Effect of Mouse and Human Tamm-Horsfall Protein on Adhesion of Uropathogens to Human Uroepithelial Cells

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Abstract

Tamm-Horsfall Protein (THP) is the normal, conserved protein excreted in mammalian urine. For experimental studies, mice are preferred animals. The present study was undertaken to compare effect of coating of human THP (hTHP) and mouse THP (mTHP) on uropathogens and uroepithelial cells (UECs). Three common species of bacteria associated with UTIs were employed in planktonic and biofilm cell forms. On the whole, effect of hTHP and mTHP was comparable. Biofilm cells of all the three strains adhered to UECs in higher numbers in comparison to their planktonic counterparts in all four set of experiments. Findings provide the basis for future *in vivo* studies to be carried-out in mice where relevant conditions matching with humans can be created.

Keywords: Tamm-Horsfall protein, biofilms, uroepithelial cells, uropathogens.

1. Introduction

Continuous flow of the urine and presence of antibacterial inhibitory factors in the bladder wall play a defensive role by entrapping and flushing uropathogens out with the flow of urine [1]. Tamm-Horsfall protein (THP; uromodulin) is the most abundant protein in urine of normal mammals [2], with a migration pattern at ~90KDa

in SDS-PAGE [3]. 70% of the THP molecule consists of polypeptide which is identical in different species and contains acidic amino acids [3]. Rest 30% consists of carbohydrates comprising of 5 N-glycosidically bound sugar chains [4]. THP is synthesized exclusively in epithelial cells of the thick ascending limb of loop of Henle (TAL) and in distal convoluted tubules (DCT) [5], with exception of macula densa [5, 6].

It is membrane bound via a glycosyl phosphatidylinositol (GPI) anchor [7], mainly localized at the apical plasma membrane of epithelial tubular cells [6]. THP is excreted in the urine at the rate of ~50mg/day but this amount can be influenced by many physiological as well as pathological factors which include urine volume, diet, exercise, pregnancy, diabetes melitus and TIN (tubulointerstitial nephritis) [8]. Because of its abundance, species conservation, and unique nephron association, THP is believed to play a critical role in urinary physiology as well as in defending the urinary tract against the bacterial infections [9].

The protective capability of THP against UTI has also been related to its concentration [10]. At physiological concentrations, THP completely abolishes the binding of type I fimbriated *Escherichia coli* to uroplankins, putative urothelial receptors [11]. THP facilitates organism's elimination from the urinary tract by coating the adhesins present on the cell surfaces [11]. Thus THP can serve as a potentially defensive factor in the urinary tract especially against type I fimbriated uropathogenic *E. coli* which is the most common organism to cause uncomplicated UTIs [12]. *Pseudomonas aeruginosa* is the third most common urinary pathogen. 35% cases are associated with indwelling catheter induced UTIs caused by this organism [13]. Less commonly other enteric gram negative bacterial species like *Klebsiella pneumoniae* has been reported to be causing UTIs [14]. Some workers have suggested a dual role of THP, since it is also found to be present in the coated form on renal tubular cell surface [15] which mediates attachment of the invading organisms at the renal level indicating that THP at the level of epithelial cells has a defensive role while presence of THP at the renal level may facilitate induction of infection. Precise role, however, of THP is yet to be defined in cases of infections caused by microbes especially in relation to complicated UTIs.

Most of the reported work has been carried out *in vitro*, using hTHP. However, results of these *in vitro* studies with hTHP have been compared with the establishment of *E. coli* induced infection in the mouse model *in vivo* where interaction of bacteria takes place in the

milieu containing mTHP. The present investigation was therefore planned to compare the effect of THP from both sources (human and mouse) on adhesion of three well recognized uropathogens in planktonic and biofilm cell forms *in vitro*. Availability of the information obtained from present study will have bearing on the interpretation of the results of *in vivo* studies for which mice are employed [16].

2. Materials and Methods

Organisms

Standard strains of *E.coli* (31-B), *P. aeruginosa* (PAO) and *K. pneumoniae* (B 5055) were used in this study. *E. coli* 31-B strain was obtained from Dr. T. Iwahi, Takeda Chemicals Ltd, Osaka, Japan. *P. aeruginosa*, PAO strain was obtained from Dr. Barbara H. Iglewski, University of Rochester, New York (U.S.A.). *K. pneumoniae*, B5055 strain was obtained from Dr. Mathia Trautman, University of Ulm, Germany. All the strains were maintained on nutrient agar slabs at 4°C (pH 7.2) and in glycerol stocks at -70°C.

Isolation and purification of Tamm-Horsfall protein from normal human and mouse urine

For preparation of human THP, 1 liter of normal urine was pooled from 6 healthy laboratory workers and refrigerated. For preparation of mouse THP, urine of 30 normal LACA mice, 6-8 weeks old, weighing 25 ± 5 gm (Central Animal House, Panjab University, Chandigarh) was collected. Animals were kept in clean polypropylene cages. Feed (Hindustan Levers Pvt. Ltd.) and water was given *ad libitum*. Pooled urine was refrigerated till 50 ml of normal urine was collected.

THP was precipitated by adding 0.58M NaCl according to Hoyer and Seiler [17] and dialyzed to remove the NaCl [18]. Purification was done by the method of Geerlings *et al* [19]. Protein content of THP was estimated by method of Lowry *et al.* [20].

For coating, 50µg/ml THP was employed based on earlier standardization done in our laboratory using microtiter well binding assay, which was within the physiological range found in normal human urine [21].

Collection of human uroepithelial cells

Uroepithelial cells were collected from the pooled morning human urine samples of 4 healthy females. UECs were pelleted by centrifuging urine at 2000 rpm for 15 minutes at 4 °C and washed thrice with PBS (pH 7.2). Number of UECs was adjusted to 10⁵ cells/ml using haemocytometer after staining with 0.01% Trypan blue [22].

Generation of Planktonic bacterial cells

Single colony of the organism was inoculated in nutrient broth and overnight incubation was done at 37 °C under shaking conditions. Bacterial cells after giving three washings with PBS (pH 7.2) were pelleted at 5000 rpm for 10 min.

Generation of Biofilm bacterial cells

Biofilms were generated on Foley's catheter (Bardia) following original method of Ladd *et al.* [23] as adopted by Yadav *et al.* [24] and Mittal *et al.* [25] in our laboratory.

Preparation of THP coated bacterial suspensions

All the three organisms were coated as employed earlier in the laboratory by Harjai *et al.* [21]. Briefly, one ml of bacteria (biofilm and planktonic cell forms, 10⁸ CFU/ml) was added to 1 ml of THP (50 µg/ml) and incubated at 37°C for 1 h. Bacterial cells were pelleted at 5000 rpm for 10 min after giving three washings with PBS (pH 7.2).

Preparation of THP coated UECs suspensions

One ml of UECs (10⁵ UECs/ml) were added to 1 ml of THP (50 µg/ml) and incubated at 37°C for 1 h. Following incubation, uroepithelial cells were pelleted by centrifugation at 3000 rpm for 10 min after 3 washings with PBS (pH 7.2).

Uroepithelial cell adhesion assay

Based on the original method of Svanborg-Eden *et al.* [26] as adopted by Sharma *et al.* [27] and Harjai *et al.* [21] was employed. UEC adhesion experiments were performed in four different sets as given below:

Set I (Control): Uncoated bacteria (UB) + Uncoated uroepithelial cells (UU)

Set II: Coated bacteria (CB) + Uncoated uroepithelial cells (UU)

Set III: Uncoated bacteria (UB) + Coated uroepithelial cells (CU)

Set IV: Coated bacteria (CB) + Coated uroepithelial cells (CU)

In the adhesion assay, mixture consisted of 1 ml each of bacteria (coated/uncoated) and UECs (coated/uncoated) as given above, in the four sets were carried out. These were then incubated at 37°C for 1 h in a shake water bath. Three washings with PBS were given to remove any unattached bacteria. Smears were prepared, fixed with methanol and stained with Giemsa stain. Bacteria adhering to 30 UECs were counted and the average number of bacteria adhering per UEC was calculated. These were carried out in triplicate in two sets for each strain and the variable of the experiments.

3. Results

THP was purified from normal human and mouse urine. Protein content of hTHP and mTHP was found to be 900 µg/ml and 600 µg/ml respectively by Lowry's method [20]. The molecular weight of hTHP and mTHP was found to be 90KDa and 85KDa respectively by SDS-PAGE electrophoresis (data not shown).

In the adhesion experiments carried out with standard strains in planktonic and biofilm cell forms, they were harvested at different time intervals (24h growth of planktonic cells, 1-10 days old biofilm cells of *E. coli*, *K. pneumoniae* and *P. aeruginosa*). In UEC adhesion cell assay with biofilm cells, it was observed that maximum adherence was shown by 3 day old biofilm cells of *E. coli* and *K. pneumoniae* whereas in case of *P. aeruginosa*, 4 day old biofilm cells showed maximum adherence (Fig. 1). For final experiments therefore, 3 day old biofilm cells of *E. coli*, *K. pneumoniae* and 4 day old biofilm cells of *P. aeruginosa* were coated with 50 µg/ml of hTHP / mTHP. Further, uroepithelial cell adhesion assay was carried out in different set of experiments.

Fig. 1. Adherence pattern of the biofilm cells of three uropathogens on day 1-10

Fig. 2. Comparative peak adhesive ability of planktonic cells of the three uropathogens to UECs in presence of hTHP and mTHP

Fig. 3. Comparative peak adhesive ability of biofilm cells of the three uropathogens to UECs in presence of hTHP and mTHP

Legend

- * *Escherichia coli* (Control)
- ** *Pseudomonas aeruginosa* (Control)
- *** *Klebsiella pneumoniae* (Control)

Fig. 4. Comparative peak adhesive ability of planktonic cells of three uropathogens to UECs in four sets

Legend

- * *Escherichia coli* (Control)
- ** *Pseudomonas aeruginosa* (Control)
- *** *Klebsiella pneumoniae* (Control)

Fig. 5. Comparative peak adhesive ability of biofilm cells of three uropathogens to UECs in four sets

Results of four different sets of experiments employing coated vs uncoated bacteria and UECs are shown in Fig. 2, 3. In all the four sets, results of adhesion experiments employing hTHP and mTHP were comparable for standard strains of *E. coli*, *P. aeruginosa* as well as *K. pneumonia*.

Fig. 6. Adherence of uncoated planktonic cells to uncoated UECs

Fig. 7. Adherence of uncoated biofilm cells to uncoated UECs

Pattern of adhesive ability of the organisms in both cell forms to UECs with hTHP and mTHP was similar (Fig. 4, 5). In comparison to planktonic cells, biofilm cells were found to be significantly more adherent to UECs in all four sets of the experiment with hTHP as well as mTHP (Fig. 6, 7). When bacterial cells were coated with hTHP/mTHP (Set II), maximum

decrease in adherence was observed (Fig. 8, 9) in case of all three Gram negative organisms in comparison to control (Set I). When UECs were coated with THP, the number of bacteria attaching were found to be in between the results obtained with Set II and Set IV (Fig. 10). On other hand, when assay was performed with both bacteria and UECs coated with hTHP/ mTHP (Set IV), minimum decrease in adherence was observed (Fig. 11) in case of all the three uropathogens.

Fig. 8. Adherence of THP coated planktonic cells to uncoated UECs

Fig. 9. Adherence of THP coated biofilm cells to uncoated UECs

Fig. 10. Adherence of uncoated planktonic cells to THP coated UECs

Fig. 11. Adherence of THP coated biofilm cells to THP coated UECs

4. Discussion

High degree of cross-species conservation has been observed in THP gene sequence studied in mice, rats, cattle, and humans [28]. Mouse and rat sequences are 90% identical, while humans and cattle are 75% identical, and those from mice and humans are only 66% identical [29]. Human and mouse THP were compared *in vitro* by Prasad *et al.* [30] on the basis of genetic analysis of constructed cDNA fragments, they found 77% homology between human and mouse THP. These two THPs have also been found to be similar on the basis of SDS-PAGE analysis except for the difference of 5Kda molecular weight between hTHP (85Kda) and mTHP (90Kda) [11]. Like its human counterpart, mouse THP expression is also

kidney tissue specific since THP gene is expressed in the mouse kidney only and not in any other epithelial or mesenchymal tissues [29]. *In vivo* studies, defensive role of THP has been reported for type I fimbriated *E. coli*. These studies are carried out in mice using two different approaches like genetic ablation (THP^{-/-}) [31] and by employing uromodulin (umod) knock out mice [9]. Results of these studies brought out that mTHP plays protective role against infection with *E. coli* [9,31] which have also been found to be applicable in case of *Staphylococcus saprophyticus* [32].

Attachment of bacteria to eukaryotic cells involves specific molecular interactions between distinct adhesins and receptors on the surface of epithelial cells [33]. Interactions between mucin and bacteria at mucosal surfaces are also important for bacterial colonization. Mucin found in saliva and on teeth is shown to bind oral bacterial flora [34], while gut mucus binds to the bacteria of gastrointestinal tract [19]. Similarly, human tracheobronchial mucin (HBM) has been shown to entrap respiratory pathogens [35]. Since THP forms the integral part of urinary mucus, all the invading pathogens in the urinary tract thus come in contact with THP. In the present study, adhesion assay carried out for both cell forms of three uropathogens has shown that effect on adhesion was comparable with hTHP as well as mTHP in all the four sets employed. Literature available for comparison is only in relation to only most common uropathogen *E. coli*. Type-I fimbriated *E. coli* has been reported to bind hTHP because of glycoprotein content which contains mannose serving as receptor [36]. *E. coli* 31B strain employed in the present study also possessed mannose sensitive type-I fimbriae. Microtiter well assay carried out by Pak *et al.* [11] showed that type-I fimbriated *E. coli* bound equally well to both hTHP and mTHP indicating that high mannose glycosylation of THP is conserved through evolution. *P. aeruginosa* lectins PA-IIL and PA-IIIL resembling P-fimbriae and type I fimbriae of *E. coli* respectively [37] have been shown to bind avidly to the THP coated renal tubular cells which secrete THP [15]. However, precise factor responsible for affecting the interaction is yet to be defined in case of *P.*

aeruginosa and *K. pneumoniae*. *In vitro* effect of mTHP on adhesion to UECs by other uropathogens has neither been studied nor has been compared with that of hTHP.

In the present study, adhesion experiments were carried out in 4 different sets using hTHP and mTHP. Maximum decrease in adherence was observed when THP coated bacterial cells interacted with UECs followed by interaction of bacterial cells with THP coated UECs. In the matching experiments carried out where THP coated bacterial cells and THP coated UECs were interacted, the fall in adherence was least. Adherence went down by 44.97%, 24.66% and 23.44% for *E. coli*, *P. aeruginosa* and *K. pneumoniae* respectively. This condition is closer to the clinical situation present in the host. Overall effect was similar with mTHP and hTHP in planktonic and biofilm cell forms of three selected organism. However, significant increased adherence potential of biofilm cells as compared to planktonic cells was observed which is in accordance with the results of Yadav *et al* [24]. These workers compared the ability of planktonic and biofilm cell forms of *P. aeruginosa* to uroepithelial cells without coating bacteria or UECs. The study of Harjai *et al.* [21] was closer to one employed in this study but employed coating of *P. aeruginosa* in both cell forms only with hTHP. They reported decreased adherence of hTHP coated planktonic cells. They further assessed effect of hTHP coating on virulence potential of planktonic and biofilm cells of *P. aeruginosa* in mouse model of urinary tract infection *in vivo*. Interestingly, with both cell forms coated with hTHP, pathogenicity was enhanced which was higher with biofilm cells of the organism.

Mouse is a well accepted model for establishment of upper as well as lower UTIs [38,9]. It is on the basis of genetic receptor similarity with humans [30] as well as due to matching binding ability of type-I fimbriated *E. coli* to human and mouse THP [11]. Present study demonstrates that three species of Gram negative uropathogens adhere equally well to UECs in presence of both hTHP and mTHP. Usefulness of mice for *in vivo* studies in relation to UTI is thus strengthened since mTHP and hTHP match in

affecting adherence of human uropathogens to UECs which is an important step in initiation of lower as well as upper urinary tract infections. This can thus help to study the effect of physiological and pathological variables which can be experimentally created in the mice. The available information can thus form the basis for further extrapolation, *in vivo* in mouse model results of which can be meaningfully extrapolated to humans.

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