Role of Kidney in the Catabolic Clearance of Human Platelet Antiheparin Proteins From Rat Circulation

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Stimulated platelets release at least two antiheparin proteins: platelet factor 4 (PF₄) and low affinity platelet factor 4 (LA-PF₄) from which β-thromboglobulin (βTG) is derived. We have found previously marked elevation of LA-PF₄/βTG antigen in platelet poor plasma of patients with chronic renal failure, whereas levels of PF₄ remained normal. Therefore, we examined the role of the kidneys in the metabolic clearance of LA-PF₄/βTG and PF₄. The supernates of aggregates of thrombin-stimulated human platelets were injected into sham operated control rats, nephrectomized rats, and into rats with acute ureteral ligation. The disappearance of human LA-PF₄/βTG antigen and PF₄ in rat plasma determined by specific radioimmunoassays followed biphasic exponential curves. The half-lives (t½) for the fast and slow components of LA-PF₄ are 6.4 and 68.4 min. Nephrectomy significantly increased these times to 9.7 and 144 min, while ureteral ligation resulted in no significant change. Comparison of the level of LA-PF₄/βTG antigen and of creatinine in aorta and in renal vein showed 25%-30% extraction of these compounds by the kidney. Less than 0.1% of the total LA-PF₄ antigen injected was recovered in the urine of control rats. In contrast to these results, the clearance of PF₄ was not affected by nephrectomy. In conclusion: (1) functional renal tissue is necessary for normal clearance of LA-PF₄/βTG, but renal excretion does not play a major role in its elimination suggesting that the protein is catabolized by the kidney; and (2) catabolic clearance of PF₄ does not depend on functioning kidney tissue.

HUMAN PLATELETS secrete at least two antiheparin proteins: platelet factor 4 (PF₄) and low affinity platelet factor 4 (LA-PF₄). These proteins are immunologically specific for human platelets and can be considered platelet markers. LA-PF₄, immunologically identical with β-thromboglobulin, is originally secreted by platelets, but it can be converted to βTG by the action of plasmin or a neutral platelet protease that cleaves a tetrapeptide from the N-terminus of LA-PF₄. It is not yet known which of these two proteins circulates in plasma in physiologic and pathologic conditions.

Several reports indicate that increased levels of PF₄ and LA-PF₄/βTG antigen may occur in the plasma of patients with enhanced platelet stimulation or destruction in vivo. We have recently reported significant elevation of LA-PF₄/βTG antigen level in plasma and in urine of patients with chronic renal failure. PF₄ levels remained normal in plasma of these patients. In vitro platelets of patients with chronic renal failure released less LA-PF₄/βTG antigen compared to normal platelets. After 3 hr of hemodialysis, there was a significant increase of LA-PF₄/βTG antigen level in plasma of these patients. By contrast, this antigen in three patients with successful kidney transplant returned rapidly towards normal levels following graft function. Independent of our research, other investigators have also found increased values of βTG antigen and normal values of PF₄ in plasma of patients with chronic renal failure.

The above data suggest that elevation of LA-PF₄/βTG in chronic renal failure may be due to deficient metabolic clearance of this protein and that the kidney plays an essential role in this process. The kidney is known to clear a number of low molecular weight protein hormones such as prolactin, glucagon, insulin, parathyroid hormone, and luteinizing hormone by a mechanism that involves glomerular filtration, luminal reabsorption, and catabolism of hormones in the renal parenchyma. The serum level of all of these proteins has been reported to increase in chronic renal failure and diminished renal catabolism has been demonstrated for each.

This paper describes experimental data on the role of the kidney in the catabolic clearance of human PF₄ and LA-PF₄/βTG antigens from the rat circulation.

MATERIALS AND METHODS

Material Released From Human Platelets by Thrombin

Washed platelets were prepared from human blood according to Mustard et al. Material released from a washed platelet suspension (5 x 10⁶ platelets per ml) was obtained following the addition of 100 U/ml of highly purified human thrombin kindly supplied by Dr. J. Fenton (Albany, N.Y.). After 3-min incubation at 37°C with occasional shaking, the platelet pellet was removed by centrifugation.
tion and discarded. The supernate was heated for 2 min at 90°C and the solution clarified by centrifugation. One ml of the supernate contained 0.7 mg protein, of which 21 µg was PF4 and 96 µg was LA-PF4/βTG antigen. This supernate, free of thrombin, was tested by clotting assay and it was stored at −20°C.

Radioimmunoassay of Human LA-PF4/βTG and PF4 Antigens

Radioimmunoassay of human LA-PF4/βTG and PF4 antigens was performed according to Rucinski et al.1 using antibodies prepared in our laboratory. Unbound tracer was separated from antigen-antibody complexes by precipitation with 35% ammonium sulfate. In some experiments, the PF4 radioimmunoassay kit supplied by Abbott Laboratories was used. Preliminary experiments established that rat platelets disrupted by Triton X-100 did not contain any material that cross-reacted immunologically with human PF4, or LA-PF4/βTG antigen using available antibodies produced in rabbits. Therefore, samples of rat blood for radioimmunoassay were collected into citrate and no precautions were taken to stabilize rat platelets during processing of blood samples. It was found, however, that rat plasma affected nonspecific binding and solubility of [125I]-PF4 in 35% ammonium sulfate. For this reason, the amount of rat plasma in the incubation mixture for PF4 radioimmunoassay was kept constant. Samples of urine for radioimmunoassay were prepared according to Dawes et al.21 Urine was dialyzed against 0.05 M phosphate buffer, pH 7.5, lyophilized and reconstituted to 20% of its original volume. Creatinine (total chromogen) in serum or plasma was determined with a Technicon Auto-Analyzer (Technicon Instruments Corp., Tarrytown, N.Y.). The level of total plasma protein was determined by the method of Lowry et al.22

Half-Life of Human Platelet Antithrombin Proteins in Rat

This study has been performed in three groups of rats including: (1) intact controls; (2) acute bilateral nephrectomy; and (3) acute ureteral ligation. Adult female Sprague-Dawley rats weighing 235–295 g were anesthetized with pentobarbital (40 mg/kg body weight) given peritoneally. Body temperature was maintained with a heating board and monitored with a rectal probe. A tracheostomy tube was placed. Bilateral nephrectomy was performed via flank incisions in group II. The other groups underwent sham operation. Ureteral ligation was performed at the ureteral vesicle junction in group III. In control rats, a bladder catheter was placed. A jugular venous catheter and carotid arterial line were inserted (PE-50 tubing). 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. Following equilibration, material released by human platelets containing LA-PF4/βTG and PF4 antigens were administered as a bolus injection. One ml of blood was drawn at various time intervals (2–240 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 reinfused into the rat to prevent volume depletion. Urine volume in control rats was replaced with normal saline.

Levels of LA-PF4/βTG and PF4 antigens were determined in the samples of rat plasma prepared as above. The half-lives of platelet proteins in the rat circulation were calculated using a two compartment model for plasma protein kinetics.22,29 The disappearance of platelet proteins in plasma was originally described by the equation:

$$c(t) = c_i e^{-k_1 t} + c_f e^{-k_2 t}$$

where $$c_i$$ is the concentration of tested substance at time zero, $$c_f$$ and $$c_i$$ are initial concentrations for the two exponentials. This equation was transformed as follows:

$$\log(c(t)) = \log(c_i e^{-k_2 t}) + \log(c_f e^{-k_1 t})$$

where $$c_i$$ is the concentration of tested substance at time t, e is the basis of natural logarithm, $$c_i$$ and $$c_f$$ are initial concentrations at time zero for slow and fast components, $$t_{1/2}$$ = half life of the slow component, and $$t_{1/2}$$ = half life of the fast component. The nonlinear least square regression Gauss-Newton computer program was applied to fit equation to this data.22

Renal Extraction of LA-PF4/βTG Antigen

The purpose of these experiments was to determine renal extraction of LA-PF4 and PF4, and to compare it with renal extraction of serum creatinine. Seven intact rats were prepared surgically as above. Following equilibration, material released by human platelets was injected via a jugular venous catheter. One hour after injection at a time when the levels of LA-PF4/βTG antigen had been demonstrated to be influenced primarily by the slow component of the disappearance process, a midline incision was made. One ml of blood was first drawn slowly from the left renal vein, immediately followed by withdrawing the same volume of blood from the abdominal aorta. This was done by a direct puncture with syringes containing 0.1 ml of 3.8% sodium citrate. By this sequential procedure, we avoided decrease of blood flow in the kidney which would occur if blood samples from both areas of circulation were collected simultaneously. The blood was immediately centrifuged and the plasma removed. The animals were sacrificed after obtaining blood samples. The levels of LA-PF4/βTG antigen and of PF4, in plasma samples were determined. In a second group of seven animals, 1.5 ml of blood was drawn from the renal vein and the abdominal aorta for simultaneous measurement of plasma creatinine and LA-PF4/βTG antigen in order to compare the renal extraction of LA-PF4/βTG to that of creatinine. Since creatinine is freely filterable at the glomerulus and not reabsorbed or secreted, it was used as a marker of renal extraction by filtration. In the same samples of blood, level of total protein was also measured to detect possible concentration of plasma proteins after passage through the kidney. Renal extraction of platelet proteins and creatinine was calculated from the formula (A-V)/A where A represents the aortic concentration and V represents the renal venous concentration of the measured substance. Statistical evaluation of the data was made by Student’s t test.

RESULTS

In Fig. 1 (A, B, and C), the disappearance rates of human LA-PF4/βTG antigen from plasma of the three experimental groups of rats are compared. AS can be seen from Fig. 1A, LA-PF4/βTG antigen injected to sham-operated rat disappeared rapidly from the circulation following a biphasic exponential curve; half-lives for the fast and slow components were 6.4 and 68.4 min. Fig 1B shows that survival of LA-PF4/βTG antigen in the circulation of nephrectomized rats was greatly prolonged although the disappearance of this protein followed the same pattern. The half-lives for the fast and slow components were 9.7 and 144 min. Both values were statistically different from values obtained in control rats ($$p < 0.001$$). Although both groups of rats had
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Fig. 1. (A) Half-life of LA-PF₄/βTG antigen in sham-operated rats. Twenty-three rats received injections of 96 µg LA-PF₄/βTG antigen. Samples of blood were collected at 2 min after injection (8 rats), at 5 min (19 rats), at 30 min (15 rats), at 60 min (13 rats), at 90 min (5 rats), at 120 min (12 rats), and at 180 min (12 rats). ---O---I represent mean values of antigen level found experimentally and their standard deviations. Broken lines represent theoretical disappearance of antigen in plasma following fast or slow process alone (calculated by computer). The initial concentration (ng/ml) of LA-PF₄/βTG at zero time was 1346 ± 147 (SD) for the fast process and 49 ± 11 (SD) for the slow process. The half-life time (min) for the fast component of LA-PF₄/βTG was 6.4 ± 0.56 (SD) and for the slow component, 68.4 ± 11(SD). (B) Half-life of LA-PF₄/βTG antigen in nephrectomized rats. Twenty nephrectomized rats received injections of 96 µg LA-PF₄/βTG antigen. Samples of blood were collected at 2 min after injection (9 rats), at 5 min (11 rats), at 15 min (6 rats), at 30 min (16 rats), at 60 min (17 rats), at 90 min (12 rats), at 120 min (18 rats), at 180 min (11 rats), and at 240 min (5 rats). The initial concentration (ng/ml) of LA-PF₄/βTG at zero time was 2899 ± 216 (SD) for the fast process and 725 ± 63 (SD) for the slow process. The half-life time (min) for the fast component of LA-PF₄/βTG antigen was 9.72 ± 1.04 (SD) and for the slow component 144 ± 13 (SD). For other explanations, see Fig. 1A. (C) Half-life of LA-PF₄/βTG antigen in rats with ureter ligation. Ten rats received injections of 96 µg LA-PF₄/βTG antigen. Samples of blood were collected at 5 min after injection (9 rats), at 15 min (5 rats), at 30 min (9 rats), at 60 min (10 rats), at 90 min (3 rats), at 120 min (8 rats), at 180 min (7 rats), and at 240 min (6 rats). The initial concentration (ng/ml) of LA-PF₄/βTG at zero time was 1804 ± 371 (SD) for the fast process and 78.3 ± 13.9 (SD) for the slow process. The half-life time (min) for the fast component of LA-PF₄/βTG antigen was 5.3 ± 0.71 (SD) and 66.3 ± 7.7 (SD) for the slow component. For other explanations, see Fig. 1A.

received identical amounts of LA-PF₄/βTG antigen, 15 min after injection, the level of LA-PF₄/βTG antigen in platelet-poor plasma of nephrectomized rats was about eight times higher than in that of normal rats. Three hours after injection, at least a 30-fold difference between LA-PF₄/βTG antigen levels in plasma of the two groups of animals was observed. Thus, the rate of clearance of LA-PF₄/βTG antigen was dependent on the presence of functioning renal tissue. However, the clearance of LA-PF₄/βTG antigen was apparently not dependent on urinary excretion. Fig. 1C shows that the rate of human LA-PF₄/βTG antigen disappearance from the circulation of rats after acute ureteral ligation was similar to the disappearance rates calculated for the sham-operated rats. The calculated half-lives for the fast and slow
components of LA-PF₄/βTG antigen were 5.2 and 66.3 min, respectively. In seven control rats, the excretion of LA-PF₄/βTG antigen in urine was measured during the clearance studies. The mean volume of urine collected during the 3-hour period was 5.7 ± 2.0 ml (SE). The amount of LA-PF₄/βTG antigen recovered in urine at that time was 75.2 ± 1.83 (SE) ng or 0.08% of the amount injected. These results suggest that the kidney eliminates LA-PF₄/βTG from the circulation by degrading the protein, rather than by eliminating it to the urine.

Figure 2A shows that PF₄ also disappeared in normal rats following a biphasic exponential curve. In this case the fast and slow components were 6.2 and 91 min. In nephrectomized rats, the clearance of PF₄ from circulation was not delayed. The half-lives for the fast and slow components were 3.9 and 87 min (Fig. 2B). In fact, initial disappearance rate of this protein was faster in nephrectomized rats for reasons that are not clearly understood at present. This result strongly suggests that the removal of all renal tissue had no significant effect on the catabolic clearance of PF₄.

Table 1 presents data on the arteriovenous difference (renal extraction) of LA-PF₄/βTG antigen, plasma creatinine, and total protein in the rats. In normal rats, 1 hr after bolus injection of the material released by human platelets. The concentration of LA-PF₄/βTG antigen in plasma obtained from the renal vein was lower by 27.3% (mean) ± 10.8 (SD) than the plasma concentration of LA-PF₄/βTG simultaneously obtained from the aorta. Plasma creatinine concentration obtained on the same plasma specimens was 30.1% ± 6.7 (SD) lower in the renal venous sample than in aortic plasma. The level of total protein was not significantly different in renal vein and aortic blood suggesting that renal extraction of water had not altered the concentration differences. In a separate group of seven rats, PF₄ levels and LA-PF₄/βTG antigen levels were simultaneously determined in aortic and renal venous plasma. LA-PF₄ extraction was 25.1% ± 5.1%, a value similar to the previously obtained extraction. However, there was no decrease in PF₄ values across the kidney, the venous level (5.52 ± 1.8 ng/ml) being slightly higher than the arterial value (4.46 ± 1.76 ng/ml). These experiments provide direct confrma-

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<th>Table 1. Renal Extraction of LA-PF₄ Antigen, Serum Creatinine, and Total Protein in the Rats*</th>
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<td><strong>Component</strong></td>
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<td>LA-PF₄ Antigen (ng/ml)</td>
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<td>Creatinine (mg %)</td>
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*Seven rats received injection of 0.6 ml of the supernate after platelet release (97 μg LA-PF₄). One hour later, samples of citrated blood were collected from aorta and renal vein. Data shown in this table represent mean values and standard deviations.
†Statistically different from aortic level, p < 0.001.
‡Statistically different from aortic level, p < 0.005.
tion that there is a significant renal extraction of LA-PF₄/βTG antigen. The similarity of the renal extraction of LA-PF₄/βTG antigen to that of creatinine suggests that like other low molecular weight proteins, LA-PF₄/βTG antigen is freely filterable at the glomerulus.

**DISCUSSION**

The pattern of disappearance of human LA-PF₄/βTG and PF₄ antigens from rat circulation was similar to that observed previously in experiments on Rhesus monkeys.²⁶ The disappearance of human LA-PF₄/βTG antigen from monkey plasma followed a biphasic exponential curve with half-lives for the fast and slow components of 7.2 and 51 min.²⁶ In normal rats, the half-life values for these components were 6.4 and 68 min (Fig. 1A). The half-lives of the fast and slow components of human PF₄ in the Rhesus monkey were 2.6 and 92 min²⁶ and in the rat 6.2 min and 91 min. Human and Rhesus monkey PF₄ and LA-PF₄/βTG, do not show species related antigenic differences.²⁶ By contrast, anti-human PF₄ and anti-human LA-PF₄ antibodies did not recognize any antigenic material in rat platelets. Nevertheless, comparison of the half-life data on platelet proteins in the rat and in the monkey suggests that similar mechanisms are involved in the clearance of these proteins from the circulation of both species. PF₄, however, appears to be cleared more slowly from rat circulation than from monkey circulation. The data on the clearance of secreted platelet proteins from human circulation are preliminary. Dawes et al.²¹ injected serum into a human volunteer and found that the clearance of PF₄ was so rapid that its half-life could not be determined. βTG antigen was cleared with a half-life of about 100 min. This value is comparable to the half-life values for the slow component of human LA-PF₄ in Rhesus monkey²⁶ and in rat (Fig. 1A).

The experimental data presented in this paper demonstrate that the kidney is a major route for the elimination of extracellular LA-PF₄/βTG antigen. Survival of LA-PF₄/βTG antigen in the circulation of nephrectomized rats was greatly prolonged (Fig. 2B). In fact, both fast and slow components of the LA-PF₄/βTG disappearance curve were significantly prolonged in this group of animals. We have previously suggested that the fast components of the disappearance process of platelet proteins reflect binding to endothelial cells as well as the rate of distribution of the body fluids, while the slow components most likely reflect true catabolic processes.²⁶ It is possible that LA-PF₄/βTG antigen binds preferentially to the endothelial cells within the kidney or that the presence of functioning kidney tissue affects endothelial cell binding of LA-PF₄/βTG antigen. Whole functioning renal tissue appears to be necessary for metabolic clearance of LA-PF₄/βTG antigen. The evidence presented suggests that LA-PF₄/βTG antigen is not normally cleared by excretion. Furthermore, the half-life of LA-PF₄/βTG antigen was not significantly prolonged by acute ureteral ligation, an experimental condition where renal blood flow and glomerular filtration continue but urinary clearance ceases.²⁷

Direct measurement showed that 27% of the LA-PF₄/βTG antigen was extracted by the kidney. This extraction ratio was similar to that found for creatinine in the same plasma samples suggesting that LA-PF₄/βTG antigen is filtered at the glomerulus. Thus, the results suggest that LA-PF₄/βTG antigen is filtered, reabsorbed by tubular epithelial cells, and catabolized within the kidney. Such a mechanism of clearance has already been described for other low molecular weight proteins such as growth hormone, insulin, glucagon and parathyroid hormone.¹⁵⁻¹⁸ Our experiments do not, however, exclude possible peritubular uptake of the protein. Since the molecular weight of LA-PF₄ is 9270 daltons and that of βTG—8800 daltons,²⁸ and there is no significant binding of these proteins to albumin in plasma, such a high rate of glomerular clearance would be expected. These proteins form tetramers in vitro,²⁹ however, in view of their rapid renal clearance this might not be true in vivo.

The half-life of PF₄ was not affected by nephrectomy and there was no extraction of PF₄ indicating that the kidney does not play a significant role in elimination of this protein from the circulation. Although the molecular weight of purified PF₄ is 7800 daltons,²⁰³¹ this protein is released from platelets in vitro as a complex with a proteoglycan carrier (350,000 daltons).³² We suggest that PF₄ also circulates in vivo as a complex with proteoglycan, and that it might not pass the glomerular filtration barrier.

Patients with chronic renal failure show significant elevation of LA-PF₄/βTG antigen in plasma but normal levels of PF₄.¹¹⁻¹⁴,³³ We proposed that this elevation is due to decreased catabolism secondary to the loss of functioning kidney tissue and not due to increased platelet release.¹¹ The experimental results described in this paper support this hypothesis.

**ACKNOWLEDGMENT**

The authors wish to thank Drs. R. G. Narins and D. Paul for their support and helpful discussions and to acknowledge the expert technical assistance of Veena Kapoor, Mary Harris, Prancee James, Peter Donisi, and Richard Piroshchak.
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