the different groups, either before (TFPI) or after (HR-TFPI) the first bolus of heparin: ET, TFPI 92.6 ± 21.5%, HR-TFPI 298.3 ± 165.8; PV-H, TFPI 91.5 ± 32.0, HR-TFPI 210 ± 1.0; PV-N, TFPI 69.4 ± 24.0, HR-TFPI 203.0 ± 79.0; C, TFPI 109.5 ± 33.5, HR-TFPI 234.0 ± 60.4. TFPI activity returned to basal values prior to the 2nd injection of heparin, which again elicited a rise in TFPI, albeit smaller due to the lower level of heparin injected. In contrast to the lack of any difference between groups with respect to TFPI, the level of heparin-releasable PF4 (HR-PF4) was significantly higher in ET and PV-H patients compared to PV-N patients or controls. However when normalized for platelet count, both PV-H and PV-N had HR-PF4 levels after the 1st heparin injection that were significantly higher than observed in ET patients (PV-H 1.163 + 0.108, PV-N 1.411 + 0.019, ET 0.737 + 0.086 ng/10/3 platelets) supporting an increased platelet activation in PV. Thus, although platelets contain approximately 5-10% of the total amount of TFPI in plasma, they do not affect the major intravascular pool of TFPI mobilizable by heparin. However, since the concentration at the site of vessel wall injury is enhanced several-fold, TFPI could play a role in competing with PF4 to limit thrombus formation in patients with high platelet count.

Keywords: Tissue Factor Pathway Inhibitor (TFPI); PF4; heparin; thrombocytosis

INTRODUCTION

In the complex interplay between processes promoting or inhibiting coagulation, an increasing number of factors are being elucidated. Two proteins, platelet factor 4 (PF4) and tissue factor pathway inhibitor (TFPI) share a number of characteristics. PF4 is a protein stored within

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TABLE I Characteristics of patients with essential thrombocytosis (ET), polycythemia vera (PV) and controls (C). Among patients with PV, 10 had high platelet count (PV-H) and 7 normal platelet count (PV-N). The values are expressed as mean ± standard deviation.

	n	age years	sex	platelets (X10/9/L)	HT%	taking drugs*
ET	17	55 ± 15.9	10F 7M	1030 ± 316	38.3 ± 1.3	13
PV-H	10	66 ± 14.2	3F 7M	770 ± 68.1	48.6 ± 1.5	9
PV-N	7	63 ± 7.4	4F 3M	256 ± 82.6	40.4 ± 1.9	3
C	10	50 ± 21.6	4F 6M	237 ± 42.0	40.7 ± 4.6	None

*Note: The patients treated with antiplatelet agents at the time of the study were taking aminosalicylic acid, 325 mg; dipyridamole, 400 mg; pentoxyfilline 1.200 mg daily alone or in combination. The patients treated with cytotoxic medication were taking hydroxyurea 10 mg/kg/day. Platelet count was significantly higher in ET in comparison with both PV-H ($p < 0.05$) and PV-N ($p < 0.001$).

platelets with a great affinity for heparin, binding and neutralizing its activity[1,2]. It is released with platelet activation. It is also stored within a "non-platelet pool", on the endothelial cell and hepatocyte surfaces from which it can be displaced following an injection of heparin[3-5]. Similarly, tissue factor pathway inhibitor (TFPI) is a glycoprotein with marked heparin affinity, stored within platelets, as well as within a large non-platelet pool, bound to vascular endothelium[6-8]. In response to an injection of heparin there is a marked and rapid increase in TFPI levels due to the displacement and redistribution of the TFPI from the endothelial surface[9-12]. However, while PF4 neutralizes the anticoagulant heparin, TFPI is a naturally occurring inhibitor of the extrinsic pathway of coagulation. Prior work has demonstrated that the number of platelets is one of the factors affecting the level of heparin-releasable PF4 (HR-PF4)[13].

The aim of our investigation was to characterize and contrast the mobilization response of these 2 proteins after heparin injection in subjects with normal and high platelet counts.

MATERIALS AND METHODS

Patients

Forty-four subjects, 34 patients and 10 controls were studied. Seventeen patients had essential thrombocytosis (ET), and the other 17 had polycythemia vera (PV). Seven of the PV patients had their platelet count controlled within normal limits (PV-N), and 10 had high platelet count (PV-H). The etiology of elevated platelet count was established in agreement with polycythemia study group criteria[14]. At the time of the study, the patients were in stable clinical states, having no thrombotic or hemorrhagic episodes. Some patients were undergoing antiplatelet therapy and/or cytotoxic medication. Among patients with PV, only two had elevated hematocrits (57% and 53%); the other patients had hematocrit values within normal limits. Ten apparently healthy subjects with normal platelet counts were used as controls. All the patients and the control subjects were informed about the study and submitted written consent. The characteristics of the patients and controls are shown in Table I.

Tissue Factor Pathway Inhibitor (TFPI) Activity

TFPI level was determined by the simplified two-stage chromogenic method on microtiter plates, described by Sandset et al.[15] Briefly, in the first step, the following mixture was incubated at 37°C for 10 minutes: 25 μl plasma 1/50 in buffer pH 8: (tris-HCl 0.05 M, NaCl 0.1 M, trisodium citrate 0.01 M, NaN_3 0.02%, containing bovine albumin 2% and polybrene 2 g ml^{-1}, [Sigma, USA] with 100 μl of combined

reagent 1: human factor VIIa 0.025 U ml^{-1} (Stago, Asnières, France), bovine thromboplastin 1/40 (Stago, Asnières, France) human factor X 0.025 U ml^{-1} (Stago, Asnières, France), $CaCl_2$ 0.075 M. In the second step, 50 μl combined reagent 2 is added: human factor X 0.3 U ml^{-1} (Stago, Asnières, France) and chromogenic substrate S-2222 1.35 mM (Kabi, Sweden). After an incubation period of 25 minutes at 37°C, the reaction was stopped by adding 50 μl of acetic acid 50%. Absorbance was read at 405 nm using a microtiter plate reader (Dynatech 6000). The results are expressed as percent of normal plasma pool. The normal plasma pool was obtained from 20 apparently healthy subjects among the hospital staff, matched by age and gender to the patients.

Heptest™

To assess the anticoagulant activity of heparin we used the commercially available Heptest clotting time (Haemachem, Inc., St. Louis, MO, USA). The method was introduced by Yin in 1985 and measures the ability of heparin to accelerate the inhibition of factor Xa[16]. It was performed as recommended by the manufacturer. It was run on a KC10 instrument. The following procedure was used: 100 μl of plasma and 100 μl of factor Xa were incubated for 120 sec. Then, 100 μl of Recalmix (TM) (calcium chloride and brain cephalin in bovine plasma) were added and the clotting time was recorded. Normal values ranged between 13 to 21 sec. heptest clotting time is prolonged not only by heparin-antithrombin III, but also by TFPI released by heparin[17].

Platelet Factor 4 (PF4)

Platelet factor 4, expressed in nanograms per milliliter (ng/ml) was determined using a commercial radioimmunoassay kit supplied by Abbott Laboratories (North Chicago, IL). The samples obtained after heparin injection were diluted using 0.01 Tris buffer with sodium chloride (0.15 M) containing in addition 0.2% of bovine serum albumin (supplied by Abbott), sufficient to fit the standard curve of the kit.

We showed in a previous study[13] that basal PF4 levels in patients with both normal and high platelet counts were similar to those in controls (approaching zero nanograms). That study also showed that subjects with normal platelet counts whose PF4 levels have been increased by intravenous heparin injection, experience no further increase from a second bolus of the glycosaminoglycan within two hours of the first. Therefore, we did not measure PF4 levels or HR-PF4 in controls.

Platelet Count

Platelet count ($\times 10/9/L$) was determined with whole blood using platelet counter HPC 32 Hycel (Amply-medical, Milan).

Protocol

All subjects were studied in a fasting state. Patients and volunteers were injected with an initial bolus of 40 I.U./Kg/body weight (mean patients and controls: 2.750 ± 542 I.U.) of a commercial mucous heparin (Liquenin Roche, Switzerland). Two hours later a second bolus of 1,000 I.U. was administered. Prior work[13] had demonstrated that a bolus of heparin ≥ 2000 units elicited a full release of PF4 from the non-platelet pool. Also, the same investigation demonstrated that a second injection of 1000 units of heparin resulted in a second, albeit lower, increase in patients with high platelet count. Blood samples were obtained from the antecubital vein, without the use of tourniquet, immediately before injection of each bolus of heparin, and from the contralateral antecubital vein 2.5 minutes after each injection. After discarding the first few milliliters of blood, 4 ml was collected through a butterfly catheter

TABLE II Tissue factor pathway inhibitor activity before (TFPI) and after (HR-TFPI) two boli of heparin in patients with Essential Thrombocytosis (ET), Polycythemia vera with high (PV-H) or normal (PV-N) platelet count and in controls (C).

	1st TFPI	1st HR-TSPI	2nd TFPI	2nd HR-TFPI
ET	92.6 ± 21.5 (60-160)	298.3 ± 165.8 (140-720)	108.3 ± 24.7 (54-160)	179.6 ± 78.8 (60-320)
PV-H	91.5 ± 32.1 (50-140)	210.0 ± 100.0 (90-270)	90.5 ± 31.6 (60-140)	135.0 ± 48.8 (110-270)
PV-N	69.4 ± 24.0 (40-100)	203.4 ± 79.4 (124-350)	72.8 ± 26.7 (45-100)	127.1 ± 43.9 (80-220)
C	109.5 ± 33.5 (63-180)	234.0 ± 60.4 (170-330)	110.0 ± 27.1 (75-150)	180.4 ± 55.0 (90-280)

The values are expressed as a percent ± standard deviation. The range is indicated in parenthesis. There were no statistically significant differences between groups (p = ns).

(21 × 3/4 12-inch tubing, LKDS, Milan, Italy) into a precooled polypropylene syringe (Terumo, Leuven, Belgium) containing 1 ml of anticoagulant mixture (Edinburg mixture)[17] in a final concentration of 30 μmol/L of theophylline, 0.077 mol/L of EDTA and 1 μg/ml of PGE1 (Sigma Chemical Co., St. Louis, MO). The syringes were kept in melting ice, and then, over a period of a few minutes, the blood was transferred into precooled plastic tubes and centrifuged at 2300 g at +4° C (Varifuge RF, Heraeus, Hanau, Germany). Platelet poor plasma (PPP) was collected from the middle layer and filtered at room temperature using the Flow pore D 26, 0.20 μm (Flow Laboratories, Meckenheim-Bonn, Germany). All samples were stored at −20°C until assaying.

Statistical Analysis

The analysis of differences was performed by the Wilcoxon, sign and F tests. Correlation coefficients were determined by Spearman rank test and from linear correlation analysis.

RESULTS

The results are shown in Table II and III.

We could not find any significant variation in basal TFPI activity between patients with high platelet count and controls. Moreover, basal TFPI activity was distributed similarly among ET, PV-H and controls, ranging 50-160% in patients and 63-180% in controls. PV-N had a lower distribution (range 40-100%), but it was not significant (Table II).

The first bolus of heparin induced increased TFPI activity (first HR-TFPI) of 2.22 ± 0.19-fold in controls. First HR-TFPI activity among patients increased 3.26 ± 0.55-fold in ET, 2.32 ± 0.22 in PV-H, and 3.20 ± 0.50 in PV-N. No statistically significant differences were noted among these.

One control and 3 patients had a minimal increase of 1.3-fold. However, as with all other patients and controls, their HEPTESTs showed a prolongation over 200 seconds, confirming the presence of the glycosaminoglycan. The 3

TABLE III Heparin-released platelet factor 4 (HR-PF4) values normalized for platelet count expressed as nanograms/10/3 platelets in patients with essential thrombocytosis (ET) and polycythemia vera with high (PV-H) or normal (PV-N) platelet count.

	1st HR-PF4	2nd HR-PF4
ET	0.737 ± 0.086	0.057 ± 0.011
PV-H	1.163 ± 0.108*	0.107 ± 0.012*
PV-N	1.411 ± 0.019*	0.056 ± 0.019

* = $p < 0.01$ as compared to ET

patients showed low levels of cholesterol, and their TFPI activity before heparin injection was 70%, 95% and 110%. In contrast, the control had a normal cholesterol level; TFPI activity was 135% before heparin injection and 170% after heparin.

Six patients showed a first HR-TFPI activity of 4-fold or more. In one patient the increase was 10-fold. None of these patients had high levels of cholesterol.

After two hours TFPI activity returned to near basal levels. It increased again after the second bolus of heparin (2nd HR-TFPI). However, as the second dose, fixed at 1.000 I.U., was lower than the first, the second increase was smaller (mean controls 1.66 ± 0.12, ET 1.66 ± 0.15, PV-H 1.55 ± 0.26, PV-N 1.86 ± 0.22-fold). Six patients had no increase in the TFPI activity following the 2nd bolus, despite having HEPTEST prolongations over 200 seconds.

A good correlation was found between the first and the second HR-TFPI activity ($r = 0.447$, $p < 0.01$).

As expected[12], the levels of HR-PF4 after the first bolus of heparin were significantly higher in ET (717.6 ± 55 ng/ml) and PV-H (844.1 ± 51 ng/ml) than in PV-N (322.2 ± 62.2 ng/ml). Furthermore, ET and PV-H showed another, though smaller, HR-PF4 increase (ET 49.3 ± 7.8 ng/ml), PV-H 80.0 ± 10.1 ng/ml) after the second bolus. In contrast, the second HR-PF4 in PV-N patients (13.6 ± 5.5 mg/ml) was insignificant. A good correlation between 1st and 2nd HR-PF4 was seen considering all the patients together ($r = 0.615$; $p < 0.001$). When HR-PF4 was normalized for platelet count, both PV-H and PV-N showed first HR-PF4 activity significantly higher than that of ET (Table IV), further supporting our previous data of increased platelet activation in polycythemia vera[12]. The hematocrit, as shown by the significantly higher values of second HR-PF4 in PV-H patients, could be one of the factors responsible for the platelet activation, which is probably due to enhanced cell-to-cell interactions. This seems to be confirmed by the observation of a significant correlation ($r = 0.366$; $p < 0.05$) between hematocrit and first HR-PF4, considering all the patients together.

We found no correlation between TFPI activity and HR-PF4 after the first bolus of heparin, nor after the second one.

DISCUSSION

Among proteins with heparin affinity, platelet factor 4 (PF4) and tissue factor pathway inhibitor (TFPI) have the characteristic of increasing quickly several fold in the plasma when a glycosaminoglycan is injected into the circulation[1-13]. They act in opposite ways, however: PF4 neutralizes heparin, and TFPI increases heparin activity[2,3].

Both PF4 and TFPI proteins are stored in platelets, though in different granules[1,19]. Both proteins are also stored on the endothelial cell surfaces[3,9] and can be mobilized from there[2,9,10]. A minimum glycosaminoglycan chain length is required to trigger this activity[20,21]. In the case of TFPI this minimum length is 5 saccharide units; for PF4 it is 7. We have previously shown that the level of PF4 mobilized by heparin (HR-PF4) is correlated not only with platelet count but also with platelet activation[4].

In contrast with PF4[13], although platelets contain approximately 5-10% of the total amount of TFPI in plasma, platelet count does not affect the major intravascular pool of TFPI which is bound to the endothelial cell surface[13]. In fact, we found no differences in basal TFPI or in HR-TFPI between patients with high and normal platelet count, or between patients and controls. Likewise, after the second administration of heparin, there

were no differences among these groups in second HR-TFPI activity.

It is not clear why some of our patients and controls had very large or very small increases in first HR-TFPI activity. Sandset et al.[15] have shown TFPI activity to be significantly correlated with total cholesterol levels. Our "poor responder" patients with small increases in first HR-TFPI activity had cholesterol levels below normal limits. However, they had TFPI activity before heparin of 70%, 95% and 110%, well within normal limits.

Six patients (3 ET and 3 PV-N) had exaggerated first HR-TFPI activity of 4-or-more times baseline. However, we could not find differences between these patients and the others to account for such an increase. Lindahl et al.[10] have shown that TFPI and HR-TFPI activity are increased in cancer patients. However, the relative increase in TFPI activity after heparin injection was the same in normals as in cancer patients. In addition, Novotny et al.[8] have shown wide variability in the TFPI antigen (150-650%) following 2,500 U. of intravenous heparin in patients undergoing cardiac catheterization. The reason for these discrepancies remains unexplained.

In contrast with HR-PF4 in subjects with normal platelet count, TFPI activity increases after a second bolus of heparin within two hours. This finding further supports the evidence that platelets are not involved in the regulation of the major intravascular pool of TFPI.

From these and previous data[13] using HR-PF4 as an "in vivo" marker, enhanced platelet activity seems to be present in patients with polycythemia vera probably due to cell-to-cell interaction. Since it has been shown that considerable amount of TFPI is present at the site of vessel wall injury, released from platelets by thrombin[7], although there is a lack of an increased TFPI activity induced by heparin, TFPI could play an important local role in competing with PF4 to limit thrombus formation in these patients.

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