Urinary 3-hydroxyproline excretion in Alport's syndrome: a non-invasive screening test?

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Abstract

Alport's syndrome is characterised by morphological and structural changes of the renal basement membranes. As the hydroxyproline content of isolated glomerular basement membranes is reduced in patients with Alport's syndrome, it is possible that the renal excretion of 3-hydroxyproline (3-OHP), a key substrate of basement membrane collagen, may be altered in such patients. The urinary excretion of 3-OHP was determined by thin layer chromatography in 20 patients with Alport's syndrome, in healthy control subjects, and in patients with other renal diseases. These included patients with post-streptococcal glomerulonephritis, lower urinary tract infection, severe reflux nephropathy, lithium induced nephropathy, polycystic kidney disease, familial benign haematuria, and renal graft rejection. Urinary excretion of 3-OHP was significantly higher in patients with Alport's syndrome compared with the patients with other renal diseases and the healthy control subjects. All other renal diseases investigated had 3-OHP values within the normal range. Urinary 3-OHP determination detected patients with Alport's syndrome with a high sensitivity (95.2%) and specificity (97.2%). We therefore suggest using urinary 3-OHP determinations as a simple non-invasive screening test for Alport's syndrome.

Alport's syndrome, a hereditary disorder, is characterised by morphological and structural changes of the renal basement membranes. Morphologically, electron lucent and electron dense areas, cloudy networks, fraying, splitting, thickening, distortion, and thinning of the lamina densa may be observed by electron microscopy.¹ However, the underlying mechanisms and molecular defects of the disease are still unclear.

Collagen type IV, an integral component of the glomerular basement membrane,² is immunologically different in patients with Alport's syndrome compared with healthy subjects.^{3–5} Hydroxyproline, a major constituent of collagens, was reported to be reduced in isolated glomerular basement membranes of patients with Alport's syndrome.⁶ Hydroxyproline exists in two stereoisomeric 'trans' forms: 4-trans-hydroxyproline (4-OHP) and 3-trans-hydroxyproline (3-OHP) but, unlike other connective tissue collagens, basement membranes normally contain large amounts of 3-OHP.⁷ As 3-OHP is excreted with the urine, changes in the collagen metabolism of the renal basement

membranes could be reflected by a different urinary excretion of 3-OHP.

In order to test this hypothesis, and to find out characteristic differences, we studied the urinary excretion of 3-OHP in patients with Alport's syndrome, in healthy control subjects, and in patients with other renal diseases.

Patients and methods

Urine samples of 20 patients with Alport's syndrome were obtained from Sint Radboudziekenhuis, Institute for Kindergeneeskunde, Nijmegen, The Netherlands. The median age of these patients was 16 years (range 4 days–57 years). There were 15 females and five males. The diagnosis was confirmed by the presence of albuminuria (20/20), by familial history (18/20), and/or by electron microscopy (10/20).

There were two groups of control subjects. The first group consisted of healthy control subjects and comprised 23 newborn infants (age 2–30 days), 11 infants (age 1–12 months), 16 toddlers (age 1–4 years), 47 children and adolescents (age 8–18 years), and 12 adults (age 22–41 years). The second group consisted of subjects with other renal diseases and comprised 10 children with acute and 10 children with chronic poststreptococcal glomerulonephritis, 10 children with haemorrhagic lower urinary tract infection, 10 children with severe reflux nephropathy (reflux grade 4), 10 adults with lithium nephropathy, five children with adult type polycystic kidney disease, seven children with familial benign haematuria, and five children with acute renal graft rejection (10 urine samples). Urine samples consisted of spontaneously voided urine or of aliquots of a 24 hour collection period.

We determined the type of proteinuria (high/low molecular weight proteinuria) by polyacrylamide gel electrophoresis (PAGE) of the urine samples from the patients with Alport's syndrome. PAGE was performed according to standard principles using 8% polyacrylamide gel, molecular weight markers, and staining with coomassie blue.

The urinary concentration of 3-OHP was determined by thin layer chromatography. Urine samples were centrifuged at 3000 g for five minutes at room temperature and the supernatant used. The urinary volume used for chromatography was determined according to its creatinine content, which was analysed by Jaffé's method (Astra Beckman (R) Automatic Analyzer, Beckman). The volume containing 1 mg creatinine was added to 1 ml of 0.1 M phosphate buffer, pH 7.0, and 1 mg of charcoal

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(Sigma C 5385). This mixture was vortexed for 20 seconds and centrifuged at 2000 *g* for 10 minutes at room temperature. The pellet was resuspended in 1 ml of 0.1 M phosphate buffer, pH 7.0, centrifuged again at 2000 *g* for 10 minutes. The pooled supernatants were applied to columns containing 3 ml of Dowex 50 W X 8 (Bio Rad 745-6441), 100–200 mesh, in hydrogen form. Amino acids were eluted with 2 ml of 4 M ammonia. The eluate was evaporated until dryness on a Pierce Reactitherm Heating Module and redissolved in 200 µl distilled water. An aliquot of 100 µl of this solution was added to 20 µl o-phthalaldehyde (30% triethylamine, 70% ethanol, 8 mg/ml, Pierce 26-010). The mixture was incubated at room temperature in the dark for 30 minutes and then evaporated until dryness. The samples were redissolved in 20 µl 0.1 M phosphate buffer pH 7.0. Adding 20 µl 7-chloro-4-nitrobenz-2-oxa-1,3 diazole (8 mg dissolved in 1 ml ethanol), the samples were heated in a waterbath for 10 minutes at 40°C. The samples were spun for 5 minutes at 2000 *g*. Ten µl of the resulting supernatant were applied to HP thin layer chromatography plates (LHP-KF Whatman 4806). The development solution consisted of acetone:chloroform:methanol:triethylamine=20:60:12.5:7.5. The plates were read at 365 nm on a Shimadzu TLC scanner CS 920; 4-trans-hydroxyproline (Sigma H 60-002), 4-cis-hydroxyproline (Sigma H 1-637), 3-trans-hydroxyproline, and 3-cis-hydroxyproline (synthesised by J Häusler⁸) were used as standards.

Specificity of the determinations on thin layer chromatography of 3-OHP was confirmed by high pressure liquid chromatography (HPLC) and by gas chromatography-mass spectrometry (GC-MS) in the standards used for the chromatography and in the urine samples obtained from the healthy adults. For the HPLC determinations checkpoint samples, taken from the thin layer chromatography, were dissolved in elution buffer and characterised on reverse phase chromatography according to Lindblad and Diegelmann.⁹ For the GC-MS determinations N-trifluoroacetyl-O-trimethylsilyl-hydroxyproline-trimethylsilyl-esters were prepared according to Donike,¹⁰ GC-MS determinations were performed on a Varian MAT 112 according to Vycudilik and Lubec.¹¹ The hydroxyprolines were characterised by two techniques: (i) monitoring of the M-15 fragment ion (*m/e*) 356, single ion monitoring (SIM) and (ii) analysis of the full mass spectra taken along with the GC separation of the derivatives at a rate of 1 sec/mass decade.

Differences between groups and within groups were analysed by the Kruskal-Wallis test.¹² Sensitivity of the 3-OHP determinations for detecting Alport's syndrome was defined as the proportion of patients with Alport's syndrome having a 3-OHP value above the range of the mean + 2 SD of the 3-OHP values of the normal control subjects. Specificity of 3-OHP determinations for detecting Alport's syndrome was defined as the proportion of control subjects and of patients with other renal diseases having a 3-OHP value within the range of the mean and 2 SD of the normal control subjects.

Results

Polyacrylamide gel electrophoresis showed non-significant unspecific high molecular weight (= glomerular) proteinuria in 12/20 patients with Alport's syndrome.

Results of the thin layer chromatography determinations of 3-OHP are shown in the table and in fig 1 and 2. They were highly significant differences between the 3-OHP values of the patients with Alport's syndrome compared with the normal control subjects and with the patients with other renal diseases but no difference between the latter and the normal control subjects.

In a single patient with Alport's syndrome the 3-OHP excretion was within the normal range (mean, 2 SD) of the healthy control subjects, and five control subjects had a 3-OHP value above the 2 sigma range of the healthy control subjects. All patients with other renal diseases had 3-OHP values within the normal range. Thus a specificity of 97.2% and a sensitivity of 95.2% of the thin layer chromatography 3-OHP determinations may be calculated for the patients with Alport's syndrome.

Results of the 3-OHP determinations

Group	No of subjects	Concentrations of 3-OHP	
		Mean (SD)	Range
Alport's syndrome	21	5.64 (2.43)*†	1.97–10.24
Normal controls:	98	0.96 (0.59)	0.06–2.55
Adults	8	0.34 (0.14)	0.06–0.55
Children	40	1.08 (0.40)	0.52–2.40
Infants	11	1.50 (0.47)	0.91–2.55
Newborns	23	0.36 (0.13)	0.20–0.60
Toddlers	16	1.44 (0.64)	0.32–2.34
Other renal diseases:	82	1.05 (0.30)	0.41–2.10
Acute glomerulonephritis	10	0.94 (0.22)	0.47–1.21
Chronic glomerulonephritis	10	1.09 (0.38)	0.74–2.10
Urinary tract infection	20	0.96 (0.21)	0.47–1.43
Reflux nephropathy	10	1.23 (0.25)	0.72–1.57
Lithium nephropathy	10	0.98 (0.29)	0.41–1.33
Polycystic kidneys	5	0.94 (0.48)	0.53–1.72
Familial benign haematuria	7	1.24 (0.30)	0.78–1.74
Graft rejection	10	1.07 (0.30)	0.54–1.48

*Significantly different from control group ($p < 0.0001$).

†Significantly different from other renal diseases group ($p < 0.0001$).

Figure 1 Determinations of 3-hydroxyproline (3-OHP) µg/mg creatinine by thin layer chromatography in patients with Alport's syndrome ($n = 20$), patients with other renal diseases ($n = 67$), and normal control subjects ($n = 109$).

Figure 2 Thin layer chromatogram of 3-hydroxyproline (3-OHP) in patients with Alport's syndrome (A), in normal control subjects (N), and of hydroxyproline standards (4-OHP=4-trans-hydroxyproline).

Figure 3 Relation between 3-hydroxyproline (3-OHP) µg/mg creatinine and age of the patients with Alport's syndrome ($n=20$), patients with other renal diseases ($n=67$), and normal control subjects ($n=109$).

Figure 4 GC-MS spectrum of 3-hydroxyproline.

Among the normal control subjects there were significant differences between the 3-OHP values of the different age groups (Kruskal-Wallis test, $\chi^2=62.98$, $p<0.0001$), owing to a higher 3-OHP excretion in children, toddlers, and infants (see table). There were no significant differences of the 3-OHP values among the patients with various renal diseases. We found no correlation between age and 3-OHP values in the patients with Alport's syn-

drome, in the normal controls, nor in the patients with other renal diseases (fig 3).

The identification of the 3-OHP bands by thin layer chromatography was confirmed by HPLC and GC-MS determination in all 12 urine samples investigated (fig 4).

Discussion

This is the first report about an increased excretion of 3-OHP in patients with Alport's syndrome. There are reports on differences of the excretion of other amino acids: Tina *et al*⁶ and Veltishev *et al*¹³ found an increased urinary excretion of glycosylated hydroxylysine, another amino acid which may be found in collagens of basement membranes in relatively low amounts. However, this was not confirmed by Schröder *et al*.¹⁴ Borel *et al* found an increased 3-OHP excretion in patients with other renal diseases—like glomerulonephritis—where proliferation and damage of the basement membranes are likely to occur.¹⁵ In the latter study the total concentration of 3-OHP was determined in samples of hydrolysed urine, however, whereas we determined the urinary concentration of free 3-OHP. Hydrolysis may also involve remnant cells, micro-organisms, and cell and other debris. Moreover, treatment with acids may cause isomerisation of trans hydroxyproline to its cis form and thus lead to erroneous results.¹⁶

HPLC and GC-MS determinations showed that the described method of thin layer chromatography accurately measured 3-OHP. We related the urinary 3-OHP concentration to the urinary creatinine excretion in order to rule out differences in renal clearance and to avoid long lasting urine collection periods. Although the urine samples of the patients with Alport's syndrome were transported frozen, we cannot rule out that freezing may have caused degradation processes. According to our experience, however, repeated freezing and thawing as well as temperature changes may result in rapid degradation of 3-OHP causing falsely decreased concentrations of 3-OHP, whereas we found raised concentrations of 3-OHP in the patients with Alport's syndrome.

The increased excretion of 3-OHP may possibly be explained as follow: after ribosomal translation of basement membrane collagen proteins, prolyl residues are subjected to the action of post-translational enzymes such as prolyl hydroxylase, lysyl hydroxylase, glycosyl transferases, etc. There is a highly specific 3 and 4 prolyl hydroxylase acting only upon non-helical collagen proteins. Cross linking and helical formation of collagens are possibly disturbed in Alport's syndrome.¹⁷ Therefore non-helical collagens could be subjected to a prolonged action of post-translational enzymes, possibly preferably to 3-prolyl hydroxylase. This could lead to an increased excretion of 3-OHP whereas other basement membrane amino acids such as 4-OHP and hydroxylysine would be excreted in normal amounts (see above).

The high specificity and sensitivity of the increased 3-OHP excretion in patients with Alport's syndrome suggest that the determination of 3-OHP could be helpful as a screening

test for this disease, especially if the non-invasiveness, simplicity, and specificity of the method is taken into consideration. Further studies are needed to confirm its clinical relevance.

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Corrosive injury to the oesophagus

Early oesophagoscopy to assess the presence or extent of damage is now routine after caustic ingestion.¹ There has been doubt about the effectiveness of steroid drugs given to prevent oesophageal stenosis from the more severe injuries.

A trial done in Washington DC (Anderson *et al*, *New England Journal of Medicine* 1990;323:637-40) has shown no benefit from steroids given within 24 hours of the ingestion. A total of 131 children were assigned randomly to receive no treatment or steroids, initially given as intravenous prednisolone and later as oral prednisone for a total of five or six weeks. Ampicillin was given concurrently with the steroid.

Oesophageal stricture developed in 10 of 31 children in the steroid group and in 11 of 29 untreated. Four steroid treated children and seven controls needed oesophageal replacement. Neither difference was statistically significant.

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