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Role of superoxide in renal scarring following infection by mannose-sensitive piliated bacteria

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Summary. The role of superoxide in scar formation following renal infection caused by mannose-sensitive (MS) piliated strains of bacteria was studied in the experimental pyelonephritis model using female Sprague-Dawley rats. The MS piliated strain stimulated renal scarring to a significantly greater extent than either the non-piliated or MR-piliated strain. Modulation of leukocytes by administering cyclophosphamide to induce neutropenia and colchicine to inhibit leukocyte migration was effective in preventing renal scarring. Treatment with superoxide dismutase during the early stage of infection was also effective in preventing scar formation. Finally, the production of superoxide by rat leukocytes was significantly larger following stimulation by MS piliated than either the non-piliated or MR piliated strains. These observations suggest that superoxide released from leukocytes plays a critical role in the development of renal scarring following a bacterial infection, especially by MS piliated strains.

Key words: Pyelonephritis – Renal scarring – Pathogenesis – *Serratia marcescens* – Piliation – Superoxide dismutase – Leukocytes

Renal scarring may be the end-stage of chronic pyelonephritis. It may also be related to renal insufficiency and renal hypertension. Such scarring is commonly found in the kidneys of patients with vesicoureteral reflux. While the mechanism of renal scar formation is poorly understood both clinically and experimentally, bacterial infection and/or inflammatory processes are thought to play important pathogenetic roles.

Many urinary pathogens bear pili on their surfaces for use in adhering to the urinary mucosa via specific receptors. Several kinds of pili have been described. Uropathogenic strains of *Escherichia coli* bear type 1 (common) pili and/or P pili [2]. Strains with type 1 agglutinate guinea pig erythrocytes in an MR fashion and have frequently been isolated from urine. In contrast, P-piliated *E. coli* aggutinate human erythrocytes in an MSR fashion and are thought to adhere to the urinary mucosa via P-specific glycolipid receptors. Other types of pili such as X and S have also been reported among uropathogenic *E. coli* [4].

Serratia marcescens is a common urinary pathogen in patients compromised with such urinary tract disorders as stones, tumors, catheters, or foreign bodies. Similar to *E. coli, S. marcescens* also possesses at least two types of pili, MS and MR [6]. We cloned the DNA gene for MS and MR pili from clinical isolate of *S. marcescens* (US 46), which bears both pili on its surface, and obtained new recombinant strains bearing either MS or MR pili. These strains were thought to differ only in their piliation – not in virulence or other characteristics [10].

Some investigators have reported a close relationship between the presence of bacterial piliation and the development of renal scarring. Using the recombinant strains mentioned, we reported previously that the MS-piliated bacteria stimulated renal scarring to a greater extent than either the non-piliated or MR-piliated bacteria [7].

Bille and Glauser [1] reported that the modulation of leukocytes was effective in preventing renal scarring, administering colchicine to inhibit leukocyte migration and cyclophosphamide to induce neutropenia. We previously reported the prevention of renal scar formation by superoxide dismutase (SOD), a specific scavenger of superoxide [8]. Our findings suggested that superoxide plays an important role in the pathogenesis of renal scar formation following kidney infection. In this experiment, we studied the role of superoxide released from leukocytes, which had been stimulated by MS-piliated (both wild and recombinant) strains of bacteria, on renal scar formation in the rat kidney.

Materials and methods

Bacteria

The US 46 strain of *Serratia marcescens* was isolated from a patient with chronic urinary tract infection. This strain has two types of pili, MS and MR, on its surface as identified by the hemagglutination (HA) test and electron microscopy. Two recombinant strains were constructed by the gene-manipulation technique described elsewhere [10]. Briefly, a high-molecular-weight chromosomal DNA of the US 46 strain was digested with *Sau*3A and subjected to 0.5% agarose gel electrophoresis. The DNA fragments were transferred to DE 81 paper (Whatman). The paper was washed and the DNA fragments of appropriate sizes were eluted from paper. After ligation of these fragments with a cosmid vector (pHC 79) which had been treated with BamHI and bacterial alkaline phosphatase, recombinant molecules were packed in vitro and transferred to a non-piliated strain of *E. coli* (p678-54), which was observed by means of the HA test and electron microscopy to have no pilus appendages on its surface. Two recombinant plasmids, pYM7 (for MSHA) and pYM122 (for MRHA), were used in these experiments. As previously reported, one recombinant strain, p678-54 (pYM7) had only MS pili and the other p678-54 (pYM122) had only MR pili. The latter was verified by means of its HA and yeast cell agglutination properties, agglutination by anti-MS or anti-MR pili specific antibody and by electron microscopic observation.

Experimental animals

Female Sprague-Dawley rats 6–8 weeks old and weighing between 200 and 250 g were used in all experiments. The rats were kept in specific pathogen-free condition at room temperature. They received a mouse diet with tap water provided ad libitum. All surgical procedures were carried out under anesthesia with ethyl ether.

Bacterial inoculation

Using a 26-ga needle, bacterial strains were inoculated directly into the parenchyma of one kidney of each rat at a dose of 9×10^7 colony forming unit (cfu) in 0.1 ml of saline. Six weeks later, the animals were killed by femoral bleeding and cervical dislocation and the inoculated kidneys were removed for study.

Renal scarring

Renal scarring was graded microscopically as follows: − no scar, + linear scar, ++ small scar, and +++ large scar with deformity. Each grade was then assigned a score of 0, 1, 2, and 3 points to −, +, ++ and +++, respectively, and the totals were calculated. The grade of renal scarring was assessed by an investigator who was unaware of the details of the experiment. Each kidney was examined histologically following staining via hemotoxylin and eosin.

Administration of cyclophosphamide, colchicine and superoxide dismutase

A 50 mg/kg dose of cyclophosphamide (Cp) was administered intraperitoneally for 3 consecutive days, beginning 2 days before the inoculation of bacterial cells. Colchicine (Col) powder (Wako, Japan) was diluted in sterile water to a concentration of 1 mg/ml and administered intraperitoneally at a dose of 0.4 mg/kg of body weight daily for 3 consecutive days. The first injection was administered the day preceding the inoculation. Two kinds of superoxide dismutase (SOD), manganese SOD (Mn-SOD) (Takeda Chemicals, Japan) from *S. marcescens* and PEG-SOD, SOD modified with monomethoxy-polyethylene glycol (PEG) (Takeda, Japan) [5, 9]. Mn-SOD was isolated from *S. marcescens* ATCC 21074, while PEG-SOD was modified from Mn-SOD using polyethylene glycol. PEG-SOD had 52% of the enzymatic activity, a lower antigenicity and a 10 times longer half-life compared with Mn-SOD. A dose of 20 mg/kg of Mn-SOD or 2 mg/kg of PEG-SOD were administered subcutaneously every 12 h for 3 days, beginning 6 h before the bacterial inoculation.

Superoxide production by rat leukocytes

Rat polymorphonuclear leukocytes (PMNL) were harvested from the peritoneal cavity 24 h following irritation by exposure to 1 ml thioglycolate medium (Gibco). After separation of the PMNL-rich fraction on a Ficol-Hypaque (Pharmacia), cells were resuspended in Hank's balanced salt solution (HBSS) (Gibco). The production of superoxide was evaluated by the cytochrome C-reduction method. PMNL were suspended at a concentration of 1.5×10^6 cells/ml in HBSS. A 1×10^{10} cfu/ml of bacteria and 30 mg/l of cytochrome C, type III (Sigma), were added to the cell suspension and incubated for 30 min at 37 °C. The opaque density (OD) was measured at 550 nmol with a spectrophotometer. The amount of superoxide producted (nmol/10^{10} PMNL/min) was calculated from the formula: (OD test − OD blank) times the volume of incubation mixture times 47.4 divided by the incubation time. Results were expressed as a percent of the control activity obtained by using the p678-54 strain as a stimulus. All experiments were performed in triplicate.

Statistical analysis

Evaluation of data was performed by the χ^2 - test according to the extent of renal scarring in each experimental group and by Student's *t*-test to evaluate the amount of superoxide product. The level of significance was $P < 0.05$.

Results

Renal scarring following bacterial inoculation

The kidneys were observed macroscopically and microscopically 6 weeks after the direct inoculation of the following bacterial strains: non-piliated (p678-54), MS- and MR-piliated (US 46), MS-piliated [p678-54 (pYM7)] and MR-piliated [p678-54 (pYM122)].

Significant scar formation was observed in the kidneys inoculated with strains US 46 and p678-54 (pYM7). In contrast, the kidneys inoculated with p678-54 and p678-54 (pYM122) had no or minimal scar formation. These findings show that the piliated strains, especially the MS-piliated strain, stimulated scarring to a significantly greater extent than the non-piliated or MR-piliated strains (Table 1).

Effect of Cp or Col administration on renal scarring

The intraperitoneal administration of a dose of 50 mg/kg of Cp for 3 consecutive days initiated 2 days before the bacterial inoculation was associated with a significant suppression of scar formation in the rats inoculated with

Table 1. Scarring of the kidney inoculated with MS- or MR-piliated strains

Strain	Piliation	Scarring[a] (number of rats)				Total score[b]	χ^2 test
		−	+	ǂ	ǂǂ		
US46	MS + MR	0	3	5	0	13	*
p678-54	None	5	3	0	0	3	* NS
p678-54 (pYM 7)	MS	0	1	6	1	16	*
p678-54 (pYM 122)	MR	4	4	0	0	4	*

[a] Scarring: renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Grading of renal scarring was assessed from − to ǂǂ, as described below. Each group consisted of 8 rats. Grade of scarring: − no scar; + linear scar; ǂ small scar; ǂǂ large scar with deformity
[b] Total score was calculated from points assigned to the grade of scar formation. Thus, the above-mentioned grades (−, +, ǂ and ǂǂ) were scored as 0, 1, 2 and 3
* $P < 0.01$

Table 2. Suppression of renal scarring by administering cyclophoshamide (Cp) to induce leukopenia

Strain	Piliation	Treatment[a]	Scarring[b] (number of rats)				Total score[b]	χ^2-test
			−	+	ǂ	ǂǂ		
p678-54 (pYM 7)	MS	None	1	4	3	0	10	$P < 0.05$
p678-54 (pYM 7)	MS	Cp	6	1	0	0	1	

[a] Treatment: 50 mg/kg dose of cyclophosphamide (Cp) was administered intraperitoneally for three consective days initiated two days before bacterial inoculation
[b, c] Renal scarring and scoring were performed as described in the footnote for Table 1

Table 3. Suppression of renal scarring with colchicine treatment

Bacteria	Piliation	Treatment[a]	Scarring[b] (number of rats)				Total score[c]	χ^2-test
			−	+	ǂ	ǂǂ		
p678-54 (pYM 7)	MS	None	0	2	5	1	15	$P < 0.05$
p678-54 (pYM 7)	MS	Colchicine	4	3	1	0	5	

[a] Treatment: 0.4 mg/kg dose of colchicine was administered intraperitoneally per day for three consecutive days initiated 1 day before bacterial inoculation
[b, c] Renal scarring and scoring were performed as described in footnote for Table 1

Table 4. Suppression of renal scarring by administering superoxide dismutase

Strain	Piliation	Treatment[a]	Scarring[b] (number of rats)				Total score[c]	χ^2 test
			−	+	ǂ	ǂǂ		
US46	MR + MS	None	0	0	8	0	16	$P < 0.01$
US46	MR + MS	PEG-SOD	5	3	0	0	3	
p678-54 (pYM 7)	MS	None	0	2	4	2	16	$P < 0.01$
p678-54 (pYM 7)	MS	SOD	3	4	1	0	6	$P < 0.01$
p678-54 (pYM 7)	MS	PEG-SOD	7	1	0	0	1	

[a] A 20 mg/kg dose of SOD or 2 mg/kg of PEG-SOD were administered s.c. every 12 h for 3 days, beginning 6 h before bacterial inoculation
[b, c] Renal scarring was evaluated and scored as described in the footnote for Table 1. SOD, Superoxide dismutase; PEG-SOD, polyethylene glycol-modified SOD

Table 5. Superoxide production by rat polymorphonuclear leukocytes stimulated by MS or MR-piliated strain

Strain	Piliation	Superoxide production[a] (%)				*t*-test
		Exp 1	Exp 2	Exp 3	Mean ± SD	
p678-54	None	100	100	100	100 ± 0	
US46	MR + MS	96.8	94.5	95.1	95.5 ± 1.2	
p678-54 (pYM 7)	MS	105.5	105.5	104.8	105.3 ± 0.4	$P<0.05$
p678-54 (pYM 122)	MR	98.8	97.8	91.2	95.5 ± 4.1	

[a] Rat polymorphonuclear leukocytes were harvested from the peritoneal cavity irritated with thioglycollate medium. Superoxide production was measured by the cytochrome C reduction method. Results (% superoxide production) were expressed as a percent of the superoxide production of the leukocytes stimulated by a non-piliated strain (p678-54)

MS-piliated bacteria [p678-54 (pYM7)] compared to the group not receiving the dose control (Table 2).

In the animals administered a 0.4 mg/kg dose of Col intraperitoneally the day before the inoculation, there was a significant reduction in renal scarring compared to the non-Col control group (Table 3). These findings suggest that the PMNL play an important role in the renal scarring following infection by MS-piliated strains of bacteria.

Suppression of renal scarring by superoxide dismutase treatment

The subcutaneous administration of 2 mg/kg of PEG-SOD at an early stage in the infection completely suppressed scarring in the kidneys of rats inoculated with either the US 46 or p678-54 (pYM7) strains. Subcutaneous Mn-SOD 20 mg/kg slightly suppressed formation of renal scarring following the inoculation of MS-piliated strains (Table 4).

Superoxide production of PMNL stimulated by piliated strains

To evaluate of superoxide production by PMNL following stimulation by four bacterial strains, as measured by the cytochrome C reduction method, showed that the cells stimulated by p678-54 (pYM7) produced a larger amount of superoxide compared to the non-piliated or MR-piliated strains (Table 5).

Discussion

Bacterial pili play an important role in the pathogenesis of a urinary tract infection, adhering to the mucosa through specific receptors and modulating the inflammatory response following infection. The *S. marcescens* US 46 strain bears two types of pili on its surface, namely MS and MR types. We cloned these pili genes to form two new recombinant strains that possessed either MS or MR pili. These two recombinant strains were identical with respect to other virulence factors. In our study of these recombinant strains, as we reported previously, the MS-piliated strain stimulates renal scarring more severely than either non-piliated or MR-piliated strains [7]. Some reports state that the renal damage following kidney infection is not due to bacterial growth but is closely related to the imflammatory process, including infiltration by PMNL [1, 3, 11]. PMNL are known to release active substances, including superoxide, following stimulation by bacteria. Therefore, in this study we evaluated the influence of PMNL and superoxide on the extent of renal scarring.

Treatment with cyclophosphamide or colchicine prevented the renal scarring mediated by the inoculation of MS-piliated bacteria. It is well known that Cp decreases the leukocyte count and that Col suppresses leukocyte migration. Therefore, the suppression of scar formation by these substances suggests an important role of PMNL in the renal scarring following renal infection. SOD also prevented renal scarring in this experiment, suggesting that the superoxide released by PMNL plays an important role in the scarring. Finally, the MS-piliated strain [p678-54 (pYM7)] markedly stimulated the PMNL, and the production of superoxide was greater than seen with either the non-piliated or MR-piliated strains.

In conclusion, these data suggest that superoxide plays an important role in the renal scarring following infection with the MS-piliated bacterial strains.

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