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A Murine Model of Inflammatory Bladder Disease: Cathelicidin Peptide Induced Bladder Inflammation and Treatment With Sulfated Polysaccharides

Siam Oottamasathien*, Wanjian Jia, Lindsy McCoard, Sean Slack, Jianxing Zhang, Aleksander Skardal, Kathleen Job, Thomas P. Kennedy[†], Randal O. Dull, and Glenn D. Prestwich[†]

Department of Surgery and Division of Pediatric Urology (SO), Department of Medicinal Chemistry and Center for Therapeutic Biomaterials (SO, WJ, LM, SS, JZ, AS, GDP) and Departments of Internal Medicine (TPK) and Anesthesia (KJ, ROD), Primary Children's Medical Center (SO), University of Utah, Salt Lake City, Utah

Abstract

Purpose—Studies show that LL-37 is a naturally occurring urinary defensin peptide that is up-regulated during urinary tract infections. Although normal urinary LL-37 levels are antimicrobial, we propose that increased LL-37 may trigger bladder inflammation. We further suggest that anti-inflammatory sulfated polysaccharides known as semi-synthetic glycosaminoglycan ether compounds can treat/prevent LL-37 mediated bladder inflammation.

Materials and Methods—C57BL/6 mice were catheterized/instilled with LL-37 (320 μ M at 150 μ l) for 45 minutes. Animals were sacrificed at 12 and 24 hours, and tissues were examined using hematoxylin and eosin. Separate experiments were performed for myeloperoxidase to quantify inflammation. GM-1111 semi-synthetic glycosaminoglycan ether treatments involved instillation of 10 mg/ml for 45 minutes directly before or after LL-37. Tissues were harvested at 24 hours. To compare semi-synthetic glycosaminoglycan ether efficacy experiments were performed using 10 mg/ml heparin. Finally, tissue localization of semi-synthetic glycosaminoglycan ether was examined using a fluorescent GM-1111-Alexa Fluor® 633 conjugate.

Results—Profound bladder inflammation developed after LL-37. Greater tissue inflammation occurred after 24 hours compared to that at 12 hours. Myeloperoxidase assays revealed a 21 and 61-fold increase at 12 and 24 hours, respectively. Semi-synthetic glycosaminoglycan ether treatment after LL-37 showed mild attenuation of inflammation with myeloperoxidase 2.5-fold below that of untreated bladders. Semi-synthetic glycosaminoglycan ether treatment before LL-37 demonstrated almost complete attenuation of inflammation. Myeloperoxidase results mirrored those in controls. In heparin treated bladders minimal attenuation of inflammation occurred. Finally, instillation of GM-1111-Alexa Fluor 633 revealed urothelial coating, significant tissue penetration and binding to endovascularity.

Conclusions—We developed what is to our knowledge a new model of inflammatory bladder disease by challenge with the naturally occurring urinary peptide LL-37. We also noted that a new class of anti-inflammatory sulfated polysaccharides prevents and mitigates bladder inflammation.

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*Correspondence: University of Utah/Primary Children's Medical Center, 100 North Mario Capecchi Dr., Suite 2200, Salt Lake City, Utah 84113 (siam.oottamasathien@hsc.utah.edu).

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Study received University of Utah institutional animal care and use committee approval.

Keywords

urinary bladder; inflammation; glycosaminoglycans; rosacea; mice

Inflammatory conditions of the bladder are a significant health concern. Neurogenic bladder disease can result from excess inflammation, leading to fibrosis. In children many diseases cause neurogenic bladder disease, including myelomeningocele/spina bifida. These processes overlap in adults, in whom chronic inflammatory bladder disorders such as IC result in debilitating urinary symptoms. More than 4 million people in the United States have IC and the cost/disease burden exceeds \$750 million dollars annually.¹ Currently IC treatment has been suboptimal due to its uncertain cause and pathogenesis.^{2–5}

We describe the development of a reproducible mouse model of bladder inflammation. The model builds on studies of epithelial cells in the human skin inflammatory disorder, rosacea. Individuals with rosacea express abnormally high epithelial skin levels of the cationic antimicrobial peptide cathelicidin and its post-enzymatic cleaved peptide product LL-37,^{6,7} resulting in profound skin inflammation. LL-37, a host defense peptide, is produced from the C-terminus of the hCAP18 precursor protein and made by a multivariate cell population, including epithelial cells and circulating neutrophils.⁸ Human and mouse urothelial cells naturally produce LL-37.⁹ Also, during pediatric urinary tract infection (pyelonephritis and/or cystitis) urinary LL-37 is significantly increased.⁹ We have preliminary data on increased levels of urinary LL-37 in noninfected children with spina bifida. LL-37 also contains immunomodulatory properties that trigger inflammation.^{7,8} Furthermore, low concentrations of LL-37 (13 to 25 μ M) can be cytotoxic against eukaryotic cells.¹⁰

We propose that increased urinary LL-37 triggers inflammatory cascades, contributing to bladder inflammation and possibly fibrosis. Numerous insults, including urinary tract infections and toxic cationic urinary metabolites, could cause injury to the protective urinary GAG layer and damage the urothelium. Consequently urothelial permeability and inflammatory cascade activation increase.

In this study we also examined a new family of anionic, partially lipophilic polysaccharide derivatives known as SAGEs. These anionic polymers mimic sulfated native urothelial GAGs. As with heparin and partially desulfated heparin,¹¹ SAGEs inhibit PMN proteases, and P and L-selectin mediated influx of leukocytes into areas of inflammation (Zhang et al, unpublished data). SAGEs also show saturable, high affinity binding to LL-37, thereby modulating the interaction with its natural targets (Zhang et al, unpublished data). We tested the hypothesis that SAGEs attenuate bladder inflammation by 1 or more modes of action, including remediation of damage to the GAG layer, charge neutralization of toxic cationic metabolites, vascular stabilization and modulation of inflammatory cell activity.

MATERIALS AND METHODS

Bladder Inflammation

LL-37 induced model—Experiments were performed in accordance with the institutional animal care and use committee at our university. We used 8 to 12-week old female C57BL/6 mice. LL-37 was obtained in high performance liquid chromatography-homogenous form (peptide sequence: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN-LVPRTEs) and dissolved in 320 μ M nanopure water. Each group consisted of 6 mice. After establishing isoflurane anesthesia a 1.5 cm silicone catheter with an inner and outer diameter of 0.30 and 0.64 mm, respectively, was introduced transurethrally. After complete urine drainage 150 μ l pyrogen-free 0.9% sodium chloride were instilled for 1 minute and emptied. LL-37 (320

μM) was instilled at a volume equal to capacity ($150\ \mu\text{l}$),^{12–16} as previously described (intravesical $t = 45$ minutes). Controls consisted of pyrogen-free 0.9% sodium chloride instillation. Substances were infused slowly to avoid trauma and vesicoureteral reflux.^{12–16} The instillation syringe was kept on the catheter to ensure no solution leakage.^{12–16} At 45 minutes the bladders were emptied to completion. Depending on the experimental group the animals were sacrificed at 12 or 24 hours.

SAGE GM-1111 and Heparin Treatment

We examined SAGE and heparin (each pH 7.0) treatment in 2 LL-37 induced bladder inflammation groups. In the 4 group 1 mice we first instilled LL-37 ($320\ \mu\text{M}$ at $150\ \mu\text{l}$) for 45 minutes and then emptied it. Immediately thereafter SAGE or heparin ($10\ \text{mg/ml}$ at $150\ \mu\text{l}$) was instilled for 45 minutes and then emptied. Bladders were harvested at 24 hours. In the 4 group 2 mice we first instilled SAGE or heparin ($10\ \text{mg/ml}$ at $150\ \mu\text{l}$) for 45 minutes, emptied the bladders and challenged them with LL-37 ($320\ \mu\text{M}$) for 45 minutes. Tissues were harvested at 24 hours. All bladders were hemisected, processed and stained with hematoxylin and eosin, or tissue MPO assays were done. Statistical analysis of MPO data on SAGE treated samples was performed with the unequal variance 2-tailed Student t test with $p < 0.05$ considered statistically significant.

Bladder Coating

We synthesized SAGE GM-1111-Alexa Fluor 633 bioconjugate as described in another series (Zhang et al, unpublished data). Bladders were instilled with GM-1111-Alexa Fluor 633 bioconjugate ($10\ \text{mg/ml}$ at $150\ \mu\text{l}$) for 45 minutes and harvested immediately thereafter ($t = 0$) or at 24 hours ($t = 24$). Tissue sections were counter-stained with 4,6-diamidino-2-phenylindole. Fluorescence imaging was done with a FV1000 Confocal IX81 microscope (Olympus®).

Tissue

Collection and histological evaluation—Bladders were removed and split longitudinally. One section was fixed in 4% paraformaldehyde and the other was processed for tissue MPO. Tissues were processed and paraffin embedded. Sections ($5\ \mu\text{M}$) were cut and stained with hematoxylin and eosin. Bladder inflammation severity was assessed via inflammatory infiltrate (PMNs) in the urothelium, submucosa, lamina propria and smooth muscle. The extent of edema, hemorrhage, urothelial erosion, ulceration and microabscess formation were observed in 10 high power fields at $10\times$ magnification per sample.¹⁷

MPO assay—Hemisected bladders were flash frozen in liquid nitrogen and stored at -80°C . Lysis buffer composed of 200 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM tris and 10% glycerin (Sigma®) and protease inhibitor cocktail (Thermo Scientific®) were added to frozen tissue samples ($200\ \mu\text{l}$ lysis buffer per 10 mg frozen tissue). Tissues were processed and MPO assays were done (Zhang et al, unpublished data).

RESULTS

LL-37 Induced Bladder Inflammation

After 1 intravesical exposure to LL-37 tissues were harvested at 12 and 24 hours, respectively. Gross inspection at 12 hours revealed moderate inflammation with focal areas of erythema, hemorrhage and global edema (fig. 1, C). At 24 hours bladders showed severe inflammation with global erythema, hemorrhage and severe tissue edema (fig. 1, D). Saline control tissues lacked inflammation (fig. 1, A and B).

We evaluated 12 and 24-hour tissues with hematoxylin and eosin. No inflammation was noted in saline controls (fig. 2, *A* and *B*). LL-37 challenged tissues at 12 hours had focal areas of urothelial ulceration, and moderate edema in the submucosa and lamina propria (fig. 2, *C*). A moderate number of PMNs were present in the urothelial, submucosa and lamina propria layers along with blood vessel margination. No microabscesses (PMN clusters) were observed. Qualitative evidence from gross and histological findings revealed that moderate tissue inflammation developed after 12 hours in all 6 mice.

In the 24-hour group histology revealed more profound inflammation (fig. 2, *D*). The qualitative amount of edema in the submucosa was similar to that in 12-hour tissues but the lamina propria showed more profound edema. Also, more PMNs were present in the urothelial, submucosa and lamina propria layers. Multiple areas of microabscesses were present. Similar patterns of PMN margination from blood vessels were seen. Gross and histological findings in the 24-hour group demonstrated severe inflammation and were consistent in all 6 mice.

The degree of inflammation was quantified using tissue MPO. Comparing 12-hour tissues showed minimal MPO activity in saline controls (11 ng/ml) and in nonmanipulated/noninstilled controls (5 ng/ml) (fig. 3). At 12 hours LL-37 challenged tissues showed a 21-fold increase in MPO activity (229 ng/ml). The 24-hour tissues showed minimal MPO activity in saline controls (14 ng/ml) but a 61-fold increase (849 ng/ml) in LL-37 challenged bladders (fig. 3).

SAGE

Abrogation of LL-37 induced bladder inflammation—Testing SAGE ability to prevent or mitigate LL-37 induced bladder inflammation was examined in 2 groups. The 4 mice in posttreatment group 1 underwent LL-37 challenge followed by immediate SAGE treatment. The 4 mice in pretreatment group 2 underwent SAGE treatment, followed by LL-37 challenge. In group 2 we evaluated whether precoating had a prophylactic effect.

In group 1 gross inspection revealed less erythema and hypervascularity but edema remained (fig. 4, *B*). Histology showed edema in the submucosa and lamina propria but the urothelium and submucosa lacked PMNs. There were also fewer PMNs in the lamina propria. Moreover, PMNs appeared to be limited to the deeper lamina propria, a finding consistent with a gradient response (fig. 4, *D*). We noted no microabscesses in SAGE treated bladders along with a paucity of PMNs rolling out of blood vessels. Inflammatory quantification by tissue MPO to compare untreated LL-37 challenged bladders vs group 1 tissues revealed a 2.5-fold decreased inflammatory response in SAGE treated bladders vs that in group 1 after SAGE treatment (LL-37, 849 vs 347 ng/ml, log scale $p = 0.220$, fig. 4, *F*).

In group 2 gross inspection showed a lack of erythema and hypervascularity with minimal edema apparent (fig. 4, *A*). Histology revealed minimal edema in the submucosa and none in the lamina propria. No evidence of PMN infiltration was observed throughout all layers and no PMNs were noted marginating out of blood vessels (fig. 4, *C*). Overall SAGE pretreated tissue almost resembled that of saline controls. Inflammatory quantification by tissue MPO to compare untreated LL-37 challenged bladders vs SAGE pretreated group 2 bladder tissues revealed a 22.3-fold decreased inflammatory response (LL-37, 849 vs 38 ng/ml, log scale $p = 0.013$, fig. 4, *E*). The histological findings coupled with statistically significant MPO results suggested that pretreatment with SAGE could serve as prophylactic anti-inflammatory therapy.

Heparin treatment showed suboptimal anti-inflammatory results. Grossly group 1 (posttreatment) showed severe inflammation with edema, hypervascularity and hemorrhage (fig. 5, *B*). Histological findings were consistent with urothelial ulceration, submucosa and lamina propria edema, abundant PMNs throughout all layers and PMNs marginating from blood vessels (fig. 5, *D*). MPO assays revealed subtle differences in inflammatory activity between untreated LL-37 challenged bladders and group 1

As bladder armor agent—To elucidate SAGE tissue coating and penetration we instilled a 10 mg/ml solution of fluorescent bioconjugate GM-1111-Alexa Fluor 633 and harvested tissues immediately ($t = 0$) or at 24 h ($t = 24$). Results in the immediate harvest group showed a uniform superficial GAG layer coating adjacent to the urothelium along with deeper penetration into the submucosa, lamina propria and superficial smooth muscle (fig. 6, *A* and *B*). Endothelial cells lining arterioles showed SAGE coating on the basal and luminal sides. No evidence of SAGE coating was observed in venules. Results in the 24-hour group revealed no evidence of SAGE along the GAG layer. SAGE was still apparent in regions of the submucosa and endothelium lining small arterioles on the basal and luminal sides (fig. 6, *C* and *D*). SAGE was visualized as intercalating in regions of bladder smooth muscle.

DISCUSSION

To create a consistent, reproducible mouse model for acute bladder inflammation we considered a potential parallel between bladder inflammation and rosacea. Normally LL-37 exists in low concentrations in urine but high levels were found during urinary tract infection episodes.⁹ Some patients with rosacea concomitantly experience symptomatology consistent with IC (www.ic-network.com). To our knowledge this clinical correlation has yet to be established but it warrants further investigation. We have preliminary data showing increased urinary LL-37 in non-infected children with spina bifida. Although more samples are necessary, these findings may substantiate the physiological importance of LL-37 in the bladder.

We first exposed bladders to LL-37 at the same concentration used for intradermal injection to reproduce rosacea-like lesions in a mouse model.⁷ We defined acute inflammation on gross and histological findings. After a single LL-37 exposure a consistent, moderate inflammatory response occurred after 12 hours with exacerbated inflammation after 24 hours. Inflammation severity was quantified by tissue MPO, further supporting gross and histological findings.

The LL-37 concentration used was increased but our goal was to establish a novel model of bladder inflammation based on a biological urinary compound. We required a model with adequate severity in which potential therapeutic agents could be tested. This model allows future investigation of inflammatory cascades induced by LL-37 and signaling pathways that are important in bladder inflammation.

To mimic physiological conditions of increased urinary LL-37, similar to what occurs during urinary tract infection episodes, chronic exposure to LL-37 is necessary. This technically challenging procedure necessitates multiple catheterizations, which is difficult based on urethral edema and small caliber of the female mouse urethra. Lastly, we extended the inflammatory period, harvesting LL-37 challenged bladders at 72 hours and noting persistent inflammation. This preliminary observation is important since few chronic bladder inflammation models exist.¹⁸ Importantly chronic bladder inflammation could show an over exuberant tissue healing response in the form of bladder fibrosis.¹⁹

Treatment for LL-37-induced inflammation with sulfated polysaccharides, heparin or SAGE was based on multiple aspects. 1) Intravesical heparin is already used to treat pelvic pain disorders with debatable benefit.^{20–23} 2) Polyanionic SAGE was effective for treating LL-37-induced inflammation of the mouse skin (Zhang et al, unpublished data). 3) Toxic cationic metabolites in urine, damage to the anionic GAG layer after an insult and influx of inflammatory cells perpetuating the initial event also support the use of GAGs to mitigate or prevent inflammation.

We first examined whether SAGE treatment after LL-37 exposure would truncate the inflammatory response. Overall we observed mild anti-inflammatory activity. On the other hand, when precoating or treating with SAGEs a profound, statistically significant anti-inflammatory effect occurred. We believe that these findings represent the ability of SAGE compounds to better mimic the GAG layer and surface coat with higher propensity, better neutralize the cationic LL-37 molecule and potentially attenuate acute inflammatory cells. We also compared SAGE GM-1111 to heparin. Despite its clinical use for bladder inflammation our heparin experiments showed poor anti-inflammatory potency. Results suggest that SAGE GM-1111 has superior anti-inflammatory activity compared to those of a standard sulfated polysaccharide such as heparin.

To elucidate SAGE coating and penetration we exposed normal bladders to fluorescently labeled SAGE. Thorough coating was evident along the GAG layer and deep tissue penetration occurred. Interestingly SAGE was clearly apparent within the endovasculture. In this model SAGE has what is to our knowledge a yet unexplained affinity for the endovasculture and it may provide endothelial cell stabilization, thereby inhibiting acute inflammatory cell activation.

CONCLUSIONS

The mechanisms perpetuating acute and chronic inflammatory events are poorly understood with even less known about pathways leading to significant bladder fibrosis. The development of novel models to explore bladder inflammation is paramount, offering the promise of unraveling new pathways perpetuating disease processes. Finally, new sulfated polysaccharides such as SAGE GM-1111 have the therapeutic potential to mitigate bladder inflammation and may serve to protect against future fibrotic events.

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LL-37 was obtained from the University of Utah Core.

Abbreviations and Acronyms

GAG	glycosaminoglycan
IC	interstitial cystitis
MPO	myeloperoxidase
NGB	neurogenic bladder
PMN	polymorphonuclear leukocyte

SAGE semi-synthetic GAG ether
t time

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Figure 1. Gross images show saline instilled control bladders harvested at 12 (A) and 24 (B) hours, and LL-37 instilled bladders harvested at 12 (C) and 24 (D) hours. Bladders were hemisected to expose inner mucosal layer. Scale bar represents 0.5 cm.

Figure 2. Histological cross section of saline instilled control bladders harvested at 12 (A) and 24 (B) hours shows no evidence of inflammation while LL-37 instilled bladders harvested at 12 (C) and 24 (D) hours show PMN infiltration (circles), microabscess (MA) formation and PMN margination out of blood vessels (rectangles). U, urothelium. SBM, submucosa. LP, lamina propria. SMC, smooth muscle cell layer. H & E, reduced from $\times 10$. Scale bar represents 75 μM .

Figure 3. Tissue MPO inflammation quantification assay of saline control (blue bars) and LL-37 (red bars) instilled bladders revealed minimal MPO activity in controls, that is 11 ng/ml at 12 and 14 ng/ml at 24 hours, but significantly increased MPO activity in LL-37 instilled bladders with continued escalation from 229 ng/ml at 12 to 849 ng/ml at 24 hours.

Figure 4. SAGE GM-1111 treatment. Gross images of bladders pre-coated/treated with 10 mg/ml GM-1111, harvested at 24 hours and hemisected to expose inner mucosal surface before LL-37 instillation (A) and those with 10 mg/ml SAGE posttreatment after LL-37 instillation (B). Scale bar represents 0.5 μ M (A and B). Tissue histological sections reveal PMNs (circles and ovals) and no significant evidence of PMN margination out of blood vessels (rectangle) in GM-111 pretreated (C) and SAGE posttreated (D) bladders. SMC, smooth muscle cell layer. LP, lamina propria. SBM, submucosa. Reduced from 10 $\times$ (C and D). Scale bar represents 75 μ M (C and D). Tissue MPO assay for GM-1111 pre-coating/treatment revealed 22-fold decrease in MPO activity in bladders instilled with heparin followed by LL-37 (purple bars) vs that in bladders instilled with LL-37 only (red bar) ($p = 0.013$) (E). Tissue MPO assay for SAGE posttreatment demonstrated 2.5-fold decrease in MPO activity in posttreated samples instilled with LL-37 only (purple bar) vs that in bladders instilled with LL-37 followed by heparin ($p = 0.220$) (F).

Figure 5.

Heparin treatment. Gross images of bladders harvested at 24 hours and hemisected to expose inner mucosal surface of bladders precoated/treated with 10 mg/ml heparin before LL-37 instillation (A) vs 10 mg/ml heparin posttreatment after LL-37 instillation (B). Scale bar represents 0.5 cm (A and B). Tissue histology shows bladders precoated/treated with 10 mg/ml heparin before LL-37 instillation (C) vs 10 mg/ml heparin posttreatment after LL-37 instillation (D). SMC, smooth muscle cell layer. LP, lamina propria. SBM, submucosa. Reduced from $\times 10$ (C and D). Scale bar represents 75 μ m (C and D). Tissue MPO assay revealed slightly decreased MPO activity in heparin precoated/treated bladders instilled with SAGE followed by LL-37 (purple bar) vs that in bladders instilled with LL-37 only (red bar) (E). Tissue MPO assay for heparin posttreatment showed no significant decrease in posttreated bladders instilled with LL-37 followed by SAGE (purple bar) vs that in bladders instilled with LL-37 only (red bar) (F). heparin posttreated tissues (LL-37, 849 vs 827 ng/ml, fig. 5, F). Grossly group 2 (pretreatment) showed similar findings (fig. 5, A). Histological findings mirrored those in group 1 (fig. 5, C). MPO assays revealed only slight differences in inflammatory activity between untreated LL-37 challenged bladders and group 2 heparin pretreated tissues (LL-37, 849 vs 759 ng/ml, fig. 5, E).

Figure 6.

SAGE coating as bladder armor. Tissues immediately harvested tissues after 10 mg/ml GM-1111-Alexa Fluor-633 instillation show uniform coating (green fluorescent area) of urinary GAG layer adjacent to urothelium (*U*) along with deeper penetration into submucosa (*SBM*), lamina propria (*LP*) and superficial smooth muscle (*SMC*) (*A* and *B*). Endothelial cells lining arterioles demonstrated significant GM-1111-Alexa Fluor 633 coating on basal and luminal sides (rectangles). Reduced from $\times 10$ (*A*) and $\times 20$ (*B*). Scale bars represent 75 (*A*) and 37.5 (*B*) μM . Tissues harvested 24 hours after 10 mg/ml SAGE instillation show no evidence of GM-1111-Alexa Fluor 633 along urinary GAG layer but coating was still strongly apparent in select submucosal regions and along endothelium lining small arterioles on basal and luminal sides (rectangles) (*C* and *D*). Coating was still noted intercalating in random smooth muscle cell layer regions. Reduced from $\times 20$ (*C* and *D*). Scale bar represents 75 μM (*C* and *D*).