Role of class II P fimbriae and cytokine response in the pathogenesis of *Escherichia coli* kidney infection in diabetic mice

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Abstract  Background: The role of class II P fimbriae (P fimbriae II) in diabetic kidney infections is uncertain, although some genetic and epidemiological studies suggest a lower prevalence of P fimbriae II genes in *Escherichia coli* strains isolated from diabetic patients with complicated kidney infections.  Methods: We inoculated a P fimbriae II deficient *E. coli* (DH5\textregistered T) or an isogenic P fimbriae II expressing transformant (DH5\textregistered TP) into the bladders of diabetic and non-diabetic BALB/C mice, and sacrificed them after 3 days. The incidence of bladder or kidney infection (\textgeq 10\textsuperscript{3} CFU of *E. coli* per bladder or kidney), bacteremia (\textgeq 10\textsuperscript{2} CFU of *E. coli* on blood culture plate), kidney pathological score, immunoreactive Histo-score (H-score), and corrected H-score (H-score adjusted for Log\textsubscript{10} CFU of bacteria in the kidney) were compared among groups.  Results: Diabetic mice were more susceptible to bladder infection than non-diabetic mice with both transformants. The geometric mean of bacteria counts in kidneys was significantly
increased only when the diabetic mice were infected with DH5αTP. Among the 4 groups of mice, diabetic mice infected with DH5αTP had the highest incidence of kidney infection and bacteremia, and the highest renal pathology scores. The IL-8 H-score and the corrected IL-6 and IL-8 H-score were significantly lower in diabetic than non-diabetic mice.

Conclusion: We concluded that P fimbriae II contribute to the pathogenesis and severity of E. coli kidney infections in diabetic mice. An impaired cytokine response may also contribute to the increased incidence and severity of kidney infections in diabetic hosts.

Methods

Ethics statement

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC Approval No: 100050) of National Cheng Kung University and all methods were performed in accordance with the relevant guidelines and regulations.

Bacterial strains, plasmids transformation, and testing for P- and type 1 fimbrial phenotypes

Non-pathogenic E. coli DH5α expresses type 1-fimbriae, but is deficient in P-fimbriae and lacks most virulence genes. The pACYC184 (GenBank/EMBL accession number X06403)-derived plasmid pPIL110-35 (kindly provided by Dr. James R Johnson, University of Minnesota), carries a 16-kb EcoRI fragment from the AD110 pap gene cluster encoding class II P-fimbriae.

Bacterial transformations were carried out according to Kushner. The strain transformed with pACYC184 (expressing only type 1-fimbriae) was designated as DH5αT, and the strain transformed with pPIL110-35 (expressing the type 1 and P fimbriae II) was designated as DH5αTP.

Expression of P fimbriae II was indicated by mannose-resistant hemagglutination to human types A1P1 and OP1 and sheep erythrocytes; expression of type 1 fimbriae was indicated by mannose-sensitive hemagglutination to guinea-pig erythrocytes.

Experimental animals

Six to eight week-old BALB/c female mice were rendered diabetic by intraperitoneal injection of Streptozotocin (STZ; Sigma Chemical Co., St Louis, USA) or carrier alone (non-diabetic controls) using a modification of the method of Rosen et al. In brief, mice were fasted for 4 h, and then given 200 mg of STZ per kg of body weight by intraperitoneal injection. Non-fasting blood glucose levels were measured daily beginning 5 days after STZ injection by monitoring glucose level in venous blood drawn from the tail (Lifescan, Inc., Milpitas, Calif). A mouse was considered diabetic after two consecutive readings greater than 250 mg/dL.
Mouse model of ascending UTI

The mouse model of ascending UTI was based on that of Johnson and Brown21 with modification.22 Female diabetic (n = 20) or non-diabetic (n = 20) mice were anesthetized with intraperitoneal pentobarbital. A polyethylene catheter was inserted through the urethral orifice into the bladder until resistance was felt, and then withdrawn several mm. Then 50 μL of bacterial suspension (5 \times 10^8 CFU/mL of E. coli) was inoculated via the catheter. Mice were sacrificed 3 days after bacterial challenge. Upon killing, the mouse blood was aspirated, and the left kidney and bladder were removed and homogenized aseptically for quantitative culture. Infection of the kidney or bladder was defined as at least 10^3 CFU of E. coli per organ. Bacteremia was defined as at least 10^2 CFU of E. coli on blood culture plate.

Renal pathology

The right kidney of diabetic mice infected with DH5αTP or DH5αT were sectioned through the central region to view the papilla, medula, and cortex. Sections were stained with hematoxylin and eosin and evaluated by a blinded pathologist. This pathologist graded the severity of renal pathology using a scoring system in which 0, 1, 2, and 3 indicated normal, mild, moderate, and severe pyelitis, respectively; and 4 and 5 indicated mild to moderate, and severe pyelonephritis, respectively.23 The mean pathological score is presented for each group.

Immunohistochemistry

A modified version of a previously described IHC protocol was used.24 Formalin-fixed, paraffin-embedded kidney sections (5 μm thick) were de-waxed and treated with ImmunoRefriger Cite (Bio SB Inc., Santa Barbara, CA, USA) in a pressure cooker for 15 min at 100 °C. Endogenous peroxidase activity was suppressed by incubating sections in a de-peroxidase reagent (Bio SB Inc., Santa Barbara, CA, USA) at room temperature for 10 min. All IHC experiments used mouse-generated primary antibodies at a dilution of 1:50 for anti-IL-6, IL-8, and 1:50 for anti-TNF-α (GeneTex Inc., Irvine, CA, USA), 1:250 for anti-IL-8 (GeneTex Inc., Irvine, CA, USA), and 1:50 for anti-TNF-α (GeneTex Inc., Irvine, CA, USA). Kidney sections were labeled with a reagent, and stained with DAB substrate-choromogen solution using the mouse/rabbit PolyDetector HRP/DAB detection system (Bio SB Inc., Santa Barbara, CA, USA). All sections were then counterstained with hematoxylin. A section of mouse kidney was used as a positive control. A negative control was performed by incubation with 1% mouse serum without the primary antibody.

Immunohistochemical evaluation

The intensity of IL-6, IL-8 and TNF-α immunostaining was evaluated semiquantitatively using the following categories: 0 (no staining), 1+ (weak, but detectable, staining), 2+ (moderate or distinct staining), and 3+ (intense staining).25 The resulting Histo-score (H-score)25 was calculated by multiplying the intensity (I) by the percentage of positive tubule cells: H-score = (percentage of positive tubule cells \times I1+) + (percentage of positive tubule cells \times I2+) + ... + (percentage of positive tubule cells \times I3+). The number of bacteria attached to the renal tubules may correlate positively with the degree of tissue cytokine expression. Therefore, we also calculated a "corrected H-score", which is the ratio of the H-score to the bacteria count (log_{10} CFU).

Urinary levels of interleukin-6 (IL-6) and interleukin-8 (IL-8)

Urine were obtained at sacrifice of mice and were immediately centrifuged, separated, frozen and stored at −70 °C until they were tested in batches. IL-6 and IL-8 levels were measured by enzyme-linked immune sorbent assay (ELISA) using commercially available kits according to the manufacturer’s instructions (eBiotechnology, San Diego, California). Cytokine levels (pg/ml) were calculated from standard curve generated with recombinant cytokines. All samples were measured in duplicated by ELISA.

Statistics

Differences in the percentage of mice with bladder or kidney infections and bacteremia were compared by Fisher’s exact test or the \chi^2 test, as appropriate. Quantitative culture results were logarithmically transformed and compared using ANOVA for within-group comparisons and Student’s t-test for inter-group comparisons. The intensity of immunostaining and urinary levels of cytokines in diabetic and non-diabetic mice was compared by the Mann–Whitney U test. Data were expressed as means ± standard errors of the mean (SEM). All statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC, USA). A p value less than 0.05 was considered significant.

Results

Characterization of E. coli phenotypes

We used E. coli strain DH5αT, which expresses the MSHA phenotype but has no MRAH activity, and strain DH5αTP, which strongly agglutinates human A1P1, OP1, and sheep RBCs with and without mannose in the medium. Thus, DH5αT expressed only the type 1 fimbriae phenotype, but DH5αTP had the type 1 phenotype and the class II P fimbriae phenotype.

Virulence in the diabetic and non-diabetic BALB/c mouse model of ascending UTI.

Bladder infection

When challenged with E. coli DH5αT, only 20% of the non-diabetic mice had bladder infections by day 3, but 90% of the diabetic mice had bladder infections (≥10^3 CFU of E. coli per bladder) (Table 1). Similarly, when challenged with E. coli DH5αTP, only 10% of the non-diabetic mice had
bladder infections by day 3, but all the diabetic mice had bladder infections.

Further analysis indicated that the geometric means of bladder bacterial counts were significantly greater in diabetic than non-diabetic mice following infection by DH5αT and DH5αTP (Table 1). Although diabetic and non-diabetic mice had different responses, DH5αT and H5αTP had similar effects on bladder infection and bladder bacterial counts within each group (Table 1 and Fig. 1a).

Kidney infection
When challenged with E. coli DH5αT, neither the diabetic nor the non-diabetic kidneys were infected by day 3 (Table 1). However, when challenged with DH5αTP, 20% of the non-diabetic mice had infected kidneys (≥10^1 CFU of E. coli per kidney) and all the diabetic mice had infected kidneys. The geometric mean bacterial count in DH5αTP-infected diabetic mouse kidneys was significantly higher than those in the other three groups (Fig. 1b).

Bacteremia
There was no bacteremia (≥10^2 CFU on blood culture plate) by day 3 in non-diabetic mice challenged with E. coli DH5αT or DH5αTP (Table 1). In contrast, bacteremia occurred in 80% of the diabetic mice infected with DH5αTP and in 10% of the diabetic mice infected with DH5αT (DH5αT vs. DH5αTP, P = 0.006) (Table 1).

Renal pathological findings and pathological scores in diabetic mice
When inoculated with E. coli DH5αT, 2 of the diabetic mice showed no inflammation in the renal pelvis and parenchyma, but the other 8 exhibited inflammatory cells beneath the epithelial lining of the pelvic cavity (pyelitis, arrows in Fig. 2a). In contrast, infection of diabetic mice with DH5αTP led to destruction of kidney parenchyma tissue architecture (within the arrowheads in Fig. 2b) and infiltration of inflammatory cells (arrows in Fig. 2b). High-power magnification of DH5αTP-infected kidneys from diabetic mice showed collections of rod-like bacteria in the renal parenchyma (arrows in Fig. 2c). The mean renal pathology score was significantly higher for diabetic mice inoculated with E. coli DH5αT than DH5αT (2.9 ± 1.9 vs. 1.1 ± 0.7, P = 0.009).

Immunohistochemistry of IL-6, IL-8, and TNF-α in mouse kidneys following DH5αTP infection
We also measured renal cytokine production following infection of diabetic and non-diabetic mice with DH5αTP.

IL-6. There was a weak expression of IL-6 in the renal tubules of diabetic mice (Fig. 3a), but greater expression in the renal tubules of non-diabetic mice (Fig. 3b). Quantification of these results by H-score indicated no significant difference (24 ± 6 vs. 65 ± 35, P > 0.05; Fig. 4a) or corrected IL-6 H-score (ratio of IL-6 H-score to log_{10} CFU in the infected kidney) was significantly lower in the diabetic than non-diabetic mice (4.8 ± 0.5 vs. 18 ± 0.1, P = 0.045; Fig. 4b).

IL-8. There was a weak expression of IL-8 in the renal tubules of diabetic mice (Fig. 3c), but greater staining in the renal tubules of non-diabetic mice (Fig. 3d). The IL-8 H-score was significantly lower in the diabetic than non-diabetic mice (25 ± 3 vs. 65 ± 25, P = 0.04; Fig. 4a) and the corrected IL-8 H-score was also significantly lower in the diabetic than non-diabetic mice (4.5 ± 0.5 vs. 18 ± 0.0, P = 0.04; Fig. 4b).

TNF-α. There was a weak-to-moderate but localized TNF-α staining in the renal tubules of diabetic mice (Fig. 3e), but stronger and more diffuse expression in the renal tubules of non-diabetic mice (Fig. 3f). However, there were no significant differences in the H-scores (20 ± 10 vs. 24 ± 5, P > 0.05; Fig. 4a) or corrected TNF-α H-scores (3.5 ± 0.5 vs. 4.4 ± 0.8, P > 0.05; Fig. 4b).

Discussion
The STZ-induced diabetic mouse model of UTI has many of the same characteristics as UTIs in humans with DM, and is therefore a powerful tool for examination of uropathogenesis in the presence of DM.

Table 1 Number and percentage of diabetic and non-diabetic mice that developed bladder infection, kidney infection, and bacteremia at 72 h after inoculation with E. coli DH5αT or DH5αTP.

<table>
<thead>
<tr>
<th>Infection site and mouse group</th>
<th>E. coli strains</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>DH5αT</td>
<td>DH5αT</td>
</tr>
<tr>
<td>n = 10</td>
<td>n = 10</td>
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<tr>
<td><strong>Bladder infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic mice</td>
<td>10 (100%) ^a</td>
<td>9 (90%) ^b</td>
</tr>
<tr>
<td>Non-diabetic mice</td>
<td>1 (10%) ^a</td>
<td>2 (20%) ^b</td>
</tr>
<tr>
<td><strong>Kidney infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic mice</td>
<td>10 (100%) ^c</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Non-diabetic mice</td>
<td>2 (20%) ^c</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Bacteremia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic mice</td>
<td>8 (80%) ^d</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Non-diabetic mice</td>
<td>0 (0%) ^d</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

^a For bladder infection in diabetic mice vs. non-diabetic mice infected with DH5αTP: P = 0.0001 or DH5αT: P = 0.006. ^b For kidney infection in diabetic mice vs. non-diabetic mice infected with DH5αTP: P = 0.0007. ^c For bacteremia in diabetic mice vs. non-diabetic mice infected with DH5αTP: P = 0.0001.
Previous research indicated that the orientation of a promoter on a 314-bp invertible element controls expression of type 1 fimbriae genes. More specifically, cystitis strains switch the invertible element to the "on" position, where it remains as infection progresses; pyelonephritis strains turn the switch to the "on" position early during infection, and then turn the switch "off". Both DH5αT and DH5αTP can express type 1 fimbriae and can cause bladder infections, but only the transformant with the class II pap gene cluster expressed P fimbriae II by switching "off" the invertible element, and this (or possibly by some other factors) allowed ascent into the renal pelvis and development of pyelonephritis.

We previously showed that P fimbriae II can enhance the early establishment (day 1 after inoculation) of E. coli infection in the kidney, but that kidney infection did not...
persist to day 3 in normal immunocompetent mice. In the present study, we also found that most of the bacteria were cleared from the kidneys of non-diabetic mice by day 3. Previous studies showed that P fimbriae II can activate receptor-bearing uroepithelial cells, and promote the release of IL-6 and IL-8. The strain DH5αTP (expressing P fimbrial II) triggers a sufficiently strong cytokine response so that it is cleared from the kidney tissues of normal mice, but not diabetic mice. In contrast, the kidneys of diabetic mice had elevated bacterial counts, and pathological examination of their infected kidneys showed significant accumulation of bacteria and tissue destruction.

Rosen et al. previously demonstrated that the effects of diabetes on the burden of UPEC (E. coli UTI89) in mouse kidneys was significantly greater in immunocompetent C3H/HeN mice than in C3H/HeJ mice, which harbor a mutation in the Toll-like receptor 4 (TLR-4) signaling domain. They therefore suggested that the defects in TLR-4-regulated innate immune factors may explain the increased colonization of UPEC in diabetic kidneys. In this study, we compared the cytokine response of diabetic and non-diabetic mice by direct measurement of IL-6 and IL-8 expression, which are the end-products of TLR4-mediated innate immunity and can be stimulated by the binding of P fimbrial adhesin II to its receptors on the surface of renal tubules. The H-score of IL-8 and the corrected H-score of IL-6 were lower in the infected kidneys of diabetic mice than non-diabetic mice, although these groups had comparable expression of TNF-α. This is compatible with the previous finding that P fimbriated E. coli triggered IL-6 and
IL-8, but not TNF-α. These findings imply that an impaired cytokine response may contribute to the increased incidence and severity of kidney infections in diabetic hosts. Geerlings et al. found that women with DM and asymptomatic bacteriuria (ASB) had lower urinary levels of IL-6 and IL-8 than non-diabetic women with ASB. Our present study also revealed lower urine levels of IL-6 and IL-8 of diabetic mice when compared with that of non-diabetic mice, although there was no statistical significance that may be due to small sample size.

In our model, the diabetic mice developed impaired cytokine responses soon after the onset of DM. Thus, it is possible that their lower cytokine production was the result of a cellular defect due to the altered cell environment in the kidney. A previous study demonstrated that high glucose-induced TGF-β1 production suppressed the immune response by inhibiting the endogenous production of cytokines, including IL-6. However, the results of other studies are inconsistent with this interpretation.

The present study also revealed that diabetic mice were more susceptible than non-diabetic mice to bladder infection with both the type 1-fimbriated strains DH5αT and DH5αTP. Geerlings et al. found that E. coli expressing type 1-fimbriae adhere better to the uroepithelial cells isolated from the urine of women with DM compared with those isolated from women without DM. Our result is consistent with epidemiological observations of an increased incidence of cystitis in diabetic patients.

In summary, our study demonstrated that the incidence of kidney infection and bacteremia and kidney pathological score were significantly increased only when the diabetic mice were infected with P-fimbriae II expressing E. coli. We also revealed a lower cytokine expression in the renal tubules of diabetic mice with kidney infection. We concluded that the P-fimbriae II contributes to the development and severity of E. coli infection in the kidneys of diabetic hosts. An impaired cytokine response to the P-fimbriated E. coli may be one of the mechanisms for the increased incidence and severity of kidney infections in diabetic hosts.

Conflicts of interest

All authors declare no conflicts of interest.

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References


