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URINARY COMPLEMENT BREAKDOWN PRODUCT, $C_{3d,g}$, IN MEMBRANOUS NEPHROPATHY – A MARKER OF DISEASE ACTIVITY?

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Summary

Paired plasma and urines were collected from 44 patients with biopsy-proven membranous nephropathy and 12 healthy controls. $C_{3d,g}$ was measured by ELISA and levels were correlated with biochemical parameters of renal function. The median plasma $C_{3d,g}$ in membranous nephropathy patients was 13 units (interquartiles 11–16) which was significantly higher than in healthy controls (9 units, interquartiles 7–11; $p<0.001$). $C_{3d,g}$ was detected in the urine of three of 10 patients with stable renal function and <3.5g protein/g urinary creatinine compared with 19 of 21 patients with stable renal function and >3.5g protein/g urinary creatinine. All six patients with declining renal function had high levels of urinary $C_{3d,g}$ (median 103 units). There was no correlation between plasma and urinary $C_{3d,g}$ levels or between urinary $C_{3d,g}$ concentration and proteinuria.

Introduction

In patients with membranous nephropathy, proteinuria may eventually remit spontaneously but often continues unabated for years, and may be associated with a variable rate of decline in GFR to end-stage renal failure [1]. Unresolved, persistent proteinuria could simply reflect previous insult(s) to the glomerular capillary wall or may indicate ongoing damage within the nephron caused either by immunological or by non-immunological mechanisms, e.g. hyperfiltration.

Glomerular C_3 deposition is a characteristic of membranous nephropathy and neo-antigens of the membrane attack complex have been described in man [2] and in animal models with the histological appearance of membranous nephropathy [3]. Furthermore, these animal models demonstrate a crucial role for complement activation in the induction of proteinuria [3]. In human membranous nephropathy, serological studies have not consistently shown evidence of significant complement activation. However, intra-renal activation of complement

would result in C_3 breakdown products which might be more readily detectable in urine. We have quantitated $C_{3d,g}$, a stable end-product of C_3 activation, in the urine of patients with membranous nephropathy, as a potential indicator of active immunological disease.

Patients

All patients had biopsy proven membranous nephropathy. Of 44 patients, six had declining renal function (median rise in serum creatinine in previous six months, 175μmol) and proteinuria (range 3.7–7.0g/g creatinine) and 38 patients with stable, normal or stable impaired renal function. Of these 38 patients, seven had no proteinuria (<0.5g protein/g urine creatinine), 10 had 0.5–3.5g protein/g urine creatinine and the remaining 22 patients had >3.5g protein/g urine creatinine.

Twelve healthy adults (hospital employees) provided control specimens of plasma and urine. None had proteinuria.

Methods

From each person, 5ml of EDTA blood and 5ml of a freshly voided urine sample collected in EDTA were transported on ice and multiple aliquots of EDTA plasma and EDTA urine were stored frozen at -70°C. Twenty-four hour urines were also collected for measurement of protein and creatinine output.

$C_{3d,g}$ assay Pre-treatment of plasma and urine samples with polyethylene glycol (PEG 6000) to remove high molecular weight D epitopes was performed [4].

An ELISA assay for $C_{3d,g}$ was developed using rabbit anti-C_{3d} (DAKO). Microtitre plates (Dynatech) were coated with anti-C_{3d} at 1/500 dilution in 0.1M sodium carbonate buffer pH 9.6 overnight at 4°C, and then the plates were blocked with phosphate buffered saline containing bovine serum albumin at 1mg/ml for one hour. A standard curve was constructed using a range of dilutions of inulin-activated pooled normal human serum, aged at 37°C for 24 hours and pre-treated with PEG 6000 as described above. An arbitrary value of 100 units $C_{3d,g}$/ml was attributed to the standard preparation and a standard curve was included in each batch. Standards and test samples in 200μl volumes were added to the plates and incubated for two hours at 4°C. The plates were washed four times with PBS and then peroxidase conjugated anti-C_{3d} was incubated on the plates for one-and-a-half hours at 4°C. Following further washing with PBS, the plates were developed with 1mM ABTS (2,2'Azinobis-3-ethylbenzthiazoline sulphonic acid) in citrate phosphate buffer pH 5.0 containing 100μm hydrogen peroxide and the absorbance at 420nm was measured by a multiscan plate reader after 40 minutes. Concentrations of C_{3d} in test samples were expressed as units/ml, and for urine samples were related to the creatinine content of the spot sample [5], i.e. $\frac{\text{(units } C_{3d,g}\text{/ml)}}{\text{(creatinine mg/ml)}}$

Results

The median plasma $C_{3d,g}$ in membranous nephropathy patients was 13.0 units/ml (interquartiles 11.0–16.0) which was significantly higher than the median in healthy controls (9.0 units/ml, interquartiles 7.0–11.0), $p<0.001$ Mann Whitney U test.

$C_{3d,g}$ was not detected in the urine of healthy controls or patients without proteinuria. $C_{3d,g}$ was detected in the urine of three of 10 patients with stable renal function and 0.5–3.5g protein/g urinary creatinine and in 19 of 21 patients with stable renal function and >3.5g protein/g urinary creatinine (Fisher test $p=0.004$). All six patients with declining renal function had high levels of urinary $C_{3d,g}$ (median 103 units/mg creatinine) (Figure 1).

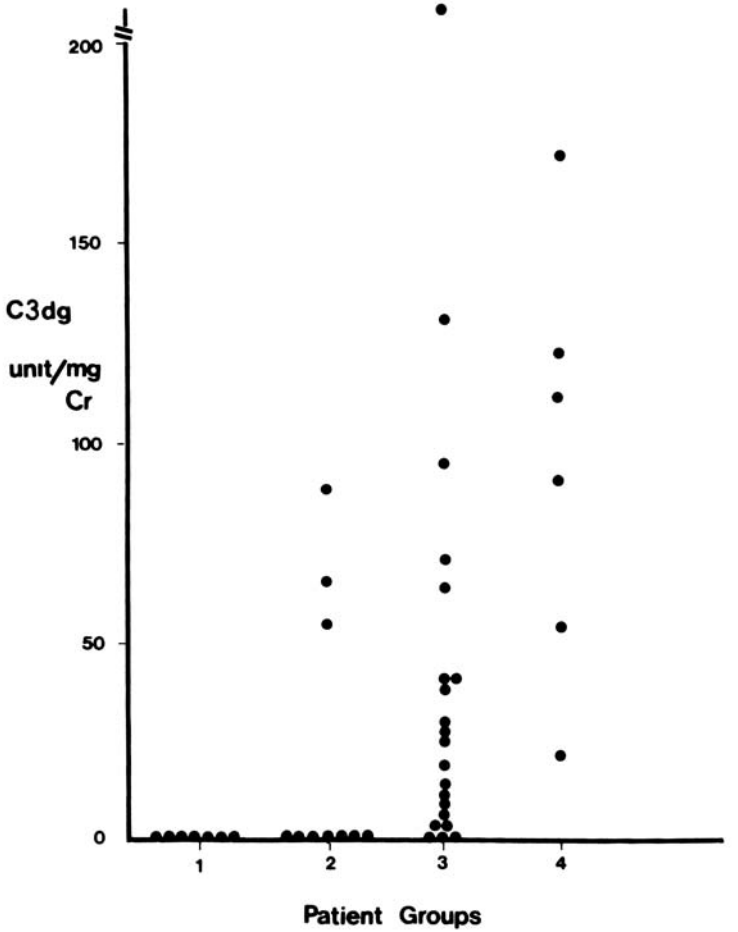

Figure 1. Urine $C_{3d,g}$ concentrations in patients with membranous nephropathy. Group 1= patients with <0.5g protein/g creatinine. Group 2=patients with 0.5–3.5g/protein/g creatinine. Group 3=patients with >3.5g protein/g creatinine. Group 4=patients with declining renal function

It is of note that all three patients in group 2 with $C_{3d,g}$ values >50 had impaired renal function (serum creatinine >170μmol/L) and that six of seven with undetectable $C_{3d,g}$ had normal renal function (serum creatinine <125μmol/L). There was no relationship between plasma and urine $C_{3d,g}$ concentrations. Urinary $C_{3d,g}$ levels showed no significant correlation with urinary protein loss (g protein/g creatinine) (Figure 2).

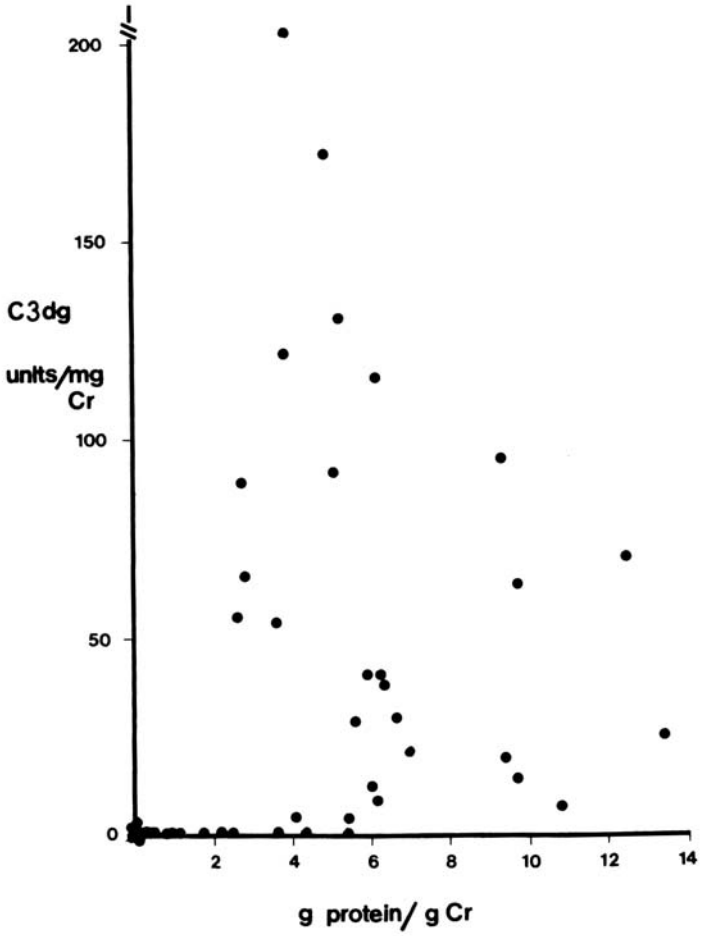

Figure 2. Urinary $C_{3d,g}$ concentrations related to proteinuria in membranous nephropathy patients

Discussion

Using a sensitive ELISA assay we have demonstrated that $C_{3d,g}$, a stable breakdown product of C_3 [7] can be detected in the urine of some patients with

persistent proteinuria, in particular in patients with declining renal function, in the majority of patients with heavy proteinuria (>3.5g protein/g creatinine) and interestingly, in a subpopulation of patients (3 of 10) with moderate proteinuria (0.5–3.5g protein/g creatinine).

In order to minimize spontaneous degradation of urinary C_3 we collected freshly voided urine samples in EDTA on ice and stored the samples at -70°C as soon as possible. Although quantitation of urinary protein excretion normally relies on 24 hour urine collections, this is an unsuitable method for measuring components that may spontaneously degrade during the collection period.

Furthermore, as the urinary creatinine excretion in the presence of a stable glomerular filtration rate is fairly constant in any patient [6], we have expressed urinary C_{3dg} in units per milligram creatinine as a means of standardizing a single urine sample for sample dilution and body size. The validity of the protein:creatinine ratio of a single urine sample in the assessment of urinary protein excretion has been demonstrated previously [5].

There are several possible explanations for the presence of $C_{3d,g}$ in urine samples of our patients. C_3, molecular weight 180,000 daltons, is not present in normal urine but is found in conditions associated with a size selective defect in the filtration unit. Thus spontaneous degradation is the finding of no $C_{3d,g}$ occurring in some patients with over 4g protein/g creatinine and minimal urinary $C_{3d,g}$ (<15 units/mg creatinine in other patients with up to 10.9g protein/g creatinine).

$C_{3d,g}$ may be generated in a site remote from the kidney producing high plasma levels resulting in filtration of $C_{3d,g}$ into the urine. Indeed we have established that membranous patients have a significantly higher plasma $C_{3d,g}$ concentration compared to normal adults. However, the lack of correlation between plasma and urine $C_{3d,g}$ values suggests the plasma source is not sufficient to account for the urinary $C_{3d,g}$ concentrations.

Finally, the $C_{3d,g}$ found in urine may be the result of intra-renal activation of complement. Certainly it is known that C_3 activation occurs on the glomerular basement membrane and $C_{3d,g}$ can be found in glomerular immune deposits [8]. Recently it has been suggested that filtered C_3 may also be activated by the proximal tubular epithelial cell membrane [9]. Complement activation, whether in the subepithelial lamina rara externa or on the proximal tubular cell membrane could be associated with continuing functional impairment of the kidney and resulting in relatively low plasma levels and relatively high urinary levels of $C_{3d,g}$. Prospective longitudinal studies are required to relate changes in $C_{3d,g}$ concentration with the patients' prognosis and further delineate the involvement of complement as a mediator of chronic renal damage.

Acknowledgments

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