Urol Res (1988) 16:49–52

Urological Research

Adherence of Urease-Induced Crystals to Rat Bladder Epithelium

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Accepted July 1, 1987

Summary. Apart from urine supersaturation with respect to struvite and calcium phosphate caused by urease-producing microorganisms, retention of formed crystals in the urinary tract is necessary for the formation of infection stones. This study was performed to investigate the role of the mucous coat lining the urothelium in the adhesion of urease-induced crystals. Removal of this glycosaminoglycan-containing layer from rat bladders increased the adherence of struvite and calcium phosphate crystals 5–6 times compared to that in intact rat bladders. Heparin completely restored the anti-adherence capacity while chondroitin sulphate had a very weak restorative effect and human urine had no restorative effect. These findings support the view that the mucous coat is of importance in preventing retention of urease-induced crystals.

Key words: Urease-induced crystals – Epithelial adherence – Mucous coat

Introduction

A prerequisite for the formation of urinary tract stones is supersaturation of the stone-forming minerals in urine. From this supersaturated urine, crystallization may occur. Many factors which promote or inhibit crystallization of calcium oxalate and calcium phosphate in human urine have been described [2, 3, 18, 21]. Recently, an inhibitory activity, which varied from one individual to another, against urease-induced crystallization of magnesium ammonium phosphate (struvite) and calcium phosphate in human urine was also reported [5, 10]. There has been considerable debate regarding the clinical importance of crystallization inhibitors in the formation of urinary tract stones [21]. It is a common experience that not all patients with "crystalluria" develop concrements. This may be explained by the so-called "washout factor", i.e. the crystals are removed from the urinary tract by the urine stream [14]. Consequently another prerequisite for stone formation is that the formed crystals are retained to allow crystal growth and aggregation to occur. Crystal retention may be achieved either by obstruction of the urine outflow or by crystal adhesion to the urothelium.

The edothelium lining the urinary tract has a surface mucous coat containing glycosaminoglycans (GAGs) [4]. When intact, this mucous coat has been shown to reduce the adherence of bacteria, proteins, calcium oxalate and urate crystals in animal experiments [4, 14, 16, 17]. This protective effect probably depends on the hydrophilic nature of the sulphated GAGs. The surface mucous coat can be digested with weak acid (0.1–0.4 M HCl), leaving the underlying epithelium intact [4, 16].

Infection stones, composed of struvite and calcium phosphate, are formed in urine supersaturated with respect to these salts due to the action of urease-producing microorganisms [7]. This study was performed to investigate the role of the mucous coat in the adhesion of urease-induced crystals. This was done by incubating rat bladders with and without an intact mucous coat with urease-induced crystals.

Material and Methods

Preparation of urease-induced crystals

Synthetic urine of a composition described by Griffith [6] was incubated under aseptic conditions at 37 °C in aliquots of 500 ml in 6 glass vessels with 7.5 units of urease (Jackbean urease E.C. 3.5.1.5; 7 units · mg^{-1}; 1 unit = 1 mg NH_3 · 5 min^{-1} at pH 7.0 and 30 °C, Sigma Chemical Company, St Louis, USA). The pH was initially 5.7 and after a 24 h incubation it reached 8.5–9.0. After incubation, the contents of each vessel were filtered (Millipore 0.22 μm) to collect the precipitated material suspended in the solution. After desiccation at 40 °C for 24 h, the precipitates were weighed, the yield of each vessel ranging from 0.40–0.61 g. The precipitates were analyzed for their magnesium and phosphate content with conventional methods [19, 22]. The magnesium content ranged from 4.8 to 5.7% and the phosphate content from 49 to 60%. Assuming that all magnesium was precipitated as struvite, 36% of the phosphate was

"struvite-phosphate" and 64% „calcium-phosphate". These calculations have previously been confirmed by infrared spectroscopy and optical crystallography [9]. Before use, all precipitates were pooled and a supersaturated slurry was prepared by adding 0.9% saline, giving a salt concentration of 25 mg · ml^{-1}.

Rats

Sixty female Wistar/Fu rats weighing approximately 250 g were used. The animals were deprived of water from midnight on the day before the experiments to restrict urine production during the test period. All animals were anesthetized and the anesthesia was induced with ether and maintained with Hypnorm^R (Janssen Pharmaceutica, Beerse, Belgium), 0.1 ml intraperitoneally every hour throughout the experiments, after which the animals were killed. A polyethylene catheter (outer diameter 0.8 mm) was introduced through the urethra into the bladder and sutured to the external urethral meatus. The catheter was used for repeated bladder installations and evacuations. The abdomen was opened and the bladder exposed to confirm the filling and emptying of the bladder.

Removal of the Mucous Coat.

All bladders were flushed 3 times with 0.4 ml of 0.9% saline to remove residual urine. In 40 rats the mucous coat was removed by instillation of 0.4 ml 0.1 M HCL in the bladders for 2 min. The acid was then aspirated and the bladders washed 3 times with 0.4 ml aliquots of 0.9% saline. In another grup of rats ($n = 10$), which served as controls, 0.4 ml 0.9% saline was instilled for 2 min instead of HCl, and the bladders then rinsed identically. The GAG content of the bladder washes obtained before acid treatment was compared with the GAG content in the HCl-aspirates and saline washes obtained after the acid treatment in 10 rats. The GAGs were measured according to Whiteman after precipitation with Alcian blue (20).

Substitution of the Mucous Coat

Immediately after the acid treatment and the rinsing procedure, a solution of sodium heparin (25,000 IU · ml^{-1}, 25 mg · ml^{-1} Loewens Chemical Industries, Balderup, Danmark) was instilled into 10 bladders in aliquots of 0.4 ml. A chondroitin sulphate solution (sodium salt type A, NO. C-4134, 10 mg · ml^{-1}, Sigma Chemical Company, St Louis, USA) or human urine respectively, was instilled into another 10 bladders in aliquots of 0.4 ml. Fresh whole human urine obtained from one healthy young adult with no history of stone disease or urinary tract infection was used. Urine from this person had previously been shown to possess inhibitory activity against urease-induced crystallization in vitro [5]. The solutions of heparin and chondroitin sulphate and the human urine were left in the bladders for 15 min and then aspirated. In the 10 control bladders and in 10 acid-treated bladders not treated with heparin, chondroitin sulphate or human urine, the same volume of 0.9% saline was instilled and left for 15 min. After the 15-min period, all bladders were rinsed twice with 0.9% saline.

Adherence of Urease-Induced Crystals

After the above-described treatment, 0.4 ml of the crystal slurry was instilled into all bladders for 1 h. After the incubations, the bladder contents were aspirated and the bladders rinsed twice with 0.9% saline in aliquots of 0.4 ml. Thereafter, the bladders were excised and opened by longitudinal incision and quickly washed in 0.9% saline in a standardized way to remove crystals not firmly adherent to the bladder mucosa.

Analysis of Adherent crystals

The washed bladders were dissolved in 15 M HNO_3 at 20 °C during 24 h. The solution was then analysed for its contents of magnesium and phosphate according to previously described methods [19, 22]. By measuring the magnseium and phosphate content in 10 untreated bladders not incubated with the salt slurry, the amounts of these ions in the normal bladder were estimated. By subtracting the mean values of the magnesium and phsophate contents in these bladders from the amount of magnesium and phosphate in the slurry-incubated bladders, it was possible to calculate the amount of "struvite-phosphate" and "calcium-phosphate" that had adhered.

Statistical Analysis

All results were calculated as $\bar{X} \pm$ S.D. Student's T-test was used to study differences between unpaired observations.

Table 1. Adherence of urease-induced crystals to rat bladder epithelium

Treatment before salt incubation	Number of rats	Bladder adherence[a]				Significance of difference compared to control
		PO_4^{--}	Mg^{++}	"struvite-phosphate"	"calcium-phosphate"	
Control	10	0.07 ± 0.03	0.004 ± 0.002	0.02	0.05	–
HCl	10	0.39 ± 0.28	0.020 ± 0.017	0.08	0.31	$p < 0.001$
HCl + heparin	10	0.09 ± 0.03	0.006 ± 0.002	0.02	0.07	n.s.
HCl + chondroitin sulphate	10	0.24 ± 0.09	0.015 ± 0.008	0.06	0.18	$p < 0.001$
HCl + human urine	10	0.38 ± 0.14	0.018 ± 0.008	0.07	0.31	$p < 0.001$

n.s. = not significant

[a] mg per bladder. Values expressed as $\bar{X} \pm$ S.D.

Results

Untreated bladders not incubated with the crystal slurry contained 0.009 ± 0.001 mg magnesium and 0.10 ± 0.02 mg phosphate. In control bladders with an intact mucous coat, the adherence of "struvite-phosphate" was 0.02 mg and the adherence of "calcium-phosphate" 0.05 mg (Table 1). The adherence in acid-treated bladders was 0.08 mg "struvite-phosphate" and 0.31 mg "calcium-phosphate" ($p < 0.001$ compared to controls). In bladders treated with heparin after acid treatment, the adherence of "struvite-phosphate" and "calcium-phosphate" was the same as in control bladders. Chondroitin sulphate had a weaker restorative effect in acid-treated bladders and human urine did not influence adherence of urease-induced crystals after acid treatment at all.

The GAG content in saline washes of intact bladders was 0.57 ± 0.10 μg. After acid treatment with 0.1 M HCl, the content of GAGs in acid aspirate and washes was 6.45 ± 1.85 μg ($p < 0.001$).

Discussion

Removal of the mucous coat of rat bladders with 0.1 M HCl increased the adherence of urease-induced crystals 5–6 times compared to that in bladders with an intact mucous coat. Heparin completely restored the anti-adherence capacity after acid treatment while chondroitin sulphate had a very weak and human urine no restorative effect.

The surface coat lining the epithelium of the bladders in laboratory animals has been shown to have anti-adherent properties [4, 14, 16, 17]. This effect has been attributed to the hydrophilic nature of the GAGs present in the urothelial mucous coat [17]. That the HCl-treatment was effective in removing the GAGs was verified by the observation that GAGs were present in high concentrations in acid aspirate and washes after acid treatment.

Heparin has been shown to adhere to the surface of acid-treated bladders but not to normal urothelium with an intact mucin layer [4, 15]. The anti-adherence capacity of heparin, found in this study and in the study by Gill et al. [4], appears to be caused by binding of heparin to the injured surface layer of the bladder. The surface characteristics are thereby probably altered, preventing crystals from adhering. Chondroitin sulphate, which is another sulphated GAG, had a very weak anti-adherence effect on epithelium devoid of its much layer, which suggests a specificity for heparin in this respect.

It must be pointed out that heparin does not naturally occur in the urinary tract, neither in man nor in animals. The main GAGs constituting the urothelial mucous coat are dermatan sulphate and heparan sulphate [12]. Heparan sulphate is probably the same substance as that reported by Allalouf et al. to be an anticoagulant acid mucopolysaccharide in rat kidney [1]. This heparin-like effect suggests that it is heparan sulphate that is responsible for the anti-adherence property of intact rat bladders.

Urease-induced crystallization has previously been studied as encrustation on solid glass rods immersed in synthetic urine, incubated with Jackbean urease [5, 9, 11]. Human urine was shown to inhibit the amount of encrustation while heparin had no effect in this respect. Human urine probably inhibited the crystal aggregation as the data obtained fitted the Langmuir adsorption isotherm [5]. In the present study, heparin prevented the adherence of preformed urease-induced crystals on injured urothelium. Human urine had no such effect. It thus seems reasonable to assume that heparin and human urine possess the ability to influence the formation of infection stones in different ways. Human urine contains substances which inhibit the crystallization process while heparin prevents the retention of preformed crystals in the urinary tract by inhibited epithelial adhesion.

The etiology of infection stones is complex, including several interrelated steps which may be influenced therapeutically in order to prevent stone formation. A prerequisite for the formation of these stones is urinary tract infection with urease-producing microorganisms [7]. It is therefore essential to find the urease-producing microorganism responsible for the stone formation in order to be able to prevent the formation of new infection stones, especially after stone surgery. It is, however, not always possible to eradicate the responsible microorganism, e.g. in patients with indwelling catheters or patients with high operative risks. The hydroxamates, which are a class of drugs with strong anti-urease activity, have become clinically available during the last few years. Some reports concerning the effect of these drugs have shown promising results [8, 13]. A high incidence of side effects of these drugs has, however, so far restricted their clinical use. We thus still have no effective treatment against the effects of urease-producing microorganisms if the infection cannot be eliminated.

Not all patients infected with urease-producing microorganisms suffer from concrement formation, even in the presence of foreign bodies such as indwelling catheters. Crystallization inhibitors or epithelial factors may be responsible for this interindividual difference. This study has shown that the mucous coat also plays a role in the adherence of urease-induced crystals. An intact GAG-layer appears to have the capacity to prevent the adherence of these crystals. The crystals can thereby be washed out by the urine stream and the risk of stone formation is reduced.

Acknowledgements. This study was supported by grants from the Swedish Medical Research Council (Project No 17X-05437), Svenska Läkaresällskapet, Göteborgs Läkaresällskap and Tore Nilson's Foundation for Medical Research. Excellent technical assistance was provided by Mrs Anita Fae and Mrs Anette Edin-Liljegren.

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