Urinary excretion of glycosaminoglycans and hydroxyproline in Paget's disease of bone, compared with neoplastic invasion of bone

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SUMMARY Urinary glycosaminoglycan and hydroxyproline excretion was studied in 11 patients with clear evidence of Paget's disease of bone. Urinary hydroxyproline, cetyl pyridinium chloride (CPC)-precipitable uronic acid and CPC-precipitable hexosamine were expressed as ratios to urinary creatinine. Urine samples were concentrated $\times 1000$ by vacuum dialysis and the glycosaminoglycans examined by electrophoresis on cellulose acetate followed by staining with alcian blue. All the cases studied showed markedly raised hydroxyproline excretion, whereas the uronic acid excretion was normal or only slightly raised in 10 of the 11 cases studied. One patient who had a raised uronic acid and raised hydroxyproline concentration was shown to have osteosarcoma as a complication of Paget's disease.

The very high hydroxyproline:creatinine ratio in all cases of Paget's disease (mean 241.8 mmol hydroxyproline/mol creatinine) contrasted sharply with the cases of disseminated neoplasm, where the ratio was either normal or slightly raised (mean 29.3 mmol hydroxyproline/mol creatinine). The ratio of hydroxyproline to CPC-precipitable uronic acid was also markedly raised in cases of Paget's disease (mean 77.3 mmol hydroxyproline/mmol uronic acid) and was lower in the neoplastic group (mean 14.1 mmol hydroxyproline/mmol uronic acid) but showed no advantage over the hydroxyproline:creatinine ratio in differentiating the two groups.

The urinary hydroxyproline:creatinine ratio promises to be of value in differentiating between Paget's disease of bone and neoplastic invasion of bone. A marked rise in CPC-precipitable uronic acid excretion alone is more suggestive of neoplastic invasion of bone, and if associated with a marked increase in hydroxyproline excretion, it raises the possibility of neoplastic change in Paget's disease of bone. The results of this study also suggest that bone collagen, rather than bone tissue in general, is primarily affected in Paget's disease.

Increased urinary excretion of hydroxyproline has been reported in cases of Paget's disease of bone.¹ Hydroxyproline is an imino acid which occurs mainly in fibrous proteins such as collagen. The majority of total body collagen is found in bone. In Paget's disease, where high hydroxyproline excretion is found in conjunction with bone lesions, it seems likely that the source of urinary hydroxyproline is bone collagen.

Glycosaminoglycans have been shown to be associated with collagen in bone.² Urinary excretion of glycosaminoglycans can be studied quantitatively by precipitation with cetyl pyridinium chloride (CPC) followed by measurement of the uronic acid

and hexosamine content of the CPC-precipitable material. Urinary glycosaminoglycans may also be concentrated by vacuum dialysis and separated by electrophoresis.^{3,4} It was decided to study a series of patients with clear evidence of Paget's disease to determine the excretion of hydroxyproline and glycosaminoglycans. The results from these patients were compared with those found in a group of patients with disseminated neoplasm on whom similar studies had been carried out.³

Material and methods

Venous blood (20 ml) and a 24-hour urine collection was taken from 11 patients with radiologically-proven active Paget's disease. The extent of skeletal activity

was assessed biochemically by serum alkaline phosphatase, calcium, inorganic phosphate and 5-nucleotidase together with 24-hour urinary hydroxyproline excretion. Twenty-four hour urinary glycosaminoglycan excretion was studied by uronic acid assay after isolation by cetyl pyridinium chloride (CPC) precipitation, and electrophoretic separation of urinary glycosaminoglycans concentrated by vacuum dialysis. Control data for glycosaminoglycan excretion was drawn from 100 normal subjects of all ages studied by identical methods and reported previously.⁴

Serum alkaline phosphatase

The method of Morgenstern *et al.*⁵ was employed, using a Technicon SMA-plus analyser.

Serum calcium

A method based on the work of Kessler⁶ and Gitelman⁷ was employed, using a Technicon SMA-plus analyser.

Serum inorganic phosphate

A method based on the work of Hurst⁸ and Kraml⁹ was employed, using a Technicon SMA-plus analyser.

Serum 5-nucleotidase

This was assayed using the method of Campbell.¹⁰

Urine creatinine

A single-channel Technicon Auto-Analyzer was used (AA1) employing method N-116 (alkaline picrate).

Hydroxyproline assay

Aliquots of 24-hour urine collections were assayed using the Hypronosticon kit (Organon (Teknika) Ltd, Cromwell Road, St Neots, Huntingdon) which is based on the method of Goverde and Veenkamp.¹¹

Isolation of glycosaminoglycans

Glycosaminoglycans were isolated from aliquots (5 ml) of 24-hour urine samples by CPC precipitation, followed by dissolution of the complex in sodium chloride and precipitation in ethanol.⁴

Uronic acid assay

Glycosaminoglycans isolated from aliquots of urine (5 ml) were dissolved in water (1 ml) and the uronic acid content was determined using the method of Bitter and Muir.¹²

Hexosamine assay

Glycosaminoglycans isolated from aliquots of urine (5 ml) were hydrolysed with HCl (4M) in sealed glass ampoules in an autoclave at 15 psi for 30 min.¹³ Hydrolysates were dried over KOH *in vacuo*, and hexosamine was assayed by Boas¹⁴ modification of the Elson-Morgan reaction, as previously described.¹⁵

Separation of glycosaminoglycans

Aliquots (50 ml) of 24-hour urine samples were concentrated $\times 1000$ by vacuum dialysis. Electrophoresis of urine concentrates (10 μ l, equivalent to 10 ml original urine) was carried out on cellulose acetate membranes in veronal-acetate buffer, pH 9.2. Marker samples of hyaluronic acid (human umbilical cord), heparan sulphate (human aorta), and chondroitin sulphate (human aorta) were run alongside urine concentrates. The glycosaminoglycans were located by staining with alcian blue and quantified by reflectance densitometry using the Joyce Chromoscan, as previously described.¹⁵

Results

The major results of this study are shown in the Table. All the cases studied showed very high serum

Biochemical data on 11 cases of Paget's disease

Case	Sex	Age	Serum alkaline phosphatase (IU/l) NR = 20-90	Serum calcium (mmol/l) NR = 2.1-2.7	Serum inorganic phosphate (mmol/l) NR = 0.8-1.4	Serum 5-nucleotidase (IU/l) NR = 2.0-17.0	Urinary hydroxyproline: creatinine (mmol/mol) NR = 3-31	Urinary uronic acid: creatinine (mmol/mol) NR = 0.3-2.8	Urinary hexosamine: creatinine (mmol/mol)	Hydroxyproline: uronic acid ratio (mmol/mmol)	Urinary electrophoresis: total alcian blue-positive material	Fraction E3	Fraction E2	Fraction E1
1	M	68	1000	2.36	1.08	9	196	3.2	4.3	61.3	53.5	5.4	41.1	25.1
2	M	71	1000	2.51	0.87	10	240	4.5	5.5	53.5	24.0	50.2	25.1	46.6
3	F	66	589	2.65	0.81	12	219	2.6	3.1	84.2	37.6	16.3	46.6	25.1
4	M	67	765	2.31	1.18	10	299	7.6	12.4	39.3	39.9	30.6	29.9	47.9
5	F	68	1000	2.51	1.19	16	418	2.4	5.8	174.2	34.3	18.1	47.9	44.4
6	M	62	544	2.44	1.09	20	143	1.9	2.2	75.3	31.9	15.5	52.6	44.4
7	M	59	539	2.56	1.08	15	201	2.5	2.7	80.4	43.8	12.0	44.4	44.4
8	M	57	1000	2.39	1.14	9	378	3.6	4.9	105.0	8.7	36.8	54.8	44.4
9	M	73	682	2.38	0.92	NA	143	1.7	2.7	84.1	34.0	20.2	45.8	44.4
10	M	89	910	2.22	1.11	7	208	4.3	4.0	48.4	32.6	28.8	38.8	44.4
11	F	81	285	2.49	0.98	NA	215	4.8	5.2	44.5	30.3	35.0	34.4	44.4

NA = Result not available.

alkaline phosphatase activities. The 5-nucleotidase activities were assayed in nine of the 11 cases, eight of these were within normal limits while one was very slightly raised. The urinary hydroxyproline results were markedly raised in every case. The uronic acid results showed five cases within normal limits, five cases with slightly raised concentrations and only one case (No 4) with a significantly raised uronic acid. The urinary hexosamine:creatinine ratio was higher than the uronic acid:creatinine ratio in all but one case.

A graph of the hydroxyproline and uronic acid results for the cases of Paget's disease and from 24 cases of disseminated neoplasm, previously published,³ are shown in Fig. 1. This shows that the pattern of excretion found in Paget's disease is markedly different from that found in disseminated neoplasm.

The urinary hydroxyproline:creatinine ratio was very high in all cases of Paget's disease, ranging from 143 to 418 mmol hydroxyproline/mol creatinine with a mean value of 241.8, and normal or only slightly

raised in cases of disseminated neoplasm, where the range was 10 to 74 mmol hydroxyproline/mol creatinine, with a mean value of 29.3 mmol hydroxyproline/mol creatinine.

The ratio of hydroxyproline to CPC-precipitable uronic acid was also greatly raised in cases of Paget's disease (range 39.3-174.2 mmol hydroxyproline/mmol uronic acid, mean 77.3) compared with cases of disseminated neoplasm, where the range was 2.7-34.8 mmol hydroxyproline/mmol uronic acid, mean 14.1. The urinary hydroxyproline:creatinine ratio in cases of uncomplicated Paget's disease compared with cases of disseminated neoplasm with clear radiological evidence of skeletal metastases is shown in Fig. 2, and the urinary hydroxyproline:uronic acid ratio is shown in Fig. 3.

Electrophoresis of urine concentrates showed three alcian blue-positive fractions in each case, as described previously.⁴ The fractions are identified as E1, E2 and E3 where E1 corresponds in electrophoretic mobility and hyaluronidase lability to chondroitin sulphates and dermatan sulphate, E2 corresponds to heparan sulphate and E3 to the glycoproteins. The proportions of these three

Fig. 1 Urinary hydroxyproline:creatinine and CPC-precipitable uronic acid:creatinine ratios in: ● 10 cases of uncomplicated Paget's disease of bone, ◐ 1 case of Paget's disease of bone with osteosarcoma and ○ 24 cases of disseminated neoplasm.³ The stippled rectangle represents the normal range.

Fig. 2 Urinary hydroxyproline:creatinine ratios in ● 10 cases of uncomplicated Paget's disease of bone and ○ 12 cases of disseminated neoplasm with clear radiological evidence of bone secondaries.³

Fig. 3 Urinary hydroxyproline:CPC-precipitable uronic acid ratios in ● 10 cases of uncomplicated Paget's disease of bone and ○ 12 cases of disseminated neoplasm with clear radiological evidence of bone secondaries.³

fractions found in each case are shown as percentages of total alcian blue-positive material in the Table. No abnormal fractions were seen in any of the electrophoretic patterns studied in this series, in contrast to the previously reported series of cases with disseminated neoplasm, where a fraction corresponding to hyaluronic acid was observed in over 50% of the cases with clear evidence of skeletal involvement.

Discussion

The object of this study was to ascertain whether there was an increase in urinary glycosaminoglycan excretion as well as urinary hydroxyproline excretion in Paget's disease. The results obtained indicate that half the cases have normal glycosaminoglycan excretion and the other half have only slightly raised excretion. These results contrast with the very high concentrations of urinary hydroxyproline excretion. All the cases studied showed the typical serum biochemistry of Paget's disease; that of normal calcium and inorganic phosphate values together with high alkaline phosphatase values and normal 5-nucleotidase activities.

The urinary excretion of hydroxyproline and

uronic acid, illustrated in Fig. 1, show interesting differences between the cases of Paget's disease and the cases of disseminated neoplasm. The majority of cases of disseminated neoplasm lie within normal limits for both parameters. For those that stay beyond the normal limits, the trend is clearly to an increase in uronic acid excretion, with only three cases showing a significant increase in hydroxyproline excretion. The pattern in Paget's disease is quite different. None of the cases lies within the normal range for both parameters, but the trend is clearly to a marked increase in hydroxyproline excretion. Only one case of Paget's disease showed a significantly raised uronic acid excretion, and this case (No 4) was found to have developed an osteosarcoma in an area of bone previously affected by Paget's disease, and so could be placed in either the Paget's or the neoplastic group.

The urinary CPC-precipitable hexosamine was slightly greater than the CPC-precipitable uronic acid in 10 of the 11 cases. The average molar ratio of hexosamine to uronic acid was 1.36, similar to that found in cases of disseminated neoplasm. The slight excess of hexosamine to uronic acid is presumably due to the presence of keratan sulphates in the CPC precipitate.

The differentiation between Paget's disease of bone and neoplastic infiltration of bone is sometimes very difficult indeed, even on radiological grounds. In this study, either the urinary hydroxyproline:creatinine ratio, or the urinary hydroxyproline:uronic acid ratio provided a clear differentiation between the two groups. The hydroxyproline:creatinine ratio is easier to measure, but the additional measurement of urinary CPC-precipitable uronic acid excretion may provide useful information in some cases. Thus a marked increase in uronic acid excretion in the presence of a normal or only slightly raised hydroxyproline excretion is more suggestive of neoplastic invasion than Paget's disease of bone, and a marked increase in both hydroxyproline and uronic acid excretion may suggest the possibility of coexistent Paget's disease and neoplastic invasion of bone. The possibility that a rising urinary uronic acid excretion may serve as an early warning of sarcomatous change in Paget's disease of bone deserves further study.

The origin of the increased CPC-precipitable uronic acid in the urine of many cases of disseminated neoplasm is not yet clear, but it seems likely that it arises from degradation of glycosaminoglycans in the ground substance of bone and other connective tissues.³ The results of this study of patients with Paget's disease, where a massive increase in urinary hydroxyproline excretion was accompanied by either no increase, or a relatively insignificant increase, in the urinary excretion of CPC-precipitable

uronic acid, adds further support to the suggestion that bone collagen, rather than bone tissue in general, is primarily affected in Paget's disease. The pattern of hydroxyproline peptides excreted in Paget's disease has been shown to be different from that of normal subjects.¹⁶ Isotopic studies of the hydroxyproline containing peptides in the urine of patients with Paget's disease have shown that these are related to collagen synthesis.¹⁷ However, the nature of any defect of collagen synthesis in Paget's disease is not clear at present.

The conclusion to be drawn from this study is that considerable increase in the urinary excretion of hydroxyproline in Paget's disease is not associated with a similar increase in the urinary excretion of CPC-precipitable uronic acid. The urinary hydroxyproline:creatinine or hydroxyproline:uronic acid ratios offer a means of differentiating Paget's disease of bone from neoplastic infiltration of bone on biochemical grounds.

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