Published in final edited form as:

J Urol. 2016 June ; 195(6): 1920–1926. doi:10.1016/j.juro.2015.12.090.

Liposome-based Intravesical Therapy targeting Nerve Growth Factor (NGF) ameliorates Bladder Hypersensitivity in Rats with Experimental Colitis

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Abstract

Purpose—Pelvic organ cross sensitization is considered to contribute to overlapping symptoms in CPPS. Overexpression of NGF in the bladder is reportedly involved in the symptom development of BPS/IC patients. This study examined whether a reduction of overexpressed NGF in the bladder by intravesical treatment with liposome and OND conjugates ameliorates bladder hypersensitivity in a rat colitis model.

Materials and Methods—Adult female rats were divided into; (a) a control group, (b) a colitis-OND group with intracolonic TNBS enema and intravesical liposomal-OND treatments, (c) a colitis-saline group with intracolonic TNBS and intravesical saline treatments, (d) a sham-OND group with intravesical liposomal-OND treatment without colitis and (e) a sham-saline group with intravesical saline treatment without colitis. Liposomes conjugated with NGF antisense OND or saline solution were instilled into the bladder, and 24 hours later, colitis was induced by TNBS enema. Effects of NGF antisense treatment were evaluated by pain behavior, cystometry, molecular analyses and immunohistochemistry 10 days after TNBS treatment.

Results—In colitis-OND rats, the NGF antisense treatment ameliorated pain behavior, and decreased a reduction in intercontraction intervals in response to acetic acid stimulation as well as NGF expression in the bladder mucosa, which were all enhanced in colitis-saline rats compared to sham rats.

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Conclusions—NGF overexpression in the bladder mucosa and bladder hypersensitivity induced after colitis were reduced by intravesical application of liposomal OND targeting NGF, suggesting that the local anti-NGF therapy could be effective for the treatment of bladder symptoms in CPPS.

Keywords

Chronic pelvic pain syndrome; Irritable bowel syndrome; Bladder pain syndrome; interstitial cystitis; Liposome; Nerve growth factor; Antisense

Chronic pelvic pain syndrome (CPPS) including bladder pain syndrome/interstitial cystitis (BPS/IC) and irritable bowel syndrome (IBS) is defined as a disease entity with painful symptoms in the pelvic region that last for at least six months and presents a major challenge to patients and health care providers ^{1, 2}. Symptoms of BPS/IC and IBS are often overlapped as one-third of patients diagnosed with BPS/IC exhibit symptoms consistent with IBS, while 25–56% of patients diagnosed with IBS also have symptoms of BPS/IC ^{3, 4}. In order to explain this complex pathology, pelvic organ “cross sensitization” has been proposed to contribute to the clinically overlapping symptoms of CPPS ⁵. We also recently demonstrated that rats with experimental colitis exhibit enhanced pain sensitivity in the bladder as evidenced by increased freezing behavior induced by intravesical nociceptive stimulation ⁶. According to the AUA guidelines, BPS/IC is defined as a symptom complex of an unpleasant sensation, which includes not only pain, but also other hypersensitive symptoms such as pressure and/or discomfort, associated with lower urinary tract symptoms of more than six weeks duration, in the absence of infection or other identifiable causes² Thus, bladder hypersensitivity seems to be a part of BPS/IC. In this study, we therefore used rats with experimental colitis as an animal model of bladder hypersensitivity.

Nerve growth factor (NGF) is known to be a complex regulator of sensory afferent plasticity in response to injury or inflammation ^{7, 8}. Increased levels of NGF are found in urine obtained from BPS/IC patients ⁹, and a recent meta-analysis study reported that urinary NGF could be a useful biomarker for the diagnosis of BPS/IC as well as a predictive biomarker to help guide treatments ¹⁰. We also recently reported that instillation of liposomes conjugated with antisense oligonucleotide (OND) targeting NGF into the bladder suppressed bladder overactivity in a rat model of acute cystitis ¹¹. However, it is still unknown if NGF contributes to bladder overactivity and enhanced bladder pain sensitivity after colonic inflammation. Therefore, this study investigated whether intravesical liposomal-OND treatment can suppress NGF expression in the bladder and bladder hypersensitivity in a rat model of experimental colitis.

Materials and Methods

Animal model

Experiments were performed in accordance with NIH guidelines, and the protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Adult female Sprague-Dawley rats were used and divided into 5 groups; (1) control group (no treatment), (2) colitis-OND group with intracolonic 2,4,6trinitrobenzen sulfonic acid (TNBS) enema and intravesical liposomal-OND treatments, (3) colitis-saline group with

intracolonic TNBS and intravesical saline treatments, (4) sham-OND group with intravesical liposomal-OND treatment without colitis and (5) sham-saline group with intravesical saline treatment without colitis.

Liposome-conjugated NGF antisense instillation

At 24hr before injection of TNBS (day 0), rats were anesthetized with 2% isoflurane (Baxter Inc., IL), and catheterized through the urethra into the bladder using a 24-gauge angiocatheter. After draining urine from the bladder, 12 μ M of phosphorothioated NGF antisense OND with the sequence 5'GCCCCGAGACGCCTCCCGA 3' complexed with liposomes or saline (vehicle) in a volume of 0.2ml was instilled and kept for 30 minutes as we previously described¹¹. Rats were then allowed to recover from anesthesia and void voluntarily to expel the solution from the bladder. In our previous study¹¹, liposomes with scramble-OND did not show the therapeutic effects on bladder overactivity and mucosal NGF expression in cystitis rats. Also, our preliminary experiments showed no effects of liposomes with scramble-OND on RTx-induced bladder overactivity in colitis rats. Therefore, we did not add plain liposome groups of sham or colitis rats in this study.

Colitis model

Colitis was induced by administration of TNBS solution (50mg/ml), which was prepared by mixing 1ml of TNBS, 1.93ml of H₂O and 2.93ml of ethanol. Rats were fasted for 24 hours before instillation, anesthetized with 2% isoflurane and inserted with a polyethylene catheter attached to a 1-ml syringe into the colon 6 cm proximal to the anus. The lower body of rats was elevated by lifting the tale, and TNBS or vehicle (50% ethanol) solution in a volume of 0.5ml was injected and kept for 3 minutes (day 1). The following behavioral study (n=24), cytometry (n=27) and tissue harvest for molecular analyses (n=25) were performed at day 10 after TNBS or vehicle treatment using separate groups of animals.

Conscious Cystometry

Twenty-seven rats (5 or 6 rats per group) were used for cystometric evaluation. After anesthesia with 2 % isoflurane, laparotomy through a lower abdominal incision was performed and a PE 50 tube (Scientific Commodities Inc., AZ, USA) with the distal end sealed by heat was inserted into the bladder dome as a cystostomy catheter. The catheter was tunneled subcutaneously and placed underneath the back skin 3 days before cystometry (CMG). Thereafter, the abdomen was closed with running sutures.

Ten days after TNBS or vehicle treatment, rats were anesthetized with 2 % isoflurane and a distal end of cystostomy tube was exteriorized from the subcutaneous space. After recovery from anesthesia, rats were placed in restraining cages (Yamanaka Chemical Ind., Japan). The cystostomy catheter was connected through a three-way stopcock to a pressure transducer (BLPR2, World Precision Instruments, Inc., Sarasota, FL, USA) and to a syringe pump (Harvard Apparatus, Holliston, MA, USA). After rats were acclimated in a cage for 1 hour, CMG was performed by filling the bladder with physiological saline (0.04ml/min) to elicit repetitive voiding. The intravesical pressure was recorded using data-acquisition software (sampling rate was 400 Hz. Chart, AD Instruments, Colorado Springs, CO, USA) on a computer system (Power Lab, AD Instruments). During CMG, at least 10 reproducible

micturition cycles were recorded after an initial stabilization period (15–30min). Intercontraction intervals (ICIs), which are the time between 2 consecutive micturition cycles, were measured 30 min after saline infusion or 60 min after acetic acid (AA) infusion and averaged from at least 3 ICIs.

Nociceptive behavioral study

Twenty-four rats (4–5 rats per group) were used for analyses of nociceptive behavior induced by bladder irritation, as previously described ¹². Briefly, ten days after TNBS or vehicle treatment, rats were acclimated in metabolic cages (Nalgene, Rochester, NY, USA) for 3 h. Then, water (30ml/kg) was administered orally, and after 15 min, animals placed in a Bollman cage were instilled with resiniferatoxin (RTX; 0.3 µM, 0.3 ml) into the bladder via a temporally inserted urethral catheter (PE-50) for 1 min. Thereafter, the urethral catheter was removed, and rats were placed back to metabolic cages. Licking and freezing behaviors were then scored for a period of 15 min with 5-s intervals by one observer (N.K.) in a blinded manner and, if licking or freezing behavior occurred during a 5-second interval, it was scored as 1 positive event as reported in our previously study ¹².

Quantification of messenger RNA

Twenty five rats (n=5 in each group) were used to measure mRNA and protein levels of NGF. The bladder was harvested at day 10 after TNBS or vehicle was given. The bladder was separated into mucosal and detrusor layers under a microscope. Total RNA was extracted by using the Rneasy kit (Quagen, Hilden, Germany), and 2 µg of total RNA was reverse-transcribed into complementary DNA using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Quantitative polymerase chain reaction (PCR) was performed with an Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) in a 25 µl volume using SYBR Green PCR Master Mix (QIAGEN, Valencia, CA, USA). Amplification of cDNA was performed using OND primers specific for NGF or β2MG as a control gene. OND primer sequences were as follows: 5'-TCCACCCACCCAGTCTTCCA-3' (forward, NGF), 5'-GCCCTCCTGCTGAGCACACA-3' (reverse, NGF), 5'-GACCGATGTATATGCTTGCAGAGT-3' (forward, β2MG), 5'-GGATCTGGAGTTAACTGGTCCAG-3' (reverse, β2MG). The protocol consisted of 40 replication cycles.

Measurement of NGF protein level

Eighteen rats (3–4 rats in each group) were used. The bladder was divided into mucosal and detrusor layers. Tissues were homogenized in RIPA lysis buffer system (Santa Cruz Biotechnology Inc., USA) in the presence of 1mM Na₃VO₄, 2mM PMSF and 10µL/mL protease inhibitor. Protein concentration was measured by using Pierce BCA protein Assay kit (Thermo Scientific, USA). Lysates of separated mucosa and detrusor tissues were stored at –80°C until assays. The samples were assayed in duplicate using enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, WI, USA), according to the manufacturer's instruction, and ELISA plates were read at 450 nm wave on an Elx800 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Tissue NGF values were

normalized against protein concentrations of each sample and expressed as picograms per microgram protein.

Immunohistochemistry

Ten days after TNBS or vehicle treatment, rats were perfused transcardially with cold physiological saline containing heparin sodium (1 unit/ml) followed by cold 4 % paraformaldehyde solution in 0.1M phosphate buffer (PFA). Then the bladder was harvested and post-fixed in 4% PFA at 4 °C for 24 h and soaked in the 20 % sucrose overnight at 4°C. The frozen tissues were then cut at 10 µm thickness (transverse sections) and mounted onto slides. Immunohistochemical staining was performed by using Dako EnVision+ System-HRP Labelled Polymer (Dako Cytomation, Glostrup, Denmark) with anti-NGF antibodies (1:250; sc-548, Santa-Cruz, Heidelberg, Germany). An antigen retrieval was performed in the reagent of HistVTone at 70°C (Nacalai, Kyoto, Japan) for 20 min. Background activities were blocked with the blocking agent (Dako, Glostrup, Denmark) at room temperature for 1 h. Reaction products were visualized by Liquid DAB+ Substrate Chromogen System (Dako, Denmark).

Statistical analysis

The data were expressed as mean $\pm$ SEM and were analyzed using GraphPad Prism 6.0 statistical software (San Diego, CA). Statistical differences among groups were determined by one-way ANOVA followed by Turkey's post-hoc test. A statistical comparison of mean values between two groups was performed using Mann-Whitney test with Bonferroni correction. P values less than 0.05 were considered to be statistically significant.

Results

Nociceptive behavior

The number of licking behavior events in the colitis-saline group (mean 68.3 ± 6.8) was significantly higher than that in the control group (mean 26.7 ± 6.7), whereas there was no significant difference in licking behavior score compared to other 3 groups including the colitis-OND group (mean colitis-OND: sham-saline: sham-OND = 44.6 ± 5.9 : 41.0 ± 8.2 : 44.7 ± 4.2 , respectively) (Figure 1A). In contrast, the number of freezing events in the colitis-saline group (mean 26.5 ± 6.1) was higher than that in other groups (mean control: colitis-OND: sham-saline: sham-OND = 0: 8.2 ± 2.6 : 11 ± 3.9 : 7.3 ± 1.9 , respectively). These results indicate that the increase of freezing events, but not licking events, after colitis was prevented in the colitis-OND group to the same level as in sham groups (Figure 1B).

Cystometry

There was no significant difference in ICIs among groups before 0.1 % AA infusion (control: colitis-saline: colitis-OND: sham-saline: sham-OND = 1216 sec: 1686 sec: 1520 sec: 1029 sec: 1564 sec, respectively) (Figure 2A). The reduction rate of ICIs after AA infusion in the colitis-saline group (0.436) was significantly greater than those in colitis-OND (0.928) and sham-saline groups (0.970) (one-way ANOVA followed by Turkey's post-hoc test) and those in control and sham-OND groups (Mann-Whitney test) (Figure 2B).

Other cystometric parameters such as maximum pressure at voiding or post-void residual volume were not significantly different among groups (data not shown).

Quantitative mRNA analysis of NGF in the bladder mucosa and detrusor

The mRNA expression of NGF in the bladder mucosa in the colitis-saline group was significantly higher compared to the colitis-OND group (one-way ANOVA followed by Turkey's post-hoc test) (Figure 3A). In addition, there was a tendency of increased NGF mRNA expression in the detrusor of colitis-saline rats vs. sham-saline rats although the difference was not significant (one-way ANOVA), and no reduction was seen in the detrusor NGF expression of colitis-OND rats compared to colitis-saline rats (Figure 3B).

NGF protein levels in the bladder mucosa and detrusor

The protein level of NGF in the bladder mucosa in the colitis-saline group was significantly higher than those in control, colitis-OND and sham-OND groups (Figure 4A). There was no significant difference in NGF protein levels of the detrusor among groups (Figure 4B).

Immunohistochemistry of NGF expression

Immunohistochemical staining of the bladder for NGF showed a high level of positive staining in the bladder urothelial layer of the colitis-saline group in contrast to faint staining in control and colitis-OND groups (Figure 5 b, a and c, respectively).

Discussion

The results of our study demonstrated that: (1) rats with TNBS-induced colitis exhibited enhanced bladder pain sensitivity and bladder overactivity in response to nociceptive bladder stimuli using intravesical infusion of RTX and AA, respectively, (2) TNBS-induced colitis increased NGF expression in the bladder mucosa and (3) intravesical instillation of liposomes with antisense OND targeting NGF ameliorated bladder pain behavior and bladder overactivity in association with a reduction of NGF overexpression in the bladder mucosa.

This study confirmed our previous findings that freezing and licking behaviors, which predominantly correspond to bladder and urethral pain induced by activation of bladder and urethral afferent pathways, respectively^{12–14}, are enhanced after colitis (i.e., enhanced pain behavior in the colitis-saline group vs. the control group)⁶. We also further demonstrated that colitis induces bladder overactivity as evidenced by the significantly larger reduction in ICIs after AA stimulation in the colitis-saline group vs. the control or sham-saline group. Our previous study using myeloperoxidase assay showed that TNBS-induced colitis elicited neutrophil infiltration in the colon but not in the bladder or the urethra⁶. Therefore, it is assumed that increased bladder pain sensitivity in rats with TNBS-induced colitis is produced by the indirect mechanisms other than inflammatory cell infiltration, which could include neurogenic inflammation due to afferent sensitization in the bladder¹⁵.

Previous studies reported that pelvic organ cross sensitization is induced by activation of dichotomized afferents innervating different pelvic organs and that activation of nociceptive

C-fiber afferents in one organ (e.g., colon) could sensitize afferent pathways in another organ (e.g., bladder) to release neuropeptides such as substance P that trigger neurogenic inflammation and mast cell activation^{6, 15, 16}. This study further demonstrated that the colon-to-bladder cross sensitization after colitis also induces overexpression of NGF in the bladder mucosa including the urothelial layer (Figs. 3–5) and that urothelially expressed NGF after colitis is a key mediator that increases bladder pain sensitivity because intravesical treatment with liposome and NGF antisense conjugates significantly reduces bladder pain behavior (i.e., freezing behavior) (Fig. 1) and bladder overactivity induced by nociceptive stimuli in the bladder (Fig. 2). Previous clinical studies showed that urinary NGF could be a useful biomarker for the diagnosis of BPS/IC^{9, 10}. NGF has also been highlighted as a chemical mediator in experimental animal models of bladder hypersensitivity^{17–19}, and chronic administration of NGF into the bladder wall or into the spinal cord induced bladder overactivity and increased excitability of bladder afferent neurons in rats^{20–22}. It has also been demonstrated that the majority of afferent nerves are located in the suburothelial layer to interact closely with urothelially excreted substances including NGF²³. Thus, NGF overexpression in the bladder mucosa induced by the colon-to-bladder cross sensitization following colitis is likely to stimulate bladder afferent pathways to enhance bladder pain sensitivity.

In clinical studies, monoclonal NGF antibody was systemically administered to treat the symptoms in patients with BPS/IC or other chronic pain syndromes²⁴. In these studies, systemic adverse events including headache, hyperesthesia, abnormal peripheral sensation and dizziness have been reported as obstacles for the systemic NGF antibody therapy to become a feasible treatment of pain, although some studies showed therapeutic effects on pain symptoms²⁴. Thus, the intravesical therapy targeting NGF expressed in the bladder could be an alternate option for the treatment of BPS/IC. We have previously shown that liposomal application is necessary to deliver NGF antisense OND to the bladder urothelium to suppress NGF overexpression and bladder overactivity in a rat model of acute cystitis¹¹. In addition, Chuang et al. reported that intravesical application of empty liposomes significantly decreased painful and urinary urgency symptoms in 24 BPS/IC patients²⁵. Recent clinical studies have also demonstrated that the intravesical treatment using liposome-encapsulated onabotulinum toxin-A significantly improved symptoms in patients with overactive bladder without severe adverse events^{26, 27}. Therefore, local suppression of NGF in the bladder using intravesical liposome-based delivery techniques could be an attractive approach for the treatment of bladder symptoms, while avoiding systemic side effects, in patients with BPS/IC and IBS.

There are some limitations of this study. First, because liposomal NGF antisense conjugates were applied one day prior to the colitis induction, further studies are needed to elucidate if the liposomal NGF antisense therapy can ameliorate bladder hypersensitivity after colitis is evoked. Secondly, this study used only one dose of NGF antisense OND based on our previous study¹¹; therefore, the dose-response relation should be examined to determine the optimal dose in a future study.

Conclusion

We showed that intravesical treatment with liposome and NGF antisense conjugates prevented NGF overexpression in the bladder and attenuated bladder hypersensitivity in a rat colitis model. Therefore, the liposome-based antisense treatment targeting NGF in the bladder could be a new, effective modality for the treatment of bladder pain in CPPS patients, in whom the cross-sensitization mechanism is involved in the emergence of overlapping symptoms from different pelvic organs.

Acknowledgments

This work was supported by grants from DOD (W81XWH-12-1-0565) and NIH (DK088836 and P01DK093424).

Abbreviations

AUA	American Urological Association
NGF	nerve growth factor
CPPS	chronic pelvic pain syndrome
OND	oligonucleotide
BPS/IC	bladder pain syndrome/interstitial cystitis
IBS	irritable bowel syndrome
TNBS	intracolonic 2,4,6-trinitrobenzen sulfonic acid

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Figure 1.

The number of nociceptive behavior events, licking (A) and freezing (B), induced by intravesical injection of resiniferatoxin (RTx).

(A) The number of licking behavior events in the colitis-saline group was significantly higher than that in the control group, whereas there was no difference in licking score compared to other 3 groups. (B) Freezing behavior events in the colitis-saline group were increased significantly, compared to controls; however, the increase of freezing events was significantly decreased in the colitis-OND group to the same level as in sham-groups. . *: $p < 0.05$, **: $p < 0.01$ (compared to each group; one-way ANOVA followed by Turkey's post-hoc test)

Figure 2.

Effects of intravesical infusion with acetic acid (AA) on intercontraction intervals (ICIs) in cystometry. (A) ICIs before AA infusion. (B) The reduction ratio of ICIs after AA infusion to ICIs before AA infusion.

There was no significant difference in ICIs before AA infusion among groups (A). The ICI reduction rate after AA infusion in the colitis-saline group was significantly higher than that in the colitis-OND group (B). * $P < 0.05$ (one-way ANOVA followed by Turkey's post-hoc test). # $P < 0.05$ (compared to the colitis-saline group; Mann-Whitney test).

Figure 3.

The mRNA expression of NGF in the bladder mucosa (A) and detrusor (B) 10 days after intracolonic injection of TNBS or vehicle (50 % ethanol). * $P < 0.05$ (one-way ANOVA followed by Turkey's post-hoc test).

Figure 4.

The protein expression of NGF in the bladder mucosa (A) and detrusor (B) 10 days after intracolonic injection of TNBS or vehicle (50 % ethanol). * $P < 0.05$ (compared to each group; one-way ANOVA followed by Turkey's post-hoc test).

Figure 5.

Photomicrographs of NGF staining in the rat bladder. There was increased positive staining for NGF in the urothelial layer of the colitis-saline group (B,b), in contrast to faint staining in the mucosal layer of the bladder from the control group (A,a) and the colitis-OND group (C,c). Magnification of pictures (A,B,C) and (a,b,c) is $\times 100$ and $\times 400$, respectively. The magnified areas of picture A, B and C, which are shown in pictures a, b and c, respectively, are indicated by rectangular boxes.