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INCREASE IN PLASMA AND URINE FIBRONECTIN CONCENTRATIONS DURING DEVELOPMENT OF PUROMYCIN AMINONUCLEOSIDE-INDUCED NEPHROTIC SYNDROME IN RATS

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SUMMARY: Nephrotic syndrome was induced in rats with 15gm puromycin aminonucleoside (PAN)/100g body weight. Urine and plasma fibronectin were analyzed in these rats by rocket immunoelectrophoresis. Fibronectin was not detected in urine between days 0 (control) and days 4 inclusive, post-PAN. At day 6 post-PAN the urine fibronectin concentration was 146.6 ± 39.9 μg/ml. The fibronection concentration further increased to 317.8 ±14.4 μg/ml urine at day 8 and 337.4 ± μg/ml urine at day 10 post-PAN. The detection of fibronection in urine coincided with significant elevation of plasma fibronectin concentration (P<0.05). We conclude that there is an increased urine fibronectin concentration in PAN- induced rat nephrotic symdrome, a result of increase in plasma fibronection concentration which has exceeded the renal threshold.

INTRODUCTION

Fibronectin is a high molecular weight glycoprotein found in a number of tissues including vascular endothelial cells (Jaffe and Mosher 1978), fibroblasts (Yamada and Olden 1978), kidney and liver (Chen *et al.*,1977). These glycoproteins were also shown to occur in normal plasma (Yamada and Olden 1978), platelets, and macrophages(Alitalo *et al.*, 1980). Fibronectins are adhesive proteins primarily involved in cell to cell and cell to surface interactions (Mosher 1980). Although plasma fibronectin is immunologically identical to fibroblast fibronectin (Engvall and Rouslahti 1977), there is still a question as to whether plasma fibronectin is identical in function to its tissue form (Hynes and Yamada 1982).

In human spontaneous nephrotic syndrome plasma fibronectin concentration was observed to moderately increase (Stathakis *et al*,1981). In primary glomerular disease there was a redistribution of fibronectin in the renal tissues to included not only the mesangial cells but also the pericapillary regions. The mechanism of the redistribution of this protein in renal damage is unclear (Ikeya *et al.*, 1985).There are no data to indicate this is a systematic process that occurs with all types of kidney failure. However, earlier studies suggested that the occurrence of fibronectin in renal tissues was, in fact, due to deposition of circulating fibronectin in the basement membrane (Linder *et al.*, 1975; Stenman and Vaheri 1978). It is possible increased renal tissues fibronectin in nephrotic syndrome may be secondary to increased plasma fibronectin, deposited in the kidney during normal glomerular filtration processes (Martinez-Hernandez *et al.*, 1981).

Fibronectin metabolites were detected in normal rat urine (Nagai *et al.*,1988). Whether or not intact fibronectin is also excreted in normal urine, is uncertain. In human patients with chronic renal failure, the plasma fibronection concentration remained in the normal range, while the plasma fibronectin concentrations of patients with proteinuria were abnormally high, and returned to normal during remission of proteinuria(Casio and Bakaletz 1984; Schena et al.,1986). Although these studies suggested that the plasma fibronectin concentrations were linked to the proteinuric state, the mechanism responsible for the elevation of plasma fibronectin concentration is unclear.

To study the fibronectin response in a renal disorder, we induced nephrotic syndrome in rats with PAN and determined the changes in concentrations of fibronectin in plasma and urine.

MATERIALS AND METHODS

Animals

Six group of male Sprague-Dawley rats were used in the study. Five groups of 5 rats were injected with a single dose of 15mg/100g body weight puromycin aninonucleoside (Sigma chemical company), intraperitoneally to produce nephrotic syndrome. A group of 5 rats which was not treated served as controls (day 0). During the study period, one individual from each group was removed and sacrificed, at 48 hour intervals,

Urine

Twenty-four hour urine was collected from individuals during each time period.

Blood

Whole blood was withdrawn by cardiac puncture and citrated (1 part 3.8% sodium citrate: 9 parts whole blood). Plasma was obtained by centrifuging citrated blood at 300 x G for 10 minutes removed from the packed cells and frozen. Fibronectin concentration was determined using thawed plasma.

Urine and plasma proteins

Urine and plasma proteins were determined by standard methods. (Savory and Hammond 1980).

Kidney tissues

The kidney were removed and placed in saline in a petri dish. A 3 mm cube of kidney tissue was cut, placed in cryomolds (Miles Laboratories), filled with O.C.T. compound (Miles Laboratories), and covered with a thin piece of wet cork. Refrigerant 22 (RIP) was chilled in liquid nitrogen for at least 3 minutes. The tissue was snap frozen by the placing of tissue-containing cryomold in the refrigerant for exactly 20 seconds. The crymold was removed from the refrigerant placed on dry ice for 10 minutes before storing in prechilled plastic storage bags at -60c until required.

Tissues fibronectin

Thin (4 to 6 microns) tissue sections were cut on a cryostat at -20C and mounted on chrome alumgelatin adhesive coated coverslips (Bourne 1983). The sections were fixed for 5 seconds in room temperature acetone and air dried. The fixed tissues were incubated with 10% normal rabbit serum in humidified chamber for 30 minutes. The serum was drained off and the edges of the coverslips wiped with paper tissue.

Goat antirat fibronectin antiserum (Calbiochem) was layered over the sections and allowed to stand overnight in a humidified chamber at 4^0C. The coverslips were then washed in 0.1M phosphate buffered saline(PBS) with agitation and blotted around sections. The

sections were incubated with biotinylated rabbit antigoat antibody (Vector Laboratories Inc.) for 30 minutes in a humidified chamber and washed in PBS with agitation. Endogenous peroxidase was blocked with 1:9 H_2O_2 in methanol for 10 minutes and the sections washed in PBS with agitation. Vectastain ABC reagent (Vector Laboratories Inc.) was applied to the sections and allowed to incubate for 30 minutes in a humidified chamber. The sections were again washed in PBS with agitation.

Colour development was obtained by incubating sections with equal volumes 0.02% H_2O_2 and 100mg% diaminobenzidine (Aldrich Chemical Company) solutions for 5 minutes. The coverslips were washed in distilled water. Dehydration of the sections was performed for 2 minutes each with 70%, 95% and 100% ethanol and finally cleared in 2 changes of xylene. The coverslips were mounted on slides with Pro-texx mounting medium (American Scientific Company).

Urine and Plasma fibronectin

Urine and plasma fibronectin was determined by rocket immunoelectrophoresis method(Laurell 1966) using 8 ml 0.7% agarose (Seakem) containing 160 goat antirat fibronectin antiserum. Fibronectin determination was performed on urine diluted to 1:10 with distilled water and plasma diluted to 1:30 with saline. Each plate included several concentrations of standard rat fibronectin. Standard curves were constructed from heights of stained

TABLE 1.

Plasma and urine protein concentrations of puromycin aminonucleoside-induced nephrotic rats

Days Post-PAN	Plasma Protein (g/dl)	Urine Protein mg/24 hours)
Day 0	6.79 ±0.18	134 ±10
Day 2	*4.58 ±0.09	*275 ±57
Day 4	*3.90 ±0.08	*283 ±47
Day 6	*3.48 ±0.37	*294 ±48
Day 8	*3.14 ±0.33	*433 ±41
Day 10	*3.39 ±0.21	*578 ±27

All values are expressed as mean ± standard error of mean

*Represents means significantly different ($p<0.05$) from control (day 0) means.

TABLE 2.

Urine and plasma fibronectin concentrations in puromycin aminonucleoside-induced rat nephrotic syndrome

Days Post-PAN	Urine Fibronectin (µg/ml)	Plasma Fibronectin (µg/ml)
Day 0 (controls)	0	733.2 ±10.6
Day 2	0	501.0 ±44.4
Day 4	0	592.0 ±25.6
Day 6	*146.6 ±39.9	*1278 ±126.0
Day 8	*317.8 ±14.4	*1230.0 ±206.6
Day 10	*337.4 ±37.8	*1377.0 ±93.0

All values are expressed as mean ±standard error of mean.

*Represents significant difference ($p<0.05$) from the control (day 0) mean.

peaks against fibronectin concentrations. Fibronectin concentrations of test animals were determined from the standard curve.

Statistical analysis

Analysis of variance and the Duncan test were applied to the data at 95% confidence level.

RESULTS

The rats developed manifestations of nephrotic syndrome, that is, hypoproteinemia and proteinuria by day 2 (Table 1), oedema and lipidaemia by day 6 post PAN.

Urine fibronectin was not detected in these rats between days 0 (controls) to 4 post-PAN (Table 2). However, small quantities (146.6 $\pm$ 39.3 μg/ml) of fibronectin were detected in urine at day 6 post-PAN. The concentration of urine fibronectin began to increase significantly ($p<0.001$) at day 10 post-PAN.

Rat plasma fibronectin in this experiment had a control (day 0) value of 733.2 $\pm$10.6 μg/ml plasma. Signigicant increases in experimental group plasma fibronectin was observed from day 6 post-PAN ($p<0.05$). Significant increases in plasma fibronectin concentrations were concurrent with the detection of fibronectin in urine. Fibronectin in frozen kidney section was not detectable, in control rats nor in PAN-induced nephrotic rats. The concentrations of antibody used ranged from 1:100 to 1:4000. Method negative controls were performed in each assay by subtituting nonimmunzied goat serum for goat antirat fibronectin antiserum. Nonspecific background staining was observed at antibody concentrations higher than 1:1000. Using lower antibody concentrations produced unpredictable stain characteristics, and lack of observable difference between control and PAN-induced nephroctic renal tissues.

DISCUSSION

In mice, fibronectin is an acute phase reactant (Dyck and Rogers 1985) which increased nonspecifically in plasma in inflammation. The increase in plasma fibronectin in human renal diseases has been documented (Stathakis *et al.* 1981., Casio and Bakaletz 1984). At this juncture, the origin of elevated plasma fibronectin in renal disease is not known. Schena *et al.* (1986) speculated that normal kidney removes or perhaps degrades some subtance or hormone controlling the release or synthesis of fibronectin. Subseqently, the inability to remove fibronectin by the damaged kidney in nephrotic rats may account for the initial increases in plasma fibronectin concentration. Subsequent appeaarence of urine fibronectin may,in fact, be an early indication of nephrosis and plasma leakage.

Fibronectin can be detected in normal human renal tissues. The distribution of fibronectin in normal human renal tissues included glomerular mesangium, glomerular and tubular basement membrances, capillaries, and interstitium (Pettersson and Colvin 1978; Oberley *et al.*, 1979; Stenman and Vaheri 1978;Madri *et al.*, 1980). In glomerular diseases, reports indicate redistribution of fibronectin to include glomerular cresents, intertubular area (Linder *et al.*, 1980) and pericapillary regions (Ikeya *et al.*, 1985). Several investigators in fact, suggested that fibronectin is an intergral part of glomerular basement membrane (Linder *et al.*, 1975; Stenman and Vaheri 1978). One study also suggested fibronectin was produced by cultured glomerular cells (Oberley *et al.*, 1979). However these views were not shared by others (Weiss *et al.*, 1979; Boselli *et al.*, 1981). Instead, another study suggested that fibronectin is not constiuent of glomerular basement membrane and that the fibronectin was

trapped in normal glomerular filtering mechanism (Martinez-Hernandez *et al.*, 1981). During the normal filtration process, the trapped fibronectin was transported to the mesangial area. This would account for the increased amount of fibronectin in the mesangium in glomerular diseases (Weiss *et al.*, 1979; Linder *et al.*, 1980). None of these studies were accomplished on rats. However, in rats, although circulating macromolecules are deposited in the mesangium of normal kidneys, there seems to be a strain difference. For example, using circulating lipids as "endogenous tracer" PAN-induced nephrotic Wistar rats showed large lipid deposits in their mesangium while PVG/c rats only showed small mesangial lipid droplets (Grond *et al.*, 1988). Since there is no comparative data on renal fibronectin deposition in different strains of rats, we can only speculate that the lack of tissue fibronectin in the kidneys of nephrotic Sprague-Dawley rats may be attributed to method insenstivity or to strain differences.

The glomerular basement membrane (GBM) contains a lattice-like netwok of anionic sites (Rennke *et al.*, 1975; Caulfield and Farquhar 1976). These anionic sites regulate the filtration of marcromolecules across GBM by restricting flow of the negatively charged macromolecules (Kanwar *et al.*, 1980). Movement across the glomerular filter is also size dependent. Molecules with radii equal or below that of insulin is filtered freely. However, the filtration of substances of increasingly greater size diminishes progessively (Brenner *et al.*, 1978). Besides molecular charge and size, shape and flexibility too seem to play important roles in the transport of macromolecules across the glomerular filter (Rennke *et al.*, 1979)

Earlier studies suggested proteinuria in aminonucleoside nephrosis rats to be the result of loss of anionic sites and alterations in size-selective properties of glomerular barriers(Caulfied and Farquhar 1978; Olson *et al.*, 1981). Others later showed that there were no significant alterations in anionic sites in the glomerular basement membrane of PAN-induced nephrotic rats (Abe *et al.*, 1988). In another study it was concluded that proteinuria in aminonucleoside nephrosis is due, not to increase of effective pore radius or pore number but rather a dimunition of the electrostatic barrier function of the glomerular capillary (Bohrer *et al.*, 1977). With these conflicting evidence, the mechanism of proteinuria remains unclear. However, in normal rats, polyethyleneimine, a polycation, seems to neutralise anionic sites allowing easier passage of cationic ferritin (pI ranging between 7.5 to 8.7, 480kd) through the glomerular filter than native ferritins (pI=4.5-4.8, 480kd) (Barnes *et al.*, 1984). A similar effect of anionic site neutralisation may be produced by other polycationic proteins. Since there is a lack of direct evidence on the glomerular flitration characteristics of fibronectin, it is still impossible to conclude that the movement of this glycoprotein (pI=5.5-6.2. 440kd) across the glomerular filter is easier than the more anionic native ferritins.

Fibronectin was detected in rat urine after six days of single administration of PAN. The presence of fibronectin in urine coincided with significant elevation of plasma fibronectin concentration. Since we were unable to demonstrate the presence of fibronectin in the renal tissues, it is most likely that the origin of urinary fibronectin is plasma fibronectin, filtered through the glomerulus. Fibronectin only began to appear in urine either after a certain degree of renal damage had occurred or after the mean plasma concentration had exceeded 600 μg/ml.

The role of fibronectin in PAN-induced rat nephrotic syndrome is uncertain. We can however conclude from this study that, the plasma fibronectin concentration in rats increased 6 days after induction of nephrotic syndrome with PAN, at which time fibronectin also became detectable in urine. These observations also suggested that the kidney regulates plasma fibronectin concentration and fibronectin is only detected in urine when the fibronectin concentration in plasma has reached a level which exceeds renal threshold for clearance of this glycoprotein.

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RINGKASAN

PENINGKATAN KEPEKATAN FIBRONEKTIN PLASMA DAN URIN SEMASA PERKEMBANGAN SINDROM NEFROSIS TIKUS TERAPUH PUROMISIN AMINONUKLEOSIDA

Sindrom nefrosis telah diaruhkan dalam tikus dengan mengguna 15 mg puromisin aminonuklesida (PAN)/100g berat badan. Fibronektin urin dan plasma dianalisiskan dalam tikus ini melalui imunoelektroforesis roket. Fibronektin tidak dikesan dalam urin paada hari 0 (Kawalan) hingga hari 4 pasca-PAN. Pada hari 6 pasca-PAN kepekatan urin ialah 146.6±39.9 µg/ml. Kepekatan fibronektin ini meningkat terus kepada 317.8±14.4 µg/mlpada hari 8 dan 337.4±37.8 µg/ ml pada hari 10 pasca-PAN. Pengesanan fibronektin dalam ini berlaku serentak dengan peningkatan tererti kepekatan fibronektin plasma ($p<0.05$). Kami mencapai kesimpulan yang peningkatan kepekatan fibronektin urin dalam sindrom nefrosis teraruh PAN merupakan akibat peningkatan kepekatan urin plasma yang melampaui ambang renal.