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TECHNICAL NOTE

Quantitative assessment of procoagulant activity in isolated rat glomeruli

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Tissue extracts from kidney of humans and other mammalians contain a procoagulant activity (PCA) that has been identified as tissue factor-like (TF) [1–3]. This activity appears to be located mainly in the renal cortex. To our knowledge, however, TF activity specifically bound to glomerular structures has not been demonstrated. The assay system described herein provides evidence of such an activity in glomeruli isolated from rat and allows its quantitative measurement. We suggest that this technique could be useful to investigate further the mechanisms of deposition of fibrin as observed in several types of experimental glomerulonephritides (GN).

Methods

Male Sprague-Dawley rats, weighing 170 to 230 g and anesthetized with ether, were used in all experiments.

Isolation of glomeruli

Following abdominal incision, the kidneys were washed until blanched with heparinized isotonic saline via a cannula inserted in the lower aorta. Isolated glomeruli were obtained according to a previously described method with minor modifications [4]; in brief, the minced cortex of the kidneys was pushed through a 106 μ-sieve and the glomeruli were washed twice and resuspended in phosphate buffered saline (PBS; 0.13 M NaCl, 0.005 M Na_2HPO_4, 0.0015 M KH_2PO_4, pH 7.2). Purity of the suspensions were checked microscopically. The suspensions contained 1 to 20 $\times$ 10^3 glomeruli per ml, determined by phase-contrast microscopy. The glomerular suspensions were used immediately after their preparation (intact glomeruli) or kept frozen at −80°C. The latter were either used immediately after being thawed or disrupted mechanically with the Lox-Press system (Biox, Sweden) before performing clotting assays; that all the glomeruli were disrupted was controlled optically.

Clotting assays

The coagulation tests were performed with citrated rat plasma as substate. To prepare rat platelet-poor plasma (PPP), blood (1.6 ml) was drawn by puncturing the abdominal aorta and was collected in 0.4 ml of 0.13 M trisodic citrate according to Lyberg, Hetland, and Prydz [5] to avoid activation of coagulation in most samples. The blood was centrifuged at 3000*g* for 15 min at 4°C. The plasma samples were screened for activation of coagulation, and all samples with a recalcification time of less than 100 sec were discarded. The plasmas were pooled and stored by aliquots at −80°C. The recalcification time of PPP was used to assess the PCA of the glomeruli as follows: 100 μl of 30 mM $CaCl_2$ was added to 100 μl PPP mixed with 100 μl of the test suspension and the clotting time recorded. Clotting times were compared to a standard curve generated using rat brain thromboplastin [6]. This standard preparation was taken arbitrarily to contain 1,000 TF units/ml. Dilutions were used to establish the calibration curve, which was linear on a bilogarithmic plot (r = 0.99, slope = 0.25). The mean results ± 1 SD obtained with five different batches of PPP were: 1,000 U/ml: 20.2 ± 0.6 sec (N = 22); 500 U/ml: 23.4 ± 0.5 sec (N = 18); 250 U/ml: 27.3 ± 1.1 sec (N = 22); 125 U/ml: 34.4 ± 0.6 sec (N = 6); 62.5 U/ml: 39.6 ± 3.5 sec (N = 22). When the clotting time of the test suspension was shorter than 20 sec, the suspension was diluted in PBS. In some experiments, human plasma deficient in factor VII, VIII or X (obtained from Dade Laboratories, Aguada, Puerto Rico) was used instead of rat PPP in order to characterize PCA. In these experiments, the clotting time of normal human PPP mixed with rat thromboplastin was used as a control to verify that species specificity of PCA did not interfere with the results. To further characterize PCA, the effects of diisopropylfluorophosphate (DFP) and phospholipase C (PLC) were assessed. DFP (Sigma Chemical Co., St. Louis, Missouri, USA) at a final concentration of 2 mM was incubated 30 min with the glomerular suspension at 22°C; after centrifugation, the DFP-containing supernatant was discarded and replaced by fresh PBS just before the recalcification time was recorded. PLC (Sigma type III from *Bacillus cereus*) at a final concentration of 5 μg/ml was incubated 15 min at 37°C with the glomerular suspension, and the recalcification time was tested as described.

The protein concentration of glomerular suspensions was measured according to Lowry et al. [7] using bovine serum albumin (Pentex Miles, Elkhart, Indiana, USA) as a standard. Differences between the TF units exhibited by intact, frozen-thawed, and Lox-Press treated glomerular suspensions were assessed statistically by the Student's t test.

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Table 1. Characterization of glomerular PCA

Sample	Clotting time, *sec*				
	Normal rat plasma	Normal human plasma	Factor-deficient plasmas VIII	VII	X
PBS	150	190	> 300	176	> 300
Thromboplastin (1:4 in PBS)	26.1	66.4	75	145	> 300
Glomerular suspension, *600 μg glom. protein/ml*	36.8	72	74	164	> 300

The recalcification clotting time of normal and deficient plasmas was determined as described in the text for each sample.

Table 2. Inactivation by phospholipase C (PLC) of the procoagulant activity of rat glomeruli

Glomerular suspension	TF units/mg of glomerular protein		
	Control (PBS)	Treated with PLC	Residual PCA in % of initial value
1	824	19	2.3
2	730	6	< 1
3	625	2	< 1
4	411	3	< 1

PCA of the glomerular suspensions was measured after incubation with PLC for 15 min at 37°C (details in text).

Results

Evidence and characteristics of PCA

Glomerular suspensions invariably shortened the recalcification time of PPP. A typical intact glomerular suspension (protein content 965 μg/ml) shortened the clotting time from 140 (for the control) to 35 sec. After Lox-Press disruption, the clotting time was 18.6 sec; serial dilutions of this disrupted glomerular suspension exhibited the following clotting times: 1:2 = 21.6 sec; 1:4 = 25 sec; 1:8 = 30.6 sec; 1:16 = 36.6 sec. The log-log plot yielded a straight line ($r = 0.99$) with a slope (0.26) identical to that obtained with serial dilutions of brain thromboplastin (0.25); this allowed us to compare different samples in terms of quantities.

The effect of suspended glomeruli was compared to that of rat brain thromboplastin, not only on rat PPP, but also on normal human PPP and human plasma deficient in factor VII or VIII. Rat thromboplastin and suspended glomeruli had no effect on the recalcification time of human plasmas deficient in either factor VII or factor X; in contrast, both shortened the clotting time of rat PPP, normal human plasma, and human plasma deficient in factor VIII. The results of a typical experiment are shown in Table 1. Incubation with DFP did not change the PCA of the glomerular suspensions (4 experiments), while incubation with PLC completely inhibited PCA, as shown in Table 2.

Quantification of rat glomerular PCA

Results are shown in Figure 1. In suspensions of intact glomeruli, the mean TF units ± SE were 0.185 ± 0.030 per μg of glomerular protein; in suspensions of frozen-thawed glomeruli this value was about twofold higher: 0.411 ± 0.035 per μg of glomerular protein. Suspensions of mechanically disrupted glomeruli exhibited a still higher PCA, about six times that of intact glomeruli: 1.18 ± 0.11 TF units per μg of glomerular protein. A significant positive correlation was found between PCA and the protein content (130 to 1820 μg/ml) of suspensions of glomeruli that were either intact ($P < 0.02$, $N = 13$), frozen-thawed ($P < 0.001$, $N = 36$), or disrupted ($P < 0.05$, $N = 15$). This shows that TF activity can be measured reliably over this range of protein concentration.

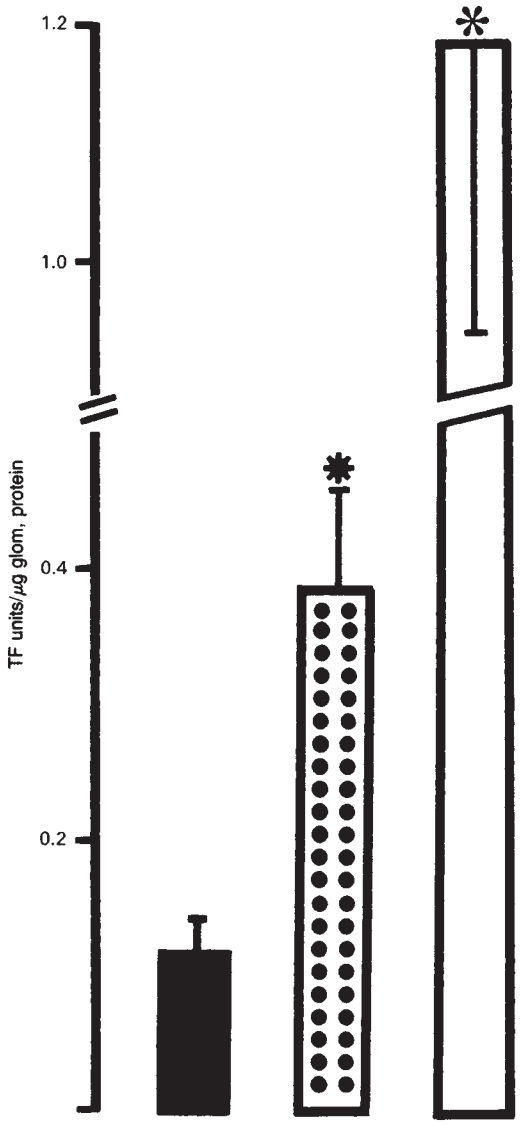

Fig. 1. *Tissue-factor units per μg glomerular protein of intact* ■ *(N = 13), frozen-thawed* ◙ *(N = 36) and mechanically disrupted* □ *(N = 15) glomeruli.* * $P < 0.001$ vs. frozen-thawed glomeruli * $P < 0.001$ vs. intact glomeruli.

Discussion

The present work shows that isolated rat glomeruli contain a PCA that exhibits the well-established characteristics of thromboplastin (tissue factor): specifically, activity depends on the presence of factors VII and X; activity is retained after incubation with DFP, an inhibitor of serine proteases, notably factor Xa [8]; and activity is suppressed by PLC, an inhibitor of the phospholipid component of tissue factor [9]. These findings confirm the data obtained by Astrup who demonstrated the presence of thromboplastin in the rat kidney [1]; our results also indicate that this activity is, at least in part, located in the glomeruli. A significant positive correlation was found between TF activity and the protein concentration of glomerular suspensions. We thus suggest that this method could be used for the accurate assessment of glomerular PCA under various conditions, notably in investigating the detailed mechanisms of fibrin deposition in experimental GN.

This study has also shown that glomerular TF activity is largely latent, as its expression increases severalfold with cellular trauma produced by freezing or mechanical disruption. This observation is consistent with previous observations that show an enhancement of approximately two- to tenfold in the TF activity of various types of cultured cells (including endothelial cells) after disruption [10–12]. Furthermore, this observation emphasizes the importance of evaluating the cell surface available PCA expressed by intact glomeruli and the total glomerular PCA which, as we have shown, is better recovered after Lox-Press treatment than after freezing and thawing.

In conclusion, our results demonstrate that PCA is present in isolated glomeruli. PCA, which has been described as tissue factor-like, is mainly intracellular. The described methodological approach might be used in further studies designed to establish, first, the type or types of glomerular cells producing this activity and, second, whether glomerular PCA could be related to the fibrin deposition that occurs in certain types of glomerulonephritides.

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