Effect of Heparin on the In Vivo Release and Clearance of Human Platelet Factor 4

By A. Koneti Rao, Stefan Niewiarowski, Pranee James, John C. Holt, Mary Harris, Bruce Effenbein, and Christine Bastl

Intravenous injection of heparin (100 U/kg) into normal volunteers resulted in an increase of platelet factor 4 (PF4) level in platelet-poor plasma from a mean value of 18.1 ± 6.6 ng/ml before the injection to 257.9 ± 68.3 ng/ml at 5 min after injection. PF4 antigen isolated from "postheparin plasma" by adsorption on heparin-agarose and elution with 2.0 M NaCl was "authentic PF4" isolated from human platelets showed identical patterns of migration as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Material released by washed human platelets was injected intravenously into rats. The clearance of PF4 followed a biphasic exponential pattern. The half-lives (T1/2) for the fast and slow components for control rats were 1.2 and 17.1 min. Heparin significantly extended the half-life of human PF4 in rat circulation. The clearance of PF4 injected together with heparin followed a single component model with a half-life of 27.6 min. Administration of heparin to rats that had been previously injected with human platelet releasate resulted in a 30-fold increase of plasma PF4 level in their circulation. The clearance of PF4 from the circulation of these rats (T1/2 = 45 min) fitted a single component model. We propose that PF4 is originally secreted by platelets into circulation and subsequently bound reversibly to vascular sites from which it can be released back into the circulation by heparin. The fast component of PF4 clearance that is abolished by heparin may reflect binding of this protein to the endothelial cells.

STIMULATED PLATELETS secrete a number of heparin-binding proteins, two of which, platelet factor 4 (PF4) and low affinity platelet factor 4 (LA-PF4), are considered to be immunologically specific for platelets. β-Thromboglobulin (βTG) is a product of limited proteolysis of LA-PF4, which is originally secreted by the platelets. Current evidence indicates that both PF4 and LA-PF4 reside in the same α granules and are released in parallel during platelet activation.

Dawes et al. observed that injection of heparin into normal volunteers resulted in a marked increase of PF4 levels in plasma, whereas the level of βTG antigen remained normal. This observation has been confirmed by other investigators. While the mechanism by which heparin increases PF4 levels in vivo is not clear, it has been suggested that this increase may be related to the mobilization of PF4 from binding sites on the vascular endothelium.

In this article we report that PF4 isolated from the "postheparin plasma" appears to be identical with PF4 isolated from human platelets. Moreover, we show that heparin significantly prolongs clearance of human PF4 from rat circulation. Our data provide experimental evidence to support a hypothesis that PF4, originally secreted by circulating platelets, accumulates on vascular sites from which it can be released into the circulation by heparin.

MATERIALS AND METHODS

Injection of Heparin

Porcine intestinal mucosal heparin (Wyeth Laboratories, Philadelphia, PA) was administered intravenously as a single bolus to 8 normal volunteers at a dose of 100 U/kg (maximum of 7,000 U). All volunteers gave informed consent in agreement with the Declaration of Helsinki. Blood samples were collected on ice into test tubes containing EDTA, prostaglandin E1 (PGE1), and theophylline before and at 5 and at 40 min following heparin injection.

Preparation of Platelet-Poor Plasma (PPP)

PPP was obtained by two-step centrifugation at 4°C (2,000 g for 60 min and 12,500 g for 2 min). The residual platelet count in PPP was less than 10³/μl.

Preparation of PPP Adsorbed by Protamine or Heparin-Agarose

Protamine-agarose was prepared as described previously. Protamine-agarose was prepared by coupling 252 mg protamine (free base, Sigma, St. Louis, MO) to 15 g CNBr-activated Sepharose at pH 8.3. Platelet-poor plasma (5 ml) was incubated with 5 ml protamine-agarose or heparin-agarose slurry for 30 min at room temperature. Supernatant plasma was drained off.

Material Released From Human Platelets Stimulated by Thrombin ("Platelet Releasate")

Suspensions of human washed platelets were prepared according to Mustard et al. The final suspensions usually contained 5 x 10⁸ platelets/ml. These suspensions were aggregated by the addition of 100 U/ml of highly purified human thrombin, kindly supplied by Dr. J. Fenion (N. Y. State Department of Health, Albany, NY) at 37°C for 3 min. Aggregated platelets were removed by centrifugation, and the supernate was incubated for 2 min at 90°C, centrifuged again.
and stored at −20°C. One milliliter of the supernate contained 0.7 mg protein, of which approximately 20 μg was PF4 and 95 μg was LA-PF4/βTG antigen.

**PF4**

Human PF4 was prepared from material released by thrombin-stimulated platelets by the method described previously.12

**Measurement of the Level of PF4 and LA-PF4/βTG by Radioimmunoassay**

This has been performed by a modified method of Rucinski et al.13 Preliminary experiments established that heparin did not interfere with the assay of LA-PF4/βTG, but it interfered with the assay of PF4 in human plasma. Subsequently, it was found that by using Tris-buffer containing 0.5 M NaCl for radioimmunoassay (instead of 0.15 M NaCl in 0.05 M Tris, pH 8.0, as described previously), the interference by heparin could be eliminated. Radioimmunoassay of human PF4 in rat plasma was performed as described previously.14

**Isolation of Human PF4 From Postheparin Plasma**

The plasma (drawn 5 min after heparin injection) was adsorbed with heparin-agarose slurry in a proportion of 70 ml PPP to 10 ml heparin-agarose. The slurry was poured into a column (1.0 cm × 15 cm) and proteins were eluted stepwise using 0.15 M, 0.5 M, and 2.0 M NaCl. PF4 levels were measured in all aliquots by radioimmunoassay. Fractons containing PF4 were pooled, dialyzed against 0.01% sodium dodecyl sulfate (SDS), and lyophilized.

**Identification of PF4 by SDS-Polyacrylamide Gel Electrophoresis**

This has been done (A) by gel slicing technique followed by radioimmunoassay, and (B) by transfer technique.15

(A) The lyophilized immunoreactive material isolated from “post-heparin plasma” by heparin-agarose chromatography was dissolved in a small volume of 0.5% SDS and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels according to Laemmli.16 Purified human PF4 was applied in parallel. The gels were then sliced (1-mm thick), and the immunoreactive material was eluted with 0.5 M NaCl, 0.02 M phosphate buffer, pH 7.4. The levels of PF4 were measured by radioimmunoassay in the eluates to compare the migration of the immunoreactive material in plasma with the migration of purified human PF4.

(B) SDS (final concentration 1%), and 1.4-dithiothreitol (DTT) (final concentration 10 mM) were added to the material eluted from heparin-agarose column with 2.0 M NaCl. Salt was then removed by dialysis against 0.1% SDS. The solution was freeze-dried and resuspended in water with 10 mM DTT, heated again at 100°C, and applied to a 20% SDS gel in 0.1 M Tris–0.1 M bicarbonate buffer, pH 8.3. After electrophoresis, the resolved bands were transferred to nitrocellulose paper, which was incubated with anti-human PF4 antiserum. Rabbit IgG reacting with PF4 was detected by means of staining with a peroxidase conjugate of goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA).

**Effect of Heparin on Clearance of Human PF4 in Rats**

Previous experiments in our laboratory have established that rat platelets disrupted with Triton X-100 did not contain any significant amount of material that cross-reacted immunologically with human PF4 or LA-PF4/βTG. Using a rat model previously reported by us,14 we have examined the effect of heparin on the clearance of PF4.

Adult female Sprague-Dawley rats weighing 235–295 g were anesthesized with pentobarbital (40 mg/kg body weight) given intraperitoneally. Body temperature was maintained with a heating board and monitored with a rectal probe. A tracheostomy tube and bladder catheter were placed. A jugular venous catheter and carotid arterial line were inserted (PE-50 tubing) for infusion and drawing blood samples, respectively. Rats were given a bolus of 0.15 M NaCl, equivalent to 1% of body weight, to replace surgical losses and then received a constant maintenance infusion of 0.15 M NaCl at 0.02 ml/min. The rats were allowed to equilibrate for 30 min. Four groups of rats were studied. In group I, 1.0 ml of “human platelet releasate” containing PF4 antigen was administered to 5 rats. One milliliter of blood was drawn at various time intervals (1–120 min) from the carotid artery into syringes containing 0.1 ml of 3.8% sodium citrate. The blood was immediately centrifuged and the plasma removed and stored at −20°C. The red cells were resuspended in an equivalent volume of normal saline and reinjected into the rat to prevent volume depletion. Urine volume was replaced with normal saline. Levels of PF4 antigen were determined in the samples of rat plasma prepared as above.

In group II, 4 rats were administered a single bolus of 500 U of porcine intestinal heparin (Wyeth) in 0.5-ml volume. As in the previous group, 1-ml samples of blood were drawn at various intervals up to 120 min from the carotid artery. Plasma was separated and frozen for PF4 assay. In group III, 2 rats were administered 1.0 ml of the “platelet releasate” along with 500 U of heparin in 0.5-ml volume and samples obtained at intervals from 1 to 120 min. In this group, the effect of heparin on the clearance of injected PF4 was studied, with the heparin being given along with the platelet releasate. To examine if heparin displaces PF4 from the sites to which it was previously bound in vivo, 4 rats (group IV) were injected with 1 ml of the “platelet releasate” first and blood samples were obtained at 1-, 2-, and 5-min intervals. Subsequently, at 5 min, a bolus of heparin (500 U) was administered intravenously and blood samples were obtained again at various time intervals following heparin injection. Levels of PF4 were measured in all samples by radioimmunoassay.

The half-lives of platelet proteins in rat circulation were calculated as described previously,14 using one- or two-compartment models for plasma protein kinetics. The Gauss-Newton nonlinear regression computer program was applied to fit these data.

**RESULTS**

Table 1 shows the effect of the administration of a single bolus of heparin (100 U/kg) intravenously on the levels of PF4 and LA-PF4/βTG antigen in human plasma. The levels of PF4 in the plasma at 5-min intervals after the injection were found to be significantly raised in the normal volunteers, but it was close to normal within 40 min after injection. In contrast, there was no significant increase in the level of LA-PF4/βTG in the plasma at 5 min after the injection. PF4 antigen was almost completely adsorbed from PPP by heparin-agarose. There was also some decrease in its levels in PPP following adsorption with protamine agarose.

Figure 1 shows the elution of the immunoreactive material adsorbed to heparin-agarose columns using increasing concentrations of sodium chloride. The radioimmunoassay performed on the eluates from the heparin-agarose columns of the plasma obtained 5 min after the heparin bolus revealed that the immunoreac-
The recovery of PF4 antigen was 30%-40%.

PF4 antigen was measured by radioimmunoassay (Fig. 2A) and by antigen transfer technique (Fig. 2B). These experiments suggest that the apparent molecular weights of both antigens are identical.

Figure 3 depicts the effect of heparin on the clearance of human PF4 from rat circulation. The clearance of PF4 following injection of the "platelet releasate" alone (group I) showed a biphasic exponential curve with a fast half-life component of 1.2 ± 0.27 min (mean ± SD) and a slow component of 17.1 ± 2.7 min (Fig. 3A). Injection of heparin alone (group II) resulted in a slight increase of immunoreactive material (51 ± 3 ng/ml after 1 min). This material disappeared with a half-life of 31.9 ± 3.5 min, fitting a single component model (data not shown in the figure). The origin of this material remains unexplained.

When "platelet releasate" was injected together with heparin (group III), the level of PF4 in plasma at zero time was several-fold higher than after injection of "platelet releasate" alone. Heparin significantly extended the half-life of human PF4, which fitted a single component model (Fig. 3B). In the fourth group of rats, heparin was injected 5 min after administration of the "platelet releasate" (Fig. 3C). In this group, the plasma level of PF4 at zero time and the initial half-life were very similar to the values observed in animals of group I. However, 1 min following the administration of heparin, approximately a 30-fold increase of plasma level of PF4 was observed. The subsequent clearance of PF4 in these animals was also increased and fitted a single component model (Fig. 3C). These studies strongly suggest that following the injection of heparin, there is a return into circulation of PF4 displaced from unidentified binding sites. In addition, these data reveal that the clearance rate of PF4 from the plasma is decreased substantially by the presence of heparin.

It should be noted that the observed rates of clearance of human PF4 from the circulation of control rats were faster (Fig. 3A) than those reported previously.14 The reason for this discrepancy is not clear, since the conditions in both sets of experiments were very similar.

**DISCUSSION**

This study demonstrates that administration of heparin to normal human volunteers results in the appearance in the circulation of a substance that seems to be identical with purified human PF4. The PF4 isolated from platelets and the antigen released into the circulation after heparin injection appear to be identical on the basis of radioimmunoassay, affinity to insolubilized heparin, and SDS-PAGE. The observation that PF4 antigen was adsorbed in part from postheparin plasma by protamine-agarose could be explained assuming adsorption of PF4-heparin complex by this reagent. At present, there is no direct confirmation of this supposition. The level of PF4 was considerably increased at 5 min following the injection and appeared to return to baseline after 40 min. Interestingly, only a slight increase was noted in the plasma levels of the LA-PF4/βTG (Table 1), despite the fact

<table>
<thead>
<tr>
<th>Antigen Assayed</th>
<th>Before Injection</th>
<th>5 Min After Injection</th>
<th>40 Min After Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA-PF4/βTG in PPP (n = 7)</td>
<td>28.6 ± 1.7</td>
<td>30.4 ± 1.7*</td>
<td>21.5 ± 4.1</td>
</tr>
<tr>
<td>PF4 in PPP (n = 4)</td>
<td>18.1 ± 6.6</td>
<td>257.9 ± 68.3†</td>
<td>24.4 ± 8.1</td>
</tr>
<tr>
<td>PF4 in PPP adsorbed with heparin-agarose (n = 3)</td>
<td>8.7 ± 2.8</td>
<td>18.6 ± 6.1</td>
<td>17.5 ± 6.3</td>
</tr>
<tr>
<td>PF4 in PPP adsorbed with protamine-agarose (n = 3)</td>
<td>14.8 ± 3.4</td>
<td>85.2 ± 10.2</td>
<td>26.2 ± 4.5</td>
</tr>
</tbody>
</table>

*These values are not significantly different from the baseline values (p > 0.05).
†Significantly different from the baseline values (p < 0.05).

**Fig. 1.** Elution of PF4 antigen from heparin-agarose column. Heparin-agarose slurry (10 ml) was added to 70 ml of postheparin plasma containing 170-270 ng PF4/ml. The column (1.0 x 15 cm) was packed and proteins were eluted by increasing salt concentration. The level of PF4 antigen was measured by radioimmunoassay. The recovery of PF4 antigen was 30%-40%.
Fig. 2. Characterization of PF4 antigen isolated from "postheparin plasma" by means of SDS-gel electrophoresis. (A) PF4 antigens (1.2 μg) obtained either from human platelets (O—O) or isolated from heparin-agarose column (●—●) were subjected to SDS-polyacrylamide using 15% gels according to Laemmli in a reduced system. The gels were sliced (1-mm thick) and the immunoreactive material was eluted with 1.5 M NaCl, 0.02 M phosphate buffer, pH 7.4, containing 1% bovine serum albumin. The molecular weight standards indicated by arrows are as follows: human serum albumin (68,000 daltons), ovalbumin (43,000), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (21,000 daltons), myoglobin (17,000 daltons), egg white lysozyme (14,000 daltons), human platelet factor 4 (7,800 daltons), trasylol (6,800 daltons). (B) PF4 antigen was subject to SDS-polyacrylamide gel electrophoresis in a reduced system using 20% SDS gels in 0.1 M Tris, 0.1 M glycine, pH 8.3. Lane A: 12.5 ng "authentic" PF4 obtained from human platelets. Lane B: PF4 antigen equivalent to 200 μl of "postheparin plasma." Lane C: PF4 antigen equivalent to 100 μl of "postheparin plasma." Lane D: myoglobin and its cyanogen bromide fragments. PF4 antigens on lanes A, B, C were detected after transfer to nitrocellulose paper by means of staining with a peroxidase conjugate (see Materials and Methods). Proteins on lane D were detected by means of staining with Coomassie brilliant blue. The gel system used gave an excellent resolution in the molecular weight range 5-15 x 10^3 daltons, but it was not reliable for estimation of molecular weight. PF4 migrated more slowly than cyanogen bromide fragments of similar molecular weight. In this system, the apparent molecular weight of PF4 was about 11,000, as compared to 7,800 molecular weight of PF4 deduced from amino acid sequence.
Fig. 3. The level of human PF4 antigen in rat plasma after injection of human “platelet releasate” alone or in combination with heparin. (A) Five rats received injection of 1 ml of “platelet releasate” containing 21 μg PF4. Samples of blood were collected at various time intervals. Solid points represent mean values of antigen found experimentally. Broken lines represent disappearance of antigen from plasma by means of fast or slow process as calculated by the computer. The initial concentration (ng/ml) of PF4 at zero time was 333.1 ± 87.2 (SD) for the fast component and 27.5 ± 6.6 (SD) for the slow component. The half-life time for the fast component of PF4 antigen was 1.2 ± 0.27 min and 17.1 ± 2.7 min for the slow component. (B) Two rats were administered with 1.0 ml of “platelet releasate” (21 μg PF4) along with 500 U of heparin. Samples of blood were obtained at various time intervals. Solid points represent mean values of antigen found experimentally. Solid lines represent values of antigen calculated by means of computer (one component program). The initial concentration of PF4 at zero time was 1,830 ± 87.1 ng/ml, and the half-life of PF4 antigen was 27.6 ± 1.2 min. (C) Four rats were administered with 1.0 ml of PF4 “platelet releasate” (21 μg PF4), and heparin (500 U) was injected 5 min later as indicated by an arrow. During the first stage of experiment, the initial concentration of PF4 antigen at zero time was 285 ± 45.9 ng/ml and its half-life time was 2.1 ± 0.3 min. During the second stage of experiment (i.e., after heparin injection), the initial concentration of PF4 antigen at zero time was 1,581 ± 112 ng/ml and its half-life was 45.4 ± 4.5 min. For other explanations see Fig. 3B.
that both these proteins are localized in the same platelet granules. Previous studies have shown that the clearance of LA-PF4/βTG is considerably slower than that of PF4 both in monkeys and in rats. This excludes the possibility that the absence of an increase in LA-PF4 level was related to a rapid clearance of this component. Such a discordance between the plasma levels of LA-PF4 and PF4 following heparin injection suggested that the immediate origin of the increase in PF4 may not be platelet release but release from other sites such as the vascular endothelium.

In order to provide evidence for this hypothesis, we carried out the experiments in rats. Rat platelets do not contain any material that cross-reacts significantly with human PF4 or LA-PF4/βTG antigens. Releasate from human platelets was administered to rats as a bolus, and 5 min later heparin was administered. These experiments revealed a 20-fold increase in plasma PF4 levels immediately following the heparin injection. Since rat PF4 does not cross-react significantly with human PF4, this material cannot be derived from rat platelets. These studies therefore confirm that the injected human PF4 bound to as yet unidentified sites and was subsequently displaced by heparin.

We have postulated previously that the fast component of the disappearance of PF4 from the circulation reflects its binding to the endothelial cells rather than the rate of distribution in the body fluids and that the slow component most likely reflects a true catabolic process. Observations described in this article provide experimental support for this hypothesis. Accordingly, the fast component of PF4 clearance from the circulation was abolished by heparin, whereas the slow component was only slightly prolonged (Fig. 3). We propose that PF4 is originally secreted by platelets into circulation and subsequently bound on the surface of endothelial cells, from which it can be brought back into circulation by heparin. We also propose that the total amount of PF4 available on the surface of endothelial cells may depend on the number of circulating platelets and on their secretory activity.

PF4 binds to heparan sulfate, a glycosaminoglycan that occurs in large quantities on the surface of endothelial cells from which it can be released by heparin. Busch et al. have demonstrated that PF4 specifically binds to cultured human umbilical vein endothelial cells in a time-dependent and saturable manner. In their experimental system heparin, heparan sulfate, dermatan sulfate, chondroitin-6-sulfate, and chondroitin-4-sulfate competed with the binding of PF4 to the endothelium with potency that decreased in the order these compounds are listed. These authors also showed that heparitinase-induced detachment of heparan sulphate from endothelium resulted in a decreased binding of PF4. Following addition of human platelet releasate to the bovine endothelial cells in culture, we have demonstrated, by means of immunofluorescence, binding of PF4 to the surface of these cells (unpublished experiments performed in cooperation with G. J. Stewart, Temple University). These pieces of evidence support the contention that injected heparin may displace PF4 from endothelium by competing with other glycosaminoglycans, most notably heparan sulfate.

Our data are compatible with the observations by Cella et al. who demonstrated that the amount of PF4 released to the circulation by heparin depends on the platelet count of the recipient. Patients with thrombocytopenia released high quantities of PF4 after heparin injection, and patients with thrombocytopenia released very little of this protein. It is possible that there is a steady-state relationship between platelet count and the amount of PF4 bound to vascular sites.

By means of immunofluorescence, Goldberg et al. demonstrated permeation of PF4 into blood vessel wall after endothelial injury in rabbits. The fate of PF4 on the uninjured endothelium is not known. Dawes et al. reported data indicating that the first dose of heparin injected to a volunteer caused a 30-fold elevation of PF4 in plasma, while subsequent heparin doses, within the next few hours, failed to elevate PF4. The full initial magnitude of the response was not regained until 144 hr after heparin was first injected.

The mechanism by which heparin releases PF4 in vivo resembles heparin-induced release of lipoprotein lipase. Injection of heparin releases two lipases into blood. One is the classical lipoprotein lipase and the other is the lipase of hepatic origin. Evidence suggests that lipoprotein lipase binds to several glycosaminoglycans, such as heparan sulfate, and is effectively displaced by heparin.

Recently Shimada et al. demonstrated that bovine endothelial cells in cell culture had no intrinsic lipoprotein lipase activity but were able to bind lipoprotein lipase in a specific and saturable manner. These authors provided direct evidence for lipoprotein lipase binding to endothelial cells through heparan sulfate on the cell surface and its release by heparin through a detachment from these binding sites. It is of interest that a semisynthetic heparin analogue administered parenterally into human subjects also released PF4 antigen and lipoprotein lipase activity. Thus, the mechanism of release of PF4 or lipoprotein lipase into circulation by heparin may be similar to displacement from vascular binding sites consisting of glycosaminoglycans.

ACKNOWLEDGMENT
The authors wish to thank Veena Kapoor for her excellent technical assistance.
REFERENCES

Effect of heparin on the in vivo release and clearance of human platelet factor 4

AK Rao, S Niewiarowski, P James, JC Holt, M Harris, B Elfenbein and C Bastl