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Role of polymeric Tamm-Horsfall protein in cast formation: Oligosaccharide and tubular fluid ions

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Role of polymeric Tamm-Horsfall protein in cast formation: Oligosaccharide and tubular fluid ions.

Background. In acute tubular necrosis (ATN), distal tubules are obstructed by casts formed by tubular debris, cells, and Tamm-Horsfall protein (THP). Since there are Arginine-Glycine-Aspartate (RGD) and Leucine-Aspartate-Valine (LDV) adhesive sequences in human THP, there may be direct integrin-mediated binding of tubular cells to THP. Alternatively, polymerization of THP may result in entrapment of the cells in its gel.

Methods. Adhesion of LLC-PK_1 cells to THP-coated wells was directly measured. THP concentrate was dissolved in solutions which mimic urine from ATN (ATN-S), distal convoluted tubule (DCT-S), collecting duct (CD-S), and monomeric buffer (M buffer). THP was also denatured by either boiling or N-glycanase digestion. Gel formation of THP was then measured. Inhibition of LLC-PK_1 cell adhesion to collagen type I was measured with each solution, as well as after the collagen was pretreated with either monomeric (mTHP) or polymeric (pTHP) THP. The effect of pTHP on the settling rate of LLC-PK_1 cells in suspension was also measured.

Results. LLC-PK_1 cells did not directly adhere to THP, a finding against integrin-mediated binding as a mechanism for in vivo tubular cell/THP cast formation. The high electrolyte concentration of ATN-S and CD-S, however, was associated with pTHP gel formation. Moreover, cells suspended in pTHP remained in suspension. In cell adhesion studies, mTHP attenuated cell adhesion by binding to the matrix, whereas pTHP attenuated cell adhesion by trapping cells in its gel. An active process was involved since both pTHP gel formation and attenuation of cell adhesion were abolished by boiling or oligosaccharide removal with N-glycanase digestion.

Conclusions. With renal ischemia and proximal tubule cell shedding, ATN and collecting duct fluid composition enhance THP gel formation and thus favor tubular cast formation and obstruction. The present in vitro results indicate the importance of oligosaccharide residues in mediating the effect of the THP on gel formation and potential cast formation in ATN.

Key words: LLC-PK_1 cells, gel formation, tubular cast, acute tubular necrosis, distal tubular obstruction, collecting duct fluid.

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Tamm-Horsfall protein (THP) is the most abundant protein in normal human urine [1–3] and is present in all mammalian kidneys [4–6]. It is synthesized and secreted from epithelial cells of the thick ascending limb of Henle's loop and probably also by the distal convoluted tubule (DCT) [7–11]. Urinary excretion of THP in rats subjected to potassium dichromate-induced acute tubular necrosis (ATN) has been shown to be higher than in normal rats [12]. THP is secreted into tubular fluid as a monomer, but subsequently may become a polymer that forms gel-like material [1, 13–18]. The THP monomer is a glycoprotein with molecular weight of 85 to 100 kD [19–22], which consists of approximately 70% protein and 30% carbohydrate by weight [20, 23, 24]. The oligosaccharide residues of THP are incorporated into the protein backbone by *N*-linked glycosylation, and at least five out of eight potential *N*-linked glycosylation sites are utilized [25–27]. There is no *O*-linked glycosylation in human THP [24]. The oligosaccharides are mannose-rich [25, 28] and are critical for binding to some cytokines [29–34] and *Escherichia coli* fimbriae [35–39] and also may be necessary for THP polymerization [40].

In ATN the distal tubules are obstructed by tubular epithelial cell casts, which consist of detached tubular epithelial cells, debris, and gel-formed THP. The potential role of detached tubular epithelial cells in the pathophysiology of renal tubular obstruction has been studied and discussed [41–45]. Since THP is a major constituent of urinary casts [14, 46–48] and casts are present in ATN [49], THP may play a crucial role in tubular epithelial cell cast formation and tubular obstruction in ATN [13, 50–53]. Patel et al demonstrated that when appropriate concentrations of THP and electrolytes in urine were passed through capillary tubes, the tubes became obstructed [13]. These authors postulated that during ATN, shed tubular epithelial cells may contribute to this obstruction by becoming tangled in or blocked by the precipitated THP. Although THP participation in cast formation and tubular obstruction in acute renal failure has

been considered for decades, the mechanism for such an effect has not yet been elucidated.

The cell adhesion molecules, integrins in particular, have been demonstrated to play a role in cell–cell adhesion, either among aggregated tubular epithelial cells or between detached tubular epithelial cells and distal tubular epithelium [44, 45, 54, 55]. Interestingly, there are two potential adhesive amino acid sequences in the human THP molecule, an Arginine-Glycine-Aspartate (RGD) sequence and a Leucine-Aspartate-Valine (LDV) sequence [26]. Both sequences are part of ligands that are recognized by integrins [56, 57]. The administration of synthetic RGD peptides have been shown to be beneficial for preservation of renal function both in vitro and in vivo acute ischemic renal failure in rats [44, 45, 55]. Toma, Bates and Kumar demonstrated that neutrophils bind to THP by an RGD-mediated mechanism [58]. It is therefore possible that adhesion of renal tubular epithelial cells to THP could contribute to cast formation during ATN. However, renal tubular epithelial cell adhesion to THP has not been examined.

Moreover, as noted, the oligosaccharide residues of THP also have adhesive properties. Therefore, these adhesive sites of THP may participate directly and/or indirectly in renal tubular epithelial cell adhesion, cast formation, and tubular obstruction in ATN as suggested by Patel et al [13]. Because detached tubular epithelial cells and THP are together in the same tubular lumen in ATN, their interactions likely occur and thus should be considered in renal tubular epithelial cell adhesion, cast formation, and tubular obstruction.

Our study therefore was designed to examine (*1*) whether renal tubular cells actively adhere to THP, (*2*) the effect of tubular fluid composition on THP polymerization and gel formation, (*3*) the interaction of renal tubular cells with monomeric versus polymeric THP, and (*4*) the role of oligosaccharides in THP gel formation and interaction with tubular epithelial cells.

METHODS

THP preparation

Tamm-Horsfall protein was directly prepared from normal human urine by salt precipitation [59] and stored at −20°C as a concentrate. A part of the THP concentrate was purified further by gel filtration chromatography on a 2.6 × 90 cm Sepharose 6B column, using chromatography buffer containing 0.05 mol/L Tris-HCl, 0.2 mol/L NaCl, 2.0 mol/L urea and pH 7.4. The THP concentrate was then dissolved in 2.0 mol/L urea solution, pH 6.0, and run on the column at 4°C. Five milliliter fractions were collected, and the THP concentration in each fraction was determined by UV absorbance at 280 nm, which

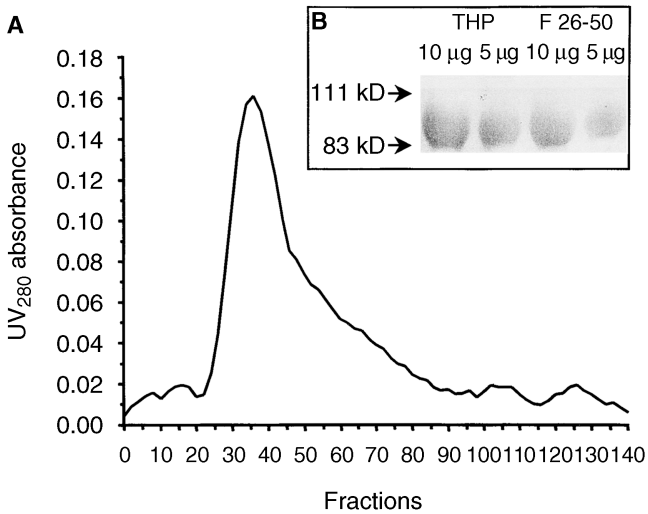

Fig. 1. Purity of the Tamm-Horsfall protein (THP) concentrate directly prepared from normal human urine by salt precipitation. (*A*) Analysis of the THP concentrate by Sepharose 6B gel filtration chromatography as described in the **Methods** section. The analysis revealed one major protein component. (*B*) 7.5% SDS-PAGE analysis of the THP concentrate (THP) in comparison to eluents from pooled-fraction 26 to 50 (F 26 to 50). Coomassie blue staining revealed one major band of protein around 85 to 100 kD in THP similar to the result in F 26 to 50. Both analyses were representative of two separate experiments ($N = 2$).

revealed one prominent peak (Fig. 1A). THP in pooled fractions 26 to 50 was salt precipitated by the same procedure, and its purity was determined by 7.5% SDS-PAGE and compared with the original THP concentrate directly prepared from urine. Coomassie blue staining of the blot revealed a major protein band between 85 and 100 kD for both the THP concentrate and the further purified pooled sample (Fig. 1B), similar to results from a previous study using the same method for THP preparation [22]. Based on these results, we conducted all experiments using the THP concentrate without further purification.

To examine its properties further, the THP concentrate was also treated to two different procedures. In the first, the THP concentrate was heat-treated (boiled, bTHP) by incubating in a 90°C water bath for 10 minutes. In the second procedure, the oligosaccharide residues of the THP molecule were removed by *N*-glycanase enzyme digestion under nondenaturing conditions according to the procedure of Muchmore, Shifrin and Decker [60]. Specifically, 1 mg THP concentrate was dissolved in 1 mL digestion buffer containing 20 mmol/L NaH_2PO_4, 50 mmol/L ethylenediamine tetraacetate (EDTA), pH 7.5. Then 2.6 units of *N*-glycanase (Oxford GlycoSciences, Wakefield, MA, USA) were added and incubated at 37°C for 48 hours with agitation. The digested THP (dTHP) was salt precipitated as described previously in this article and then dialyzed against water in the same manner as for the original preparation from

urine. The dTHP concentration was determined by protein measurement and kept frozen at −20°C. Sham-digested THP (sTHP) was prepared by incubating the THP concentrate in digestion buffer without *N*-glycanase using the above digestion procedure. From our preliminary experiments, the sTHP had similar adhesive and gel-forming properties to the THP concentrate (data not shown). Protein concentration in this study was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

Cells and cell culture

Porcine renal proximal tubular epithelial cells (LLC-PK_1) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in 100 mm diameter cell culture dishes in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1) containing 2 mmol/L glutamine, 15 mmol/L HEPES, 1.5 g/L $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, pH 7.4, supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA). The cells were grown to confluence in an atmosphere of 95% air/5% CO_2 and 90% humidity at 37°C. The cells were allowed to become quiescent in serum-free medium for 24 hours before the experiments.

Protein coating of cell culture plates

Tamm-Horsfall protein or collagen type I from rat tail tendon (Upstate Biotechnology, Lake Placid, NY, USA) was diluted in calcium-free phosphate-buffered saline (PBS) to a final concentration of 100 to 400 μg/mL (THP) or 40 μg/mL (collagen). Human serum albumin (HSA; 400 μg/mL) was used as a negative control coating. Then 50 μL of the protein solution was added to each well of 96-well polystyrene cell culture plates and incubated overnight at 4°C. After overnight coating, the protein solution was aspirated, and the wells were washed twice with calcium-free PBS. Prior to cell adhesion, nonspecific adhesion was blocked with 1% bovine serum albumin (BSA) in calcium-free PBS at room temperature for two hours. The blocking solution was then removed, and the wells were washed twice with calcium-free PBS.

Cell adhesion assays

Measurement of direct adhesion of cells to THP or collagen-coated wells. The cell monolayer was trypsinized with 0.1% trypsin/0.04% EDTA in Hank's balanced salt solution. The trypsin was inactivated by soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, USA), and then the cells were washed three times with calcium-free PBS, counted, and resuspended to 100,000 cell/mL in DMEM/F12 at 37°C; 200 μL of cell suspension (20,000 cells/well) were allowed to adhere in each precoated well for 90 minutes at 37°C. At the end of the 90 minutes, the supernatant, which contained nonadherent cells, was gently aspirated, and the wells were washed twice with calcium-free PBS. The number of adherent cells was determined by hexosaminidase enzyme assay as described previously [61]. In addition, the cell adhesion results determined by hexosaminidase activity were confirmed by cell counting in a series of independent experiments. Cell adhesion was expressed as a percentage of the number of cells seeded in each well. Each experiment (*N*) represents the mean adhesion of three wells.

Measurement of THP inhibition of cell adhesion to collagen. To test the effect of THP on LLC-PK_1 cell adhesion to collagen, two types of experiments were performed. In each, the cells were trypsinized and washed as described previously in this article and resuspended to 100,000 cell/mL in each experimental solution described later in this article at 37°C. Collagen type I was chosen as the matrix for these studies because of its avid binding to LLC-PK_1 cells.

(*1*) Either the THP concentrate, bTHP or dTHP (80 μg/mL each) was preincubated in each experimental solution for 60 minutes at 37°C before adding cells. The cells were then added to the preincubated THP solution, and 200 μL of cell suspension (20,000 cells/well) were allowed to adhere to collagen-precoated wells for 90 minutes at 37°C. Control LLC-PK_1 cells were treated exactly the same as treated cells, but without addition of the THP. Adherent cells were quantitated as described previously in this article.

(*2*) THP concentrate (80 μg/mL) was preincubated in each experimental solution (described later in this article) for 60 minutes at 37°C. The experimental solutions preincubated with THP were then pipetted into the collagen-coated wells and incubated for 90 minutes at 37°C. The solutions were then aspirated, and the wells were washed twice with calcium-free PBS. LLC-PK_1 cells (20,000 cells/well) in the same experimental solution without THP were then added to the preincubated wells for a 90-minute adhesion. Cell adhesion was assessed as described previously in this article.

Cell adhesion of the treated group was expressed as the percentage of adhesion in the untreated control wells for the same experimental solution (% control). Adhesion of untreated control cells was defined as 100%. Each experiment (*N*) represents the mean adhesion of three wells. In addition, the aspirated solution (nonadherent cells) was examined microscopically.

In these THP experiments, four solutions were used, which differed mainly in concentration of salts. One of these was representative of urine from patients with ATN (ATN-S). The other two solutions were representative of tubular fluids in normal distal convoluted tubule (DCT-S) or collecting duct (CD-S). The monomeric buffer (M buffer) was a solution in which THP is in monomeric form due to 0.72 mol/L urea and minute amounts of salts. The compositions of each solution are

Table 1. Composition of the experimental solutions

	M buffer[a]	ATN-S[b]	DCT-S[a]	CD-S[b]
Na^+ *mmol/L*	0.0	60.0	35.0	170.0
K^+ *mmol/L*	0.0	80.0	12.0	60.0
Ca^{2+} *mmol/L*	3.5	3.5	3.5	3.5
Mg^{2+} *mmol/L*	5.0	5.0	5.0	5.0
Cl^- *mmol/L*	7.0	131.0	44.5	179.0
SO_4^{2-} *mmol/L*	5.0	8.0	5.5	19.0
PO_4^{3-} *mmol/L*	0.0	10.0	8.5	30.0
Mannitol *mmol/L*	262.0	0.0	0.0	0.0
Urea *mmol/L*	720.0	46.5	19.5	320.0
D-Glucose *mmol/L*	17.5	17.5	17.5	17.5
pH	6.0	6.0	6.0	6.0
Total osmolarity *mOsm*	1020.0	361.5	151.0	804.0
Tonicity *mOsm*	300.0	315.0	131.5	484.0
Electrolytes *mmol/L*	20.5	297.5	114.0	466.5

The solutions were made freshly for each experiment with simplified concentration of the compositions estimated from the literature. Ca^{2+} and Mg^{2+} are necessary for cell adhesion, D-glucose for the energy resource. Mannitol was for tonicity compensation in M buffer. The pH of all solutions was kept at 6.0. Abbreviations are: M buffer, monomeric buffer; ATN-S, acute tubular necrosis solution; DCT-S, distal convoluted tubule solution; CD-S, collecting duct solution.

[a] THP was mainly a monomer in these solutions

[b] THP was mainly a polymer in these solutions

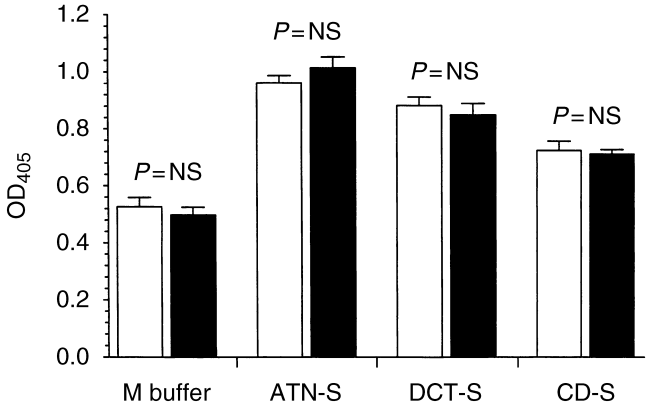

Fig. 2. Absence of effect of THP (80 μg/mL) on hexosaminidase activity of LLC-PK_1 cells in each experimental solution. LLC-PK_1 cells were incubated in each experimental solution either with (■; 80 μg/mL) or without (□) the THP concentrate as described in the **Methods** section. The incubated cells were washed twice to remove THP, resuspended in the same solution, and 8000 cells were pipetted to each well to assess their hexosaminidase activity in comparison to cells without exposure to THP. The hexosaminidase activity was expressed as light absorbance at 405 nm (OD_{405}), $N = 6$ for each experimental solution.

shown in Table 1. Since Ca^{2+} and Mg^{2+} are necessary for cell adhesion and D-glucose for cell energy, they were included in all solutions. Mannitol was added to the M buffer to compensate for tonicity of the solution. The pH of all solutions was kept equal at 6.0. All solutions were freshly prepared for each experiment at room temperature.

None of the experimental solutions alone had any effect on the hexosaminidase assay (data not shown). This was tested by incubating each experimental solution with hexosaminidase substrate for 90 minutes; then the reaction was stopped and light absorption at 405 nm was measured. In addition, THP alone (80 μg/mL) in each experimental solution had no effect on LLC-PK_1 cellular hexosaminidase activity at 8000 cells ($N = 6$ in each solution; Fig. 2). This was tested by incubating the LLC-PK_1 cells with or without the THP concentrate (80 μg/mL), in each experimental solution similar to the procedure described previously in this article. After the incubation, the cell suspension was centrifuged, the supernatant was removed, and cells were washed twice and resuspended in the same experimental solution. The hexosaminidase activities of THP-treated and untreated cells, at an 8000 cell concentration, were compared. The hexosaminidase activity was expressed as light absorbance at wavelength 405 nm (OD_{405}).

Measurement of THP gel formation by spectrophotometry

Either the THP concentrate, bTHP, dTHP, or sTHP (80 μg/mL) was dissolved in each experimental solution. After 60 minutes of incubation, gel formation in each THP solution was measured by ultraviolet light absorption at 280 nm by using BSA (80 μg/mL) in the same experimental solution as a blank. The results were expressed as the difference between the absorbance of the samples and their blanks (ΔOD_{280}). The greater the ΔOD_{280}, the greater the gel formation. Each experiment (N) represents the mean ΔOD_{280} of three samples.

Cell suspension experiment

An experiment was performed to determine the settling rate of LLC-PK_1 cells suspended in solution where THP was in the polymeric (gel) form. In this experiment, the LLC-PK_1 cell monolayer was trypsinized and the cells washed as before. The cells were suspended in 400 μL of a high salt urine-like solution containing in mmol/L: 75 NaCl, 14 Na_2SO_4, 15 KCl, 30 KH_2PO_4, 3.5 $CaCl_2$, 5 $MgSO_4$, 300 urea, 17.5 D-glucose, pH 6.0, at a concentration of 2,500,000 cells/mL. THP concentrate (100 μL) was added at a concentration of 80 μg/mL. The tubes were vortexed and allowed to stand at room temperature for 20 minutes. At the end of the 20-minute incubation, the suspension was visually and microscopically examined.

In another experiment, the cells were suspended in cuvets as described previously in this article. As the cells were allowed to settle, absorbance readings (300 nm) were taken as an index of number of cells remaining in suspension. THP solution alone or cells suspended without THP served as controls.

Cell viability

Cell viability was determined by trypan blue dye exclusion. Cell adhesion assays were performed as usual; non-

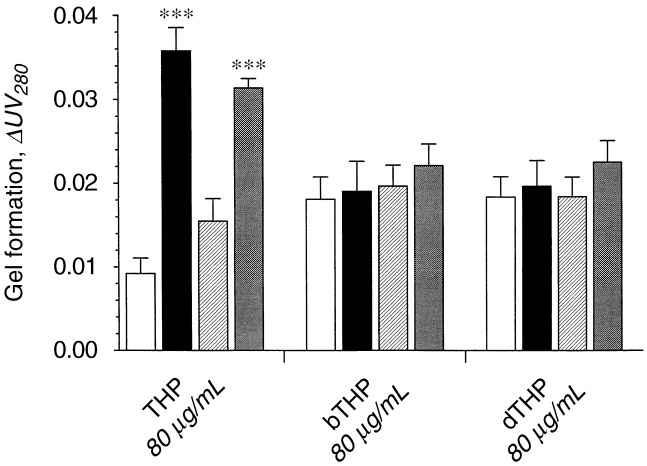

Fig. 3. Polymeric THP (pTHP), not monomeric THP (mTHP), formed a gel and its effect was abolished either by boiling or removal of oligosaccharide residues after N-glycanase digestion. Eighty μg/mL of either the THP concentrate (THP), boiled THP (bTHP), or *N*-glycanase digested THP (dTHP) was incubated in each experimental solution for one hour and then gel formation in the solution was assessed by ultraviolet (280 nm) light absorption. The gel formation was assessed in triplicate samples and expressed as the difference between absorbance of the THP solution and blank, bovine serum albumin (80 μg/mL) in the same experimental solution (ΔUV_{280}). Symbols are: (□) M buffer; (■) ATN-S; (▨) DCT-S; (▩) CD-S; ***$P < 0.001$ vs. M buffer and DCT-S; $N = 8$ for each experimental solution.

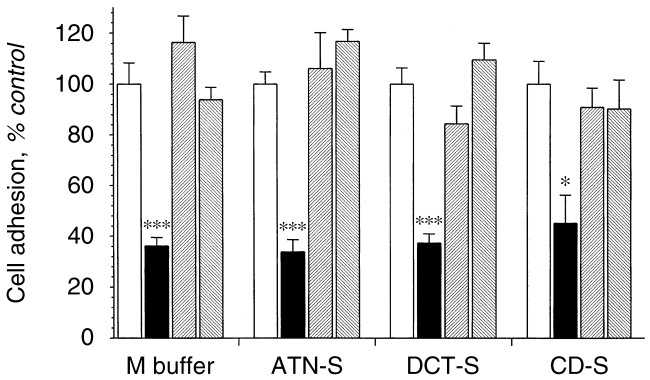

Fig. 4. Boiling and digestion by N-glycanase abolished the effect of THP on LLC-PK$_1$ cell adhesion to collagen type-I. The experiments were performed with no THP (□), or 80 μg/mL of either the THP concentrate (THP; ■), boiled THP (bTHP; ▨), or *N*-glycanase digested THP (dTHP; ▧) in each experimental solution as described in the **Methods** section. Cell adhesion was assessed in triplicate wells and expressed as percentage Control. *$P < 0.05$; ***$P < 0.001$ vs. no THP, bTHP, and dTHP; $N = 6$ for each experimental solution.

adherent cells were collected by aspiration and centrifugation and were then washed twice with calcium-free PBS. Adherent cells were trypsinized and collected, and then trypsin/EDTA inactivated as described previously in this article. Both nonadherent and adherent cells were stained with 0.4% trypan blue and counted.

Statistical analysis

The results were analyzed by using either Student *t* test or analysis of variance (ANOVA) and Tukey's multiple comparison post-test (Prism 3.00; GraphPad Software, San Diego, CA, USA). A *P* value of less than 0.05 was considered statistically significant. Data are presented as means ± SEM.

RESULTS

Direct adhesion of LLC-PK$_1$ cells to THP or collagen

When LLC-PK$_1$ cells were allowed to adhere to culture plates whose wells were precoated with THP, the resultant adhesion (0.87 ± 0.11%, $N = 7$) was not different from the negative control (HSA, 0.44 ± 0.03%, $N = 7$) as compared with considerable LLC-PK$_1$ adhesion to collagen (48.8 ± 2.8%, $N = 7$). Moreover, adhesion of the cells to THP was not increased by increasing the concentration of THP in the coating solution to 400 μg/mL.

Polymeric THP, not mTHP, formed a gel and this effect was abolished either by boiling or removal of oligosaccharide residues (Fig. 3).

The THP concentrate (THP, 80 μg/mL) formed a gel when exposed either to the ATN-S, or CD-S (Fig. 3). In contrast, THP did not form a gel when exposed to either the M buffer or DCT-S ($P < 0.001$ for both ATN-S and CD-S vs. M buffer and DCT-S, $N = 8$). Denaturation of THP by boiling (bTHP) or removal of its oligosaccharide residues after *N*-glycanase enzyme digestion (dTHP) abolished THP gel formation in all solutions (P = NS for all solutions, $N = 8$; Fig. 3).

Effect of THP on LLC-PK$_1$ cell adhesion to collagen

Tamm-Horsfall protein (80 μg/mL) attenuated LLC-PK$_1$ cell adhesion to collagen to about 30 to 40% of control in all solutions ($P < 0.001$ in M buffer, ATN-S, and DCT-S; $P < 0.05$ in CD-S; Fig. 4). Moreover, THP (80 μg/mL) had no effect on cell viability, as assessed by trypan blue dye exclusion (data not shown). In addition, as shown in Figure 4, either heat treatment or oligosaccharide removal prevented this inhibitory effect of THP on LLC-PK$_1$ cell adhesion to collagen in all solutions (P = NS for either bTHP or dTHP compared to no THP, $N = 6$ each). The loss of the effect of THP on cell adhesion after the oligosaccharides were removed revealed the importance of the oligosaccharides for the effects of THP on tubular cell adhesion.

The inhibitory effect of THP on cell adhesion occurred with both monomeric (mTHP in M buffer or DCT-S) and polymeric (pTHP in ATN-S or CD-S) forms. To examine the mechanism of this effect, cell adhesion was determined in collagen-coated wells that were pretreated with either mTHP (THP, 80 μg/mL in M buffer or DCT-S) or pTHP (THP, 80 μg/mL in ATN-S and CD-S) and washed, as described in the **Methods** section. As shown

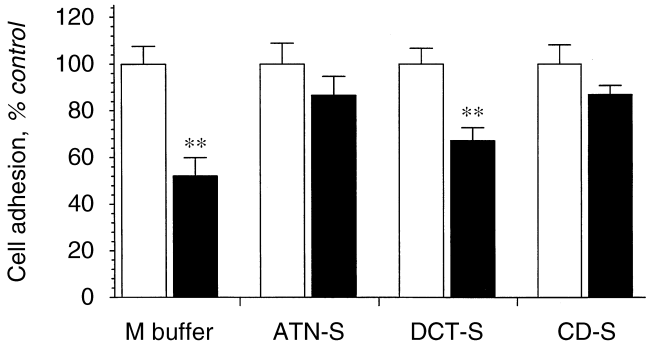

Fig. 5. Pretreatment of collagen-coated wells with mTHP, but not pTHP, blocked LLC-PK$_1$ cell adhesion. Collagen type I–coated wells were pretreated with THP concentrate (80 μg/mL) in each experimental solution without LLC-PK$_1$ cells for 90 minutes. After the wells were washed, LLC-PK$_1$ cells in the same solution, without THP, were allowed to adhere to the collagen as described in the Methods section. Cell adhesion was assessed in triplicate wells and expressed as percentage Control. Symbols are: (□) no THP; (■) THP; $^{**}P < 0.01$ vs. no THP incubated to collagen type I-coated wells; $N = 6$ for each experimental solution.

in Figure 5, cell adhesion was attenuated only by pretreatment of the collagen with mTHP (M buffer or DCT-S, $P < 0.01$ each vs. control, $N = 6$ for each experimental solution). In contrast, cell adhesion was not attenuated by pretreatment of the collagen with pTHP (in ATN-S or CD-S, P = NS for both ATN-S and CD-S, $N = 6$ for each experimental solution). These results indicated that mTHP, but not pTHP, bound to the collagen and interfered with subsequent cell-matrix contact. In contrast, the pTHP gel did not bind to the collagen, was removed by washing and thus did not decrease subsequent LLC-PK$_1$ cell adhesion.

Microscopic examination of nonadherent cells

After each cell adhesion experiment, the solution of cells that had not adhered was gently removed (before washing and quantitating the adherent cells). This solution was examined microscopically. In every experiment, microscopic examination revealed that in high ionic solution where THP existed as a gel, the nonadherent cells were seen floating in suspension without cell–cell contact. In contrast, in the low ionic strength solutions, the nonadherent cells remained in the well, having settled on top of the matrix and adherent cells (these cells would be subsequently washed off before quantitation of adherent cells).

Effect of pTHP on LLC-PK$_1$ cells in suspension

LLC-PK$_1$ cells were suspended in solution with or without pTHP gel. After 20 minutes, cells could be visualized microscopically in suspension only in the pTHP solution. Figure 6 shows the time course of this effect determined by absorbance readings as described in the **Methods** section. When no pTHP was present, the cells quickly settled out of solution, whereas in the presence of pTHP, the cells settled at a much slower rate, indicating that they were hindered or entrapped by the pTHP gel.

DISCUSSION

Jean Oliver dissected nephrons from deceased patients with the clinical syndrome of ATN [62, 63]. He found an increased frequency of casts in the medullary collecting ducts. Since thousands of nephrons empty into a single collecting duct, tubular obstruction was suggested to be involved in the pathogenesis of ATN. Subsequent experimental rat studies using micropuncture technique confirmed increased intratubular pressures after acute renal ischemia [64–66]. The tubular casts after renal ischemia were shown to consist not only of tubular epithelial cells but also THP [13].

Since many of the tubular epithelial cells which are shed into the lumen with renal ischemia are still viable [43] and have integrins that have redistributed from the basolateral to apical surfaces [67], the suggestion was proposed that cell–cell adhesion may play a role in the intraluminal formation of casts. Cell matrix adhesion also becomes a possibility since increased luminal fibronectin was also found after a renal ischemic insult [68]. Further support for a role of adhesion molecules, particularly integrins, was found when either antibodies or antisense oligonucleotides against intercellular adhesion molecule-1 or intercellular adhesion molecule-1–deficient mice were found to be protective against renal ischemic injury [69–74]. Moreover, synthetic cyclical RGD molecules were found to normalize the increased intratubular pressure in an experimental model of rat renal ischemia [45]. This observation suggested that these adhesive amino acid sequences were involved in the intraluminal cast formation and tubular obstruction. Human THP is known to possess both RGD and LDV adhesive amino acid sequences [26] and, as already noted, THP is a consistent component of tubular casts after a renal ischemic insult.

The present in vitro study therefore was undertaken to assess whether THP may play an active or passive role in cell adhesion and thus tubular cast formation. LLC-PK$_1$ cells were used since they are of proximal tubule origin [75], the major nephron site of renal ischemic injury [76]. The adhesion of these cells to THP was initially examined. These experiments demonstrated very little or no direct adhesion of LLC-PK$_1$ cells to THP, thus providing evidence against integrin-mediated binding as a mechanism of tubular epithelial cell/THP cast formation. In contrast, LLC-PK$_1$ cells adhered strongly to collagen-type I.

Since LLC-PK$_1$ cells did not directly bind to THP-coated plates, we postulated that the polymerization state of THP may affect its ability to trap cells and thereby contribute to cast formation in vivo. To test this

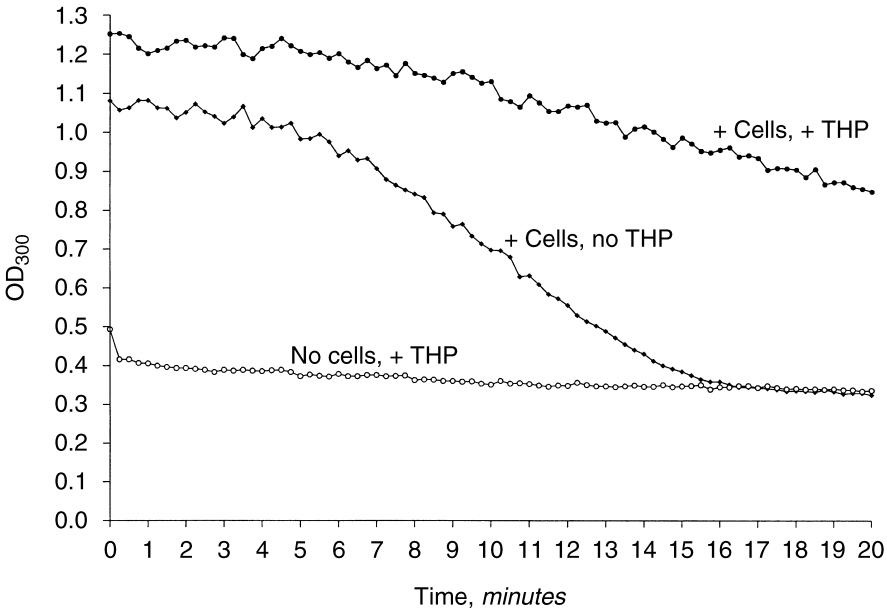

Fig. 6. Time course of effect of pTHP on LLC-PK$_1$ cells in suspension. LLC-PK$_1$ cells were suspended in solution with (+ cells, + THP) or without (+ cells, no THP) pTHP (80 μg/mL). As the cells were allowed to settle, absorbance readings were taken over 20 minutes as an index of number of cells remaining in solution. The pTHP solution without cells (no cells, + THP) served as control.

hypothesis, we examined the polymerization state (gel formation) of the THP concentrate in different solutions, the composition of which mimicked different nephron sites, that is, distal convoluted tubule, collecting duct or ATN urine, as well as a solution where THP was in monomeric form. It was confirmed that THP formed a gel in the high ionic strength ATN-S and CD-S solutions, whereas it did not form a gel in the lower ionic strength DCT-S or M buffer. This gel forming property of pTHP was abolished by either boiling or removal of its oligosaccharide component.

In the next experiments, we examined whether the polymerization state of THP had an effect on the ability of THP to prevent cell-matrix adhesion of LLC-PK$_1$ cells to collagen. If THP in gel form trapped the cells in suspension, then it should prevent the cells from contacting and adhering to the collagen, while the monomeric form of THP should not have this effect. Unexpectedly, however, the THP concentrate was found to attenuate LLC-PK$_1$ cell adhesion to collagen in all of the solutions, that is, both monomeric and polymeric forms, in the absence of any effect on cell viability. Moreover, the inhibitory effect of THP on LLC-PK$_1$ cell adhesion was abolished by either boiling or removal of the oligosaccharide residues. Thus, the effect of THP on cell adhesion appeared to be an active process, dependent on the adhesive properties of its oligosaccharides. Since the RGD and LDV sequences should be intact with the enzymatic removal of oligosaccharides, it would appear in the present in vitro setting that the adhesive properties of the oligosaccharides are important for the effects of THP on tubular cell adhesion and gel formation.

To examine why cell adhesion was attenuated by both polymeric and monomeric forms of THP, experiments were performed in which the collagen coated wells were pretreated with either mTHP (in M buffer or DCT-S) or pTHP (in ATN-S and CD-S) and then the THP was washed away. Subsequent cell adhesion measurements demonstrated that cell adhesion was attenuated only by pretreatment of the collagen with mTHP, not by pretreatment of the collagen with pTHP. These results indicated that mTHP, but not pTHP, had bound to the collagen, possibly forming a film that interfered with subsequent cell-matrix contact. In contrast, the pTHP gel apparently did not bind to the collagen and was removed by washing. Thus, pTHP pretreatment of the collagen-coated wells did not attenuate subsequent LLC-PK$_1$ cell adhesion.

In the cell adhesion studies, microscopic examination of the nonadherent cell solution demonstrated that whenever THP existed as a gel, the nonadherent cells were seen floating in suspension without cell-cell contact. We therefore measured the settling rate of LLC-PK$_1$ cells suspended in pTHP. This experiment demonstrated that when no pTHP was present, the cells quickly settled out of solution, whereas in the presence of pTHP, the cells settled at a much slower rate, indicating that they were hindered or entrapped by the pTHP gel.

In conclusion, renal tubular LLC-PK$_1$ cells did not directly adhere to THP, thus providing evidence against integrin-mediated binding as a mechanism for in vivo tubular cell/THP cast formation. The high electrolyte concentrations of ATN-S and CD-S, however, were associated with pTHP gel formation. Moreover, cells suspended in pTHP tended to remain in suspension. In cell adhesion studies, mTHP attenuated cell adhesion by binding to the matrix, whereas pTHP attenuated cell

adhesion by trapping cells in its gel. Both the gel formation and attenuation of cell adhesion by pTHP were abolished by boiling or oligosaccharide removal. The present in vitro results therefore favor the importance of the oligosaccharide residues in mediating the effect of the polymeric THP on gel formation, cell trapping and thus potential cast formation in ATN.

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APPENDIX

Abbreviations used in this article are: ATN, acute tubular necrosis; ATN-S, solution mimicking urine from ATN; BSA, bovine serum albumin; bTHP, heat treated (boiled) THP concentrate; CD, collecting duct; CD-S, solution mimicking urine from the collecting duct; DCT, distal convoluted tubule; DCT-S, solution mimicking urine from the distal convoluted tubule; d-THP, digested Tamm-Horsfall protein; EDTA, ethylenediamine tetraacetate; HAS, human serum albumin; LDV, Leucine-Aspartate-Valine adhesive sequence; LLC-PK_1, porcine renal proximal tubular epithelial cells; mTHP, monomeric Tamm-Horsfall protein; PBS, phosphate-buffered saline; pTHP, polymeric Tamm-Horsfall protein; RGD, Arginine-Glycine-Aspartate sequence; sTHP, sham digested Tamm Horsfall protein; THP, Tamm-Horsfall protein.

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