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Experimental Analysis of Bacterial and Host Factors Involved in Renal Scarring

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Summary. **The effects on renal scarring of bacterial pili and superoxide released by polymorphonuclear leukocytes (PMN) were examined using a rat experimental pyelonephritis model. Bacteria which possessed mannose-sensitive (MS) pili were found to promote renal scarring following their direct inoculation into the renal parenchyma. Treatment with cyclophosphamide to induce leukopenia, colchicine to modulate PMN migration, and superoxide dismutase or an experimental antioxidant to reduce PMN superoxide release suppressed renal scarring following infection with MS-piliated bacteria. In contrast, treatment with phorbol myristate acetate significantly enhanced renal scarring. In addition, the production of superoxide by PMN was significantly greater following stimulation by MS-piliated bacteria than after stimulation with either non-piliated or MR-piliated strains. These findings suggest that MS-piliated bacteria stimulate renal scarring more markedly than do non-piliated or MR-piliated bacteria, and that superoxide released from PMNs plays an important role in the development of renal scarring following infection by MS-piliated strains.**

INTRODUCTION

Renal scarring is a frequent complication of vesicoureteric reflux and may lead to both renal insufficiency and renal hypertension. Despite the importance of this condition, the actual mechanism of scar formation remains poorly understood. There are two main factors involved in the production of scars in the kidney, i.e., the invading bacteria and the host defenses. With regard to bacterial invasion, piliation or fimbriation are both thought to be extremely important.

Many investigators have noted that scar formation occurs following bacterial infection and the subsequent inflammatory response. Glauser et al.[1] and Slotki and Asscher[2] both reported the prevention of chronic pyelonephritis and renal scarring by the initiation of antibiotic therapy within 1 to 3 days of the onset of bacterial infection. These reports suggested that there may be a close relationship between scar formation and the occurrence of suppuration during the early stage of pyelonephritis. In other words, renal scarring is probably not directly related to bacterial growth, but rather to subsequent inflammatory reaction in which polymorphonuclear leukocytes (PMN) appears to play a major role.

Almost all bacteria have pili that enable them to adhere to the urinary mucose and these are usually classified as mannose-sensitive (MS) and mannose-resistant (MS) pili. *Serratia marcescens* is one of the commonest urinary pathogens in patients with an underlying cause for urinary tract infection such as stones, tumors, catheters, or foreign bodies. This bacterium also possesses MS and MR pili. We have previously cloned the DNA genes for the MS and MR pili from a clinical isolate of *S. marcescens* (US 46) which has both types of pili.[3] These DNA genes were then transferred to non-piliated *E. coli* to produce new transformants bearing either MS or MR pili, and we found that MS-piliated bacteria produced renal scarring in a pyelonephritis model, while MR-piliated bacteria did not.

When stimulated by bacteria or chemicals, PMNs release active oxygen radicals, including superoxide, which are known to produce tissue damage. Although active oxygen radicals are bactericidal, they may also induce damage in the surrounding host tissue. In this report, we describe the suppressive effect of cyclophosphamide, colchicine, superoxide dismutase (SOD), and a novel synthetic antioxidant on renal scarring following infection by MS-piliated bacterial strains.

MATERIALS AND METHODS

Bacterial strains

Serratia marcescens (strain US46) was isolated from a patient with complicated urinary tract infection. This bacterial strain expresses two types of pili (MS and MR) that can be identified by hemagglutination (HA) testing and electron microscopy. Non-piliated *E. coli* (strain p 678-54) was also used, a bacterium which has no pili on its surface. Two *E. coli* strains were derived from p678-54 by gene manipulation, as was previously reported.[3] Briefly, high-molecular weight DNA from the US46 strain was partially digested with Sau 3A and subjected to 0.5% agarose gel electrophoresis. The DNA fragments thus obtained were transferred to DE 81 paper (Whatman) by electrophoresis, the paper was washed, and fragments of specific sizes were eluted. Following ligation of these fragments with a cosmid vector (pHC79), which had been pretreated with Bam HI and bacterial alkaline phosphatase, recombinant molecules were packed in vitro and introduced into strain p678-54. Several transformants that reacted to the HA test for MS or MR when exposed to chicken erythrocytes were selected from approximately 3,000 transformants. Two recombinant plasmids, pYM7 for MS pili and pYM122 for MR pili, were then used to produce two p678-54 derivatives harboring the respective plasmids i.e., p678-54 (pYM7) and p678-54 (pYM122). p678-54 (pYM7) exhibited only MS pili and p678-54 (pYM122) had only MR pili, as shown by the HA test, yeast cell agglutination, anti-MS or anti-MR antibody agglutination, and electron microscopy.

Rat pyelonephritis model

Female Sprague-Dawley rats aged six to eight weeks and weighing 200 g to 250 g were used in all experiments. The rats were kept in a specific pathogen-free environment at ambient room temperature and maintained on laboratory chow with tap water ad libitum. Eight rats were used for each experimental group. All surgical procedures were performed with the animals under ethyl ether anesthesia.

Bacterial inoculation

Each bacterial strain was inoculated directly into the rat renal parenchyma with a 26-G needle at a dose of 9×10^7 colony forming units (cfu). The animals were killed by exanguination from the femoral artery and cervical dislocation 6 weeks after bacterial inoculation, and the inoculated kidneys were removed for examination.

Renal scarring

Renal scarring was graded marcoscopically as follows: −, no scarring; +, linear scars; ++, mildly depressed scars; and +++, massive scarring associated with renal deformity. These grades were then assigned point scores of 0, 1, 2 and 3, respectively, and the total score was calculated for each group of rats.

Administration of cyclophosphamide, colchicine, SOD and synthetic antioxidant

A 50 mg/kg dose of cyclophosphamide was administered intraperitoneally for 3 consecutive days from 2 days before the inoculation of bacteria. Colchicine 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 daily for 3 consecutive days. The first injection was administered the day preceding the inoculation. Manganese SOD (Mn-SOD) and monomethoxypolyethlene glycol-modified SOD (PEG-SOD) (Takeda Chem, Japan) were used inactivate superoxide.[4] PEG-SOD was modified from Mn-SOD with PEG, which resulted in a 52% retention of enzymatic activity and a lower antigenicity, and had a half-life of ten times that of Mn-SOD.[5] A 20 mg/kg dose of Mn-SOD or 2 mg/kg of PEG-SOD were administered subcutaneously every 12 hours for 3 days. The initial injection was given 6 hours before bacterial inoculation.

We also examined a novel antioxidant, 2-O-octadecyle ascorbic acid (CV3611), which is known to be a free radical scavenger (Takeda Chem, Japan).[6,7] CV3611 or ascorbic acid were administered every day for 4 days at a dose of 15 mg/kg. These compounds were solubilized with 1% dimethylsulfoxide and diluted with saline.

Administration of phorbol myristate acetate

A 500 μg/kg of phorbol myristate acetate (PMA) was administered intravenously for 3 consective days initiated 2 days after bacterial inoculation.

Statistical analysis

Differences in renal scarring between the groups were assessed using the Wilcoxon signed-rank test

and were considered statistically significant at $p<0.05$.

RESULTS

Renal scarring following bacterial inoculation

The kidneys were observed macroscopically and microscopically at 6 weeks after the direct inoculation of the following bacterial strains: non-piliated p678-54, MS- and MR-piliated US46, MR-piliated p678-54 (pYM122), MS-piliated p678-54 (pYM7). Significant scar formation was observed in the kidneys inoculated with US46 and p678-54 (pYM7). In contrast, the kidneys inoculated with p678-54 and p678-54 (pYM122) showed little or no scar formation. The piliated strains, especially the MS-piliated strains, stimulated scarring to a significantly greater extent than the non-piliated or MR-piliated strains (Table 1).

Effect of cyclophosphamide and colchicine on renal scarring

Intraperitoneal administration of cyclophosphamide (50 mg/kg) for 3 days from 2 days before bacterial inoculation significantly suppressed scar formation in the rats inoculated with MS-piliated bacteria when compared to the control animals (Table 2).

Intraperitoneal colchicine (0.4 mg/kg) for 3 days from the day before inoculation also produced a significant reduction in renal scarring when compared to the control group (Table 3). These findings suggest that PMNs play an important role in renal scarring following infection by MS-piliated bacterial strains.

Effect of SOD on renal scarring

When the transformant p678-54 (pYM7) which expressed only MS pili was inoculated into rat kidneys,

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

Bacteria	Piliation	Scarring[a] (Number of rats)				Wilcoxon signed rank test	Total score[b]
		−	+	++	+++		
p 678-54	−	5	3	0	0	−	3
US 46	MS+MR	0	3	5	0	p=0.006	13
p 678-54 (pYM 122)	MR	4	4	0	0	ns	4
p 678-54 (pYM 7)	MS	0	1	6	1	p=0.006	16

[a] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.

[b] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

Table 2. Suppression of renal scarring by administering cyclophosphamide (Cp) to induce neutropenia

Bacteria	Piliation	Treatment[a]	Scarring[b] (Number of rat)				Wilcoxon signed rank test	Total score[c]
			−	+	++	+++		
p 678-54 (pYM 7)	MS	−	1	4	3	0	−	10
p 678-54 (pYM 7)	MS	Cp	6	1	0	0	p=0.014	1

[a] A 50 mg/kg dose of cyclophosphamide (Cp) was administered intraperitoneally for 3 consective days initiated 2 days before bacterial inoculation.

[b] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.

[c] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

Table 3. Effect of colchicine on renal scarring following infection with MS-piliated bactreia

Bacteria	Piliation	Treatment[3)]	Scarring (Number of rats)[b)]				Wilcoxon segned rank test	Total score[c)]
			−	+	++	+++		
p 678-54 (pYM 7)	MS	−	0	2	5	1	−	14
p 678-54 (pYM 7)	MS	colchicine	4	3	1	0	p=0.006	10

[a)] A 0.4 mg/kg of cochicine was administered intraperotoneally every day for 3 days initiated 1 day before bacterial inoculation.
[b)] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.
[c)] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

Table 4. Suppression of scarring in rat kidneys inoculated with MS-piliated bacteria by treating with SOD.

Bacteria	Piliation	Treatment[a)]	Scarring[b)] (Number of rats)				Wilcoxon signed rank test	Total score[c)]
			−	+	++	+++		
US46	MS+MR	−	0	0	8	0	−	16
US46	MS+MR	PEG-SOD	5	3	0	0	p=0.006	3
p 678-54 (pYM 7)	MS	−	0	2	4	2	−	16
p 678-54 (pYM 7)	MS	SOD	3	4	1	0	p=0.006	6
p 678-54 (pYM 7)	MS	PEG-SOD	7	1	0	0	p=0.006	1

[a)] A 20 mg/kg of Mn-SOD or 2 mg/kg of PEG-SOD were administrated subcutaneously every 12 h for 3 days. First administration was done 6 hours before bacterial inoculation.
[b)] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.
[c)] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

severe scar formation occurred. However, renal scarring was completely suppressed in SOD- and PEG-SOD-treated rats at 6 weeks after bacterial inoculation. PEG-SOD was more effective in suppressing scarring than Mn-SOD (Table 4).

Effect of antioxidants on scar formation

The effect of CV3611 and its parent compound, ascorbic acid, were examined on scar formation following the inoculation of MS-piliated bacteria. Intravenous CV3611 significantly reduced renal scarring following infection with US46 (MS- and MR-piliated) and p678-54 (pYM7) (MS-piliated). Ascorbic acid had less effect than CV3611 (Table 5).

Effect of PMA on scar formation

PMA (500 μg/kg) was administered intravenously for 3 consecutive days from 2 days after bacterial inoculation. Renal scar formation following infection with the MS-piliated transformant was significantly more severe in the PMA-treated rats than in the control animals (Table 6).

Suppression of PMA-enhanced renal scarring by PEG-SOD

PEG-SOD (2 mg/kg) was administered subcutaneously at 12-h intervals for 3 days to rats inoculated with MS-piliated bacteria, and PMA was also administered for 3 days from 2 days after infection. PEG-SOD significantly suppressed renal scar formation when

Table 5. Effect of subcutaneous administration of ascorbic acid or CV3611 on the renal scarring mediated by the piliated bacteria

Bacteria	Piliation	Treatment[a]	Scarring[b] (Number of rats)				Wilcoxon signed rank test	Total score[c]
			−	+	++	+++		
US 46	MS+MR	−	0	1	2	5	−	20
US 46	MS+MR	CV 3611	1	7	0	0	p=0.006	7
p678-54 (pYM7)	MS	−	0	0	3	5	−	21
p678-54 (pYM7)	MS	ascorbic acid	2	1	3	2	p=0.014	13
p678-54 (pYM7)	MS	CV 3611	1	5	2	0	p=0.006	9

[1] A 15 mg/kg of ascorbic acid or CV3611 was administered subcutaneously every day for 4 days, First administration was done a day before bacterial inoculation.
[b] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.
[c] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

Table 6. Enhanced renal scarring by administering phorbol myristate acetate (PMA)

Bacteria	Piliation	Treatment[a]	Scarring[b] (Number of rats)				Wilcoxon signed rank test	Total score[c]
			−	+	++	+++		
p678-54 (pYM7)	MS	−	0	7	1	0	−	9
p 678-54 (pYM7)	MS	PMA	0	4	3	1	p=0.034	13

[1] A 50 0 μg/kg dose of phorbol myristate acetate (PMA) was administered intravenously for 3 consective days initiated 2 days after bacterial inoculation.
[b] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.
[c] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

Table 7. Suppressive effect of PEG-SOD on enhanced renal scarring by phorbol myristate acetate (PMA) following infection with MS-piliated bacterial strain

Bacteria	Piliation	Treatment[a]	Scarring[b] (Number of rats)				Wilcoxon signed rank test	Total score[c]
			−	+		+++		
p678-54 (pYM7)	MS	PMA	0	2	6	0	−	14
p678-54 (pYM7)	MS	PMA+PEG-SOD	3	2	2	0	p=0.034	6

[a] A 500 μg/kg dose of phorbol myristate acetate (PMA) was administered intravenously for 3 consective days initiated 2 days after bacterial inoculation. A 2 mg/kg of PEG-SOD were administrated subcutaneously every 12 h for 3 days. First administration was done 3 h before bacterial inoculation.
[b] Scarring; Renal scarring was observed macroscopically 6 weeks after bacterial inoculation. Renal scarring was assessed from − to +++ according to severity. Every group was consisted of 8 rats. Grade of scarring; −: No scarring, +: Linear scarring, ++: Mild scarring, +++: Large scar with deformity.
[c] Total score was calculated by assigning a point value to each scar grade and finding the sum for each rat group. Grading −, +, ++ and +++ were assigned points 0, 1, 2 and 3, respectively.

compared with the control group receiving only PMA (Table 7).

DISCUSSION

Almost all bacteria possess surface pili or fimbriae. These pili react with host cell receptors,enabling bacteria to adhere to a mucosal surface. *S. marcescens* is frequently found in patients with complicated urinary tract infections. This species possesses both MS and MR pili, which are distinguishable by HA testing,antibody agglutination studies, and electron microscopy.

We previously cloned the MS and MR pili genes of *S. marcescens* strain US46 and transfer them to the non-piliated *E. coli* strain p678-54. Recombinant strains expressing either MS or MR pili were identified by HA testing, antibody agglutination, and electron microscopy.[3] The current study showed that MS-piliated bacteria stimulated renal scarring more than MR-piliated bacteria in a rat pyelonephritis model. O'Hanley et al.[9] have reported that a recombinant *E. coli* strain possessing MR-pili colonized the kidney, but not an MS-piliated strain. In addition,a bacterial strain possessing both pili colonized and invaded the kidneys of mice after the intravesicular inoculation of lower numbers of bacteria compared with the strain possessing only MR-pili. They suggested that MR-pili had an important role in colonizing the renal epithelium through interaction with a specific recepter, but that MS-pili had a significant role only in pyelonephritis associated with vesicoureteric reflux. This was because the MS-piliated bacteria could only colonize the kidney at a larger inoculum size that caused acute reflux. We also confirmed in our direct inoculation model that MS-pili had an important role in promoting renal tissue damage and scarring.

A few investigators have reported that renal parenchymal damage following acute pyelonephritis is not directly related to bacterial multiplication in the kidney, but is instead closely related to the host inflammatory response including PMN infiltration.[1,2] PMNs are known to release many active substances when stimulated by bacteria, including superoxide, singlet oxygen, hydroxyl radical, and hydroxy peroxide molecules, as well as other lysozomal enzymes. Superoxide can induce severe damage in normal renal tissue.

In this study, cyclophosphamide, colchicine, SOD, and a new synthetic antioxidant suppressed renal scarring in the rats inoculated with an MS-piliated transformant. In addition, treatment with PMA significantly increased scarring in the rats inoculated with the MS-piliated transformant. PMA is known to strongly stimulate PMNs to release superoxide and other mediators, and is commonly used as the stimulator in assays of PMN superoxide production. This enhanced scarring was inhibited by the administration of PEG-SOD. Finally, MS-piliated bacteria stimulated more superoxide production by PMNs than MR-pliliated bacteria. These findings indicate that PMNs and the superoxide that they release an important role in scar formation following renal infection by MS-piliated bacteria.[10,12]

REFERENCES

1) Glauser MP, Lyons JM, Braude AT: Prevention of chronic experimental pyelnephritis by suppression of acute suppuration. *Clin Invest* **61**: 403-407, 1978.
2) Slotki IN, Asscher AW: Preventeion of scarring in experimental pyelonephritis in the rat by early antibiotic therapy. *Nephron* **30**: 262-268, 1982.
3) Mizunoe Y, Nakabeppu Y, Sekiguchi M, Kawabata S. Moriya T, Amako K: Cloning and suquence of the gene encoding the major structural component of mannose-resistant fimbriae of Serratia marcescens. *J Bacteriol* **170**: 3567-3574, 1988.
4) Maejima K, Miyata K, Tomoda K: A manganese superoxide dismutase from Serratia marcescens. *Agric Biol Chem* **47**: 1537-1543, 1983.
5) Miyata K, Nakagawa Y, Nakamura M, Ito T, Sugo K, Fujita T, Tomoda K: Altered properties of Serratia superoxide dismutase by chemical modification. *Agric Biol Chem* **52**: 1575-1581, 1988.
6) Kato K, Terao S, Shimamoto N, Hirata M: Studies on scavengers of active oxygen species. I. Synthesis and biological activity of 2-0-alkylascorbic acids. *J Med Chem* **31**: 793-798, 1988.
7) Tanabe M, Kito G: Effects of CV-3611, a new free radical scavenger, on ischemic heart failure in conscious Beagle dogs. *Jap J Pharm* **50**: 467-476, 1988.
8) Matsumoto T, Mizunoe Y, Sakamoto N, Tanaka M, Kumazawa J: Increased renal scarring by MS piliated bacteria. *Urol Res* **18**: 299-303, 1990.
9) O'Hanley P, Lark D, Falkow S, Schoolnik G: Molecular basis of Escherichia coli colonization of upper urinary tract in BALB/c mice. Gal-Gal pili immunization prevents Escherichia coli pyelonephritis in the BALB/c mouse model of human pyelonephritis. *J Clin Invest* **75**: 347-360, 1985.
10) Matsumoto T, Mizunoe Y, Ogata N, Tanaka M, Kumazawa J: Role of superoxide in renal scarring following infection by mannose-sensitive piliated bacteria. *Urol Res* **19**: 229-233, 1991.

11) Matsumoto T, Mizunoe Y, Sakamoto M, Kumazawa J: Suitability of colchicine and superoxide dismutase for the suppression of renal scarring following an infection with bacteria showing mannose-sensitive pili. *Nephron* **56**: 130-135, 1990.

12) Matsumoto T, Mizunoe Y, Ogata N, Tanaka M, Takahashi K, Kumazawa J: Antioxidant effect on renal scarring following infection of Mannose-sesitive piliated bacteria. *Nephron* **60**: 310-215, 1992.