Lysosomal enzymes in human urine: evidence for polymorphonuclear leucocyte proteinase involvement in the pathogenesis of human glomerulonephritis

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Summary

1. Lysosomal proteinase activity was assayed in human cadaver kidney, urine, granules from polymorphonuclear leucocytes of normal persons, and urine samples from 154 patients with renal disease.
2. Granules from polymorphonuclear leucocytes showed proteinase activity at acid and neutral pH, whereas cadaver kidney showed proteinase activity at acid pH only.
3. The urine from 13 patients with glomerulonephritis showed proteinase activity at both acid and neutral pH as well as increased amounts of antigenic glomerular basement membrane fragments. The properties of the urinary proteinases suggested that they had originated in polymorphonuclear leucocytes.
4. Only the urine samples containing these proteinases were capable of degrading isolated human glomerular basement membrane in vitro.
5. Clinical recovery, where it occurred, was accompanied by the disappearance of urinary proteinase activity, and reduction in glomerular basement membrane antigen excretion.

Key words: basement membrane, glomerulonephritis, lysosomes, polymorphonuclear leucocytes, proteinases.

Abbreviation: GBM, glomerular basement membrane.

Introduction

Animals with nephrotoxic nephritis (Hawkins & Cochrane, 1968; Cochrane & Aikin, 1966) excrete acid proteinases of lysosomal origin in their urine during the first (heterologous) phase of the disease process, which suggests that the glomerular injury may be due to lysosomal enzymes released from polymorphonuclear leucocytes. However, there is no direct evidence to confirm that this mechanism occurs in human glomerulonephritis. We have therefore studied the possible role of polymorphonuclear leucocyte lysosomal enzymes in human renal disease by examining the characteristics of proteinase excretion in normal and pathological urine, together with similar assays for plasma, kidney and leucocyte enzymes. We have also studied isolated human glomerular basement membrane in vitro to identify the potential pathogenicity of the urinary proteinases.

Materials and methods

Patients and urine samples

Of the 154 patients with renal disease, 97 had glomerulonephritis. Diagnosis was based upon clinical and/or histopathological criteria. Renal biopsy was only undertaken when there was a specific clinical indication. Standard techniques of fixation and staining for light and electron microscopy and immunofluorescence were used. In 18 of those with glomerulonephritis, diagnosis was based upon clinical and biochemical findings alone. Of the remaining 79 with glomerulonephritis, 31 had...
diffuse proliferative disease, 25 had membranous glomerulonephritis, five minimal change, 16 mesangiocapillary and two mesangiopathic (IgA) nephropathy. The other 57 patients without glomerulonephritis had a variety of diagnoses including polycystic kidneys, pyelonephritis and renal transplant rejection.

Both 24 h and random samples, taken throughout the day from the patients and from 21 healthy control subjects, were concentrated up to 15-fold by using an Amicon thin-channel separator with UM 10 membranes. The concentrated samples were stored at −20°C until assayed. More than one sample was taken from both patients and control subjects and assayed.

Polymorphonuclear leucocyte

Intact granules from the polymorphonuclear leucocytes of healthy laboratory personnel were prepared as described by Davies, Barrett, Travis, Sanders & Coles (1978).

Analytical methods

Protein. Total protein concentrations were determined by a Folin-Lowry technique on a Technicon Auto-analyzer with crystalline bovine serum albumin (factor V Armour) used as reference standard.

Acid proteinase. This was measured by the method of Anson (1939) as modified by Barrett (1967). The effect of pH on acid proteinase activity was determined over the range pH 2-1-6-0 (Barrett, 1967). The liberated peptides, soluble in trichloroacetic acid, were determined by their Folin-Lowry reaction.

Neutral proteinase. Total neutral proteinase activity was assayed with azocasein as substrate (Starkey & Barrett, 1976). Elastase (EC 3.4.21.11) activity was assayed with elastin from bovine nuchal ligament (Sigma) as substrate over the range pH 6–10 (Ohiisson & Olsson, 1974) and with N-benzyloxycarbonyl-L-alanine 2-naphthyl ester (Z-Ala-2-O-Nap) (kindly donated by Dr C. G. Knight, Strangeways Research Laboratories, Cambridge). Cathepsin G (EC 3.4.21.20) activity was assayed against N-benzoyl-DL-phenylalanine 2-naphthyl ester (BZ-DL-Phe-2-O-Nap) (Sigma) as substrate (Starkey & Barrett, 1976). The effects of enzyme activators and inhibitors were investigated by the addition of the appropriate compound in the standard assay mixture.

Enzyme activities are defined as units of product liberated per unit time.

Glomerular basement membrane

Preparation of antigen. Anti-(human glomerular basement membrane) was produced in male New Zealand White rabbits by intramuscular injection of human glomerular basement membrane (GBM) isolated from cadaver kidneys (Krakower & Greenspon, 1951). Serum was stored at −20°C. GBM antigen in urine was assayed by a double-diffusion technique in agarose [1-0 g/100 ml (Calbiochem)] in phosphate-buffered saline, pH 7-2, over 72 h. Results were expressed as the minimum concentration of urine which produced a precipitin line on completion of the incubation period. The antigenic fragments present in the urine of two patients were further investigated by separation on a Sephadex G-200 column.

Degradation. Freeze-dried human GBM was incubated with urine (2 mg/ml) as enzyme source. The release of soluble hydroxyproline was used as a measure of the extent of degradation as previously described by Davies et al. (1978).

Results

Urinary enzyme excretion

Normal subjects. All normal subjects excreted various amounts of an acid proteinase (mean = 50-26 ± 42-63 units/ml of urine, n = 43); the pH profile is shown in Fig. 1. The enzyme had a maximum activity at pH 2-1 and this, together with the finding of complete inhibition of activity by pre-incubation in alkaline conditions, suggests that the enzyme was pepsin (EC 3.4.23.1). The activity in healthy persons at pH 3-4, the optimum for cathepsin D (EC 3.4.23.5), a lysosomal proteinase, was always less than 50% of that at pH 2-1. Therefore, for screening purposes, urine proteinase activity was measured at pH 2-1 and 3-4. Those samples in which activity at pH 3-4 was equal to or greater than 50% of activity at pH 2-1 were further investigated with a full profile of activity over the range pH 2-1–6-0.

Patients. Total acid (pH 2-1) proteinase was significantly reduced in renal disease. Mean activity was 2-56 ± 2-76 units/ml of urine, n = 252 (n = total number of assays performed) when compared with normal subjects, and by Student’s t-test this was highly significant (P < 0-001).

The pattern of proteinase excretion was normal in all but 13 patients, who all showed additional activity at pH 3-4 equal to or greater than 50% of activity at pH 2-1, with a bimodal distribution of
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FIG. 1. Proteinase activity of normal urine at different pH values. Results have been expressed as % of activity at pH 2.1. Each point is the mean value and SD for 21 subjects.

Acid proteinase activity with varying pH. Activity at pH 3.4 = 3.2 ± 3.22 units/ml, n = 54. In addition, all 13 excreted a third proteinase (3.03 ± 3.68 units/ml, n = 55) with optimum activity at neutral pH (Fig. 2) when assayed against elastin or azocasein. Neutral proteinase activity could not be demonstrated in urine samples from any other patients, despite the presence of numerous leucocytes in some urine samples.

Excretion of GBM antigen

GBM antigen was detectable in normal urine only after concentrations to at least 75-fold. However, all the previously mentioned 13 patients with glomerulonephritis had demonstrable antigenic fragments when the urine was concentrated only 15-fold, and serial dilution revealed similar fragments, in some patients when samples were concentrated only 7.5- to 1.5-fold. Antigen titres in all other patients were similar to those in normal subjects. On immunodiffusion, there appeared to be at least three distinct antigens present in both normal urine and pathological urine. Subsequent examination of two of these samples by gel chromatography indicated that the molecular weights of these three antigens were 80 000, 150 000 and in excess of 200 000.

Clinical results

Of the 13 patients with abnormal patterns of enzyme and GBM excretion, one had a steroid-responsive nephrotic syndrome. The remaining 12 had acute oliguric, or rapidly progressive glomerulonephritis. In all 12 renal biopsy revealed a diffuse proliferative glomerulonephritis with many polymorphonuclear leucocytes present in the glomeruli. Whereas in normal kidneys only the occasional polymorphonuclear leucocyte is seen in any glomerular tuft, these 12 patients had at least three and often as many as 15 polymorphonuclear leucocytes per glomerulus. In all other biopsy samples from patients the counts of polymorphonuclear leucocytes per glomerulus were less than three with an average of 1.5. The biopsies from the 12 patients with proliferative glomerulonephritis showed extracapillary crescents in six; immunofluorescence studies in eight of them showed linear IgG deposits in five, and linear β1C

FIG. 2. Profile of urinary proteinase activity from pH 1 to pH 10 in patient O.S., who had rapidly progressive glomerulonephritis with cellular proliferation and glomerular crescents.
in four (two had both linear IgG and β1C) and granular deposition of IgG in one.

Subsequent, spontaneous improvement (as judged by determinations of blood urea and glomerular filtration rate) was seen in three patients, and was accompanied by the disappearance of acid (pH 3-4) proteinase and neutral proteinase from the urine, together with a fall in GBM antigen titre. These changes are shown for one patient in Fig. 3.

Properties of urinary proteinases

Incubation of the urine samples in the presence of known activators and inhibitors of proteinase activity indicated that the acid (pH 3-4) proteinase was a carboxyl proteinase, since it was unaffected by metal ions, cysteine, mercaptoethanol, iodoacetamide, soyabean trypsin inhibitor, Trasylol and 4-chloromercuribenzoate. Its molecular weight was approximately 50 000, as determined by column chromatography. The neutral proteinase activity contained two enzymes, elastase and cathepsin G, as shown by activity against specific substrates. The elastase was inhibited by Trasylol, serum and soyabean trypsin inhibitor, and had a molecular weight of 28 000. These results, taken together, suggest that the proteinase activity is derived from lysosomal cathepsin D, elastase and cathepsin G.

GBM degradation

The incubation of urinary neutral proteinase (0.250 unit/ml) with freeze-dried human GBM resulted in the solubilization of up to 60% of the total available hydroxyproline. The release of hydroxyproline was linear over the incubation period of 72 h. All other urine samples failed to release hydroxyproline from GBM, as did urinary acid (pH 3-4) proteinase.

Tissue enzyme studies

Normal human kidney contained an acid proteinase (pH 3-4) (0.53 ± 0.046 unit/g wet weight, n = 9) but no neutral proteinase. Polymorphonuclear leucocyte granules were shown to have both an acid (pH 3-4) proteinase (4.16 ± 0.49 units/10⁹ polymorphonuclear leucocytes, n = 6) and neutral proteinase activity (0.386 ± 0.053 unit/10⁹ polymorphonuclear leucocytes, n = 6).
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Discussion

There was a highly significant ($P < 0.001$) reduction in uropepsinogen excretion in renal disease. This did not seem to correlate in any way with either the type of disease or with glomerular filtration rate. Neutral proteinases were detected in the urine of 13 of 31 patients with severe, diffuse, proliferative glomerulonephritis, but not in the urine of normal subjects or in the urine of patients with other forms of renal disease. The origin of these enzymes, and their ability to initiate glomerular damage, may be important to the understanding of human glomerulonephritis.

The enzyme activity could enter urine from serum, the urinary tract or the cellular elements of blood. Serum contains no detectable free proteinase activity, and kidney contains cathepsin D but no detectable neutral proteinase. Polymorphonuclear leucocytes and platelets contain neutral proteinases (Janoff & Scherer, 1968; Legrand, Caen, Booyse, Rafelson, Robert & Robert, 1973; Ohlsson & Olsson, 1974; Starkey & Barrett, 1976) as well as cathepsin D, and are therefore the most likely source of this urine enzyme activity.

With the exception of the one nephrotic patient mentioned, only those patients with more than three polymorphonuclear leucocytes per glomerulus had detectable urinary neutral proteinase activity. In all other biopsies taken, glomerular counts of these leucocytes were less than three per glomerulus, and although some patients with rejecting transplants, or severe pyelonephritis, were studied, none of these had elastase or cathepsin D in their urine. This would suggest that the enzymes are released from glomerular polymorphonuclear leucocytes and that enzyme activity is not merely the result of contamination of urine by leucocytes along the urinary tract, as may occur in severe infection. Our failure to detect activity in the urinary deposit may arise from the death of such cells with subsequent neutralization of proteinase activity by cytoplasmic inhibitors. The presence of proteinases and GBM fragments in the urine of the nephrotic patient remains unexplained.

Polymorphonuclear leucocytes contribute to tissue damage in many disease states. Cochrane & Aikin (1966) showed that these leucocytes were necessary for tissue injury in experimental cutaneous Arthus reactions. Hawkins & Cochrane (1968) delayed the onset of proteinuria in experimental nephrotoxic nephritis by preliminary depletion of the leucocytes, an observation that was confirmed by Naish, Thomson, Simpson & Peters (1975). These effects of polymorphonuclear leucocytes were attributed to acid proteinases. However, later studies have shown that these leucocytes contain at least three neutral proteinases capable of degrading vascular and glomerular basement membranes (Malemud & Janoff, 1976; Oransky, Ignarro & Perper, 1973). Davies et al. (1978) have shown that cathepsin G and elastase damage GBM in vitro, whereas cathepsin D had no effect. It therefore seems unlikely that cathepsin D is directly implicated in the pathogenic mechanism, despite previous suggestions to the contrary (Cochrane & Aikin, 1966; Hawkins & Cochrane, 1968; Henson, 1972; Karan, Saatci & Bakkaloglu, 1976).

The studies by Hawkins & Cochrane (1968), Weissman, Zurier & Spieler (1971) and Henson (1972) suggest that the simplest explanation for the damage to the GBM in immunologically induced glomerulonephritis is the selective release of polymorphonuclear leucocyte lysosomal enzymes directly on to the membrane. Our results support this idea, since neutral proteinase was found only in the urine of patients with severe glomerulonephritis, and was accompanied by GBM damage, as shown by the excretion of high concentrations of antigenic fragments. When the patients recovered enzyme and antigen disappeared from the urine. In addition, only urine containing neutral proteinase was capable of causing damage to GBM in vitro.

We suggest therefore that polymorphonuclear leucocyte lysosomal neutral proteinases have a significant role in the pathogenic process of rapidly progressive or acute oliguric glomerulonephritis, whether the initial injury is due to the deposition of immune complexes or anti-GBM antibody.

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References


