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Nerve Growth Factor Levels are Increased in Urine but Not Urothelium in Patients With Detrusor Overactivity

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Abstract

Objective: Urinary nerve growth factor (NGF) has gained great interest in detecting detrusor overactivity (DO) in patients with overactive bladder syndrome. However, the source of urinary NGF has not been fully elucidated. We investigated the relationship of urinary NGF levels and NGF expression in the urothelium and suburothelium of patients with idiopathic DO.

Materials and Methods: Bladder tissues not involving the muscle layer were obtained from 18 patients with urodynamic DO with or without bladder outlet obstruction. Urine samples at full bladder were also collected for urinary NGF measurement. Fourteen bladder and urine samples obtained from patients with stress urinary incontinence but no lower urinary tract symptoms served as controls. Urinary NGF levels were measured by ELISA. Expression of NGF in the urothelium was measured by immunohistochemical staining using anti-human antibody, and stained sections were captured by a digital image system. Correlation analysis between the urothelial NGF levels in the bladder tissue and urinary NGF/creatinine (Cr) were performed.

Results: Urinary NGF/Cr levels were significantly higher in DO patients (0.78 ± 1.26) compared with the control group (0.01 ± 0.02; $p=0.02$). However, NGF expression in the DO urothelium (125.87 ± 21.79) were not significantly higher than the controls (135.60 ± 13.50; $p=0.142$). Correlation between urinary and urothelial NGF levels were not significant in both controls and patients with DO (Spearman's $r=-0.32$, $p=0.26$ and Spearman's $r=0.28$, $p=0.22$, respectively).

Conclusion: DO patients have higher urinary NGF/Cr levels than controls. However, urothelial NGF expression was no different between the two groups. The results suggest elevated urinary NGF levels in DO patients might not be due to urothelial NGF overproduction. (*Tzu Chi Med J* 2010;22(4): 165–170)

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1. Introduction

Nerve growth factor (NGF) has been implicated as a chemical mediator of pathology-induced changes in C-fiber afferent nerve excitability and reflex bladder activity [1,2]. The levels of neurotrophic factors including NGF increase in the bladder after spinal cord injury [1,3], and increased levels of NGF have been detected in the lumbosacral spinal cord and dorsal root ganglia of rats after spinal cord injury [4]. It has been demonstrated that chronic administration of NGF into the spinal cord or chronic administration of NGF into the bladder of rats induces bladder hyperactivity and increases the firing frequency of dissociated bladder afferent neurons [2,4–7].

The stimulus for NGF production in the bladder is due in part to stretch of the bladder after bladder outlet obstruction (BOO). Stretching bladder smooth muscle cells *in vitro* increases mRNA for NGF and stimulates the secretion of NGF [8]. Protein synthesis inhibitors suppress the stretch-evoked secretion. These results indicate that mechanical stretch activates cellular machinery for the production and secretion of NGF which in turn acts on sensory nerves in the bladder to enhance afferent input to the spinal cord and enhance reflex bladder activity.

Human bladder tissue obtained from subjects undergoing suprapubic prostatectomy for outlet obstruction had more than twice the level of NGF than tissue obtained by cystoscopy from patients who were being evaluated for conditions other than obstruction [9]. There could be a difference of NGF concentration in the detrusor and superficial bladder tissue. Increased levels of urinary NGF have also been detected in patients with BOO exhibiting overactive bladder (OAB) symptoms. Following successful medical treatment that reduced the symptoms, the urinary NGF levels were reduced [10]. It was concluded that urinary NGF levels can be used as a biomarker for OAB and detrusor overactivity (DO) and as a method for assessing successful therapies.

There are only a few clinical studies of NGF concentrations in bladder tissue. Lowe et al found that the levels of NGF were higher in samples from patients with painful bladder conditions than in controls. Immunostaining showed increased NGF expression in the urothelium, which was most marked in patients with idiopathic sensory urgency [11]. However, a recent study measuring NGF concentrations (using ELISA) in superficial bladder biopsies from 12 women with DO and 15 who did not have urodynamic DO did not show a significant correlation between tissue NGF levels and DO [12].

Urothelial dysfunction is known to act as a key contributing factor in interstitial cystitis/painful bladder syndrome (IC/PBS). Whether urothelial dysfunction exists in OAB has not been elucidated. A previous study has shown that adherens junctions are decreased in patients with DO and BOO [13,14]. It is likely that urothelial dysfunction also plays a role in DO and OAB. Whether the elevated urinary NGF in the bladders of patients with DO comes from the urothelium or detrusor remains undetermined. How the NGF protein is excreted into the vesical lumen has also not been explored. We thought that urothelial dysfunction could be a cause for the increased NGF level in the urine of patients with DO. This study investigated the relationship of urinary NGF levels and NGF expression in the urothelium of patients with DO.

2. Materials and methods

2.1. Patients and specimens

Bladder tissue not involving the muscle layer was obtained from 18 patients with urodynamic DO who underwent intravesical botulinum toxin A injection for urinary incontinence. Urine samples at full bladder were also collected for urinary NGF measurement. Fourteen bladder and urine samples obtained from patients with stress urinary incontinence but no lower urinary tract symptoms served as controls. This study was approved by the institutional review board of the hospital. Informed consent was obtained before the surgical procedure was performed.

2.2. Measurement of urinary NGF levels

Measurement of urinary NGF levels was performed by the ELISA method using nondiluted urine samples. Voided urine was put on ice immediately and centrifuged at 3000*g* for 10 minutes at 4°C. The supernatant was separated into aliquots in 1.5 mL tubes and preserved in a –80°C freezer. At the same time, 3 mL urine was taken to measure the urinary creatinine (Cr) level. The urinary NGF concentration was determined using the Emax ImmunoAssay System (Promega, Madison, WI, USA) with a specific and highly sensitive ELISA kit, which has a minimum sensitivity of 7.8 pg/mL. The amount of NGF in urine samples which had levels below the detection limits of the NGF assay was extracted from an NGF standard curve. We ran samples in triplicate. If the urinary NGF levels were not consistent after three measurements, the assay was repeated and the values averaged. When the urinary NGF concentration was higher than the upper detection limit (250 pg/mL), the urine samples were diluted to fit the detection limit. For urine samples with NGF concentrations lower than the detectable limit but above zero, a concentration method was performed using a column-protein concentration kit (Amicon Ultra-15; Millipore, Billerica, MA USA). The total urinary NGF

levels were further normalized by the concentration of urinary creatinine (NGF/Cr) level [15].

2.3. *Immunohistochemistry staining of urothelium NGF*

The urinary bladder specimens were immersed and fixated for 1 hour in an ice-cold solution of 4% formaldehyde in phosphate buffered saline (pH 7.4) and rinsed with ice-cold phosphate buffered saline containing 15% sucrose overnight. Biopsy specimens were embedded in OCT medium (Miles, Elkhart, IN, USA) and stored at –80°C. Four sections per specimen were cut in a cryostat at 8 μm and collected on polysine slides. Sections were post-fixed in acetone at –20°C and blocked with blocking solution (BioGenex Laboratories Inc., San Ramon, CA, USA). The sections were incubated overnight at 4°C with primary antibodies of anti-human NGF antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After rinsing the sections with 1% Tween-20 phosphate-buffered saline, DAB staining was performed by the Super Sensitive Polymer-HRP IHC Detection System (BioGenex Laboratories Inc.). NGF stained bladder sections were captured by digital image system and the images were converted to gray-level. Density of NGF expression was quantified with the Image J software, developed by the National Institutes of Health. Three tissue sections per patient were quantified and the mean values were used in the subsequent statistical analyses.

2.4. *Statistical analyses*

Correlation analysis between the bladder tissue NGF in the urothelium and urinary NGF/Cr were performed by Pearson's correlation. The concentrations of urinary NGF and bladder tissue NGF were compared by the Mann-Whitney test. A p value <0.05 was considered significant.

3. Results

3.1. *Urinary and urothelial NGF in DO and controls*

Urinary NGF/Cr levels were significantly higher in patients with DO (0.78±1.26 pg/mg) than controls (0.01±0.02 pg/mg; $p=0.02$). However, NGF expression in the DO urothelium (125.87±21.79) were not significantly higher than controls (135.60±13.50; $p=0.142$; Table 1). Expression of NGF in the urothelium of the bladder of DO and controls are shown in Fig. 1.

Table 1 — Bladder tissue nerve growth factor (NGF) expressions and urine NGF in patients with detrusor overactivity (DO) and controls*

	Controls ($n=14$)	DO ($n=18$)	p (control *vs.* DO)
Urothelial NGF	135.60±13.50	125.87±21.79	0.142
Urine NGF (pg/mL)	0.85±1.68	40.70±56.60	0.006†
Urine NGF/Cr (pg/mg)	0.01±0.02	0.78±1.26	0.020†

*Data presented as mean±standard deviation; †$p<0.05$. Cr=creatinine.

3.2. *Correlation between urine NGF and bladder tissue NGF expression*

The urinary NGF/Cr levels were not correlated with urothelial NGF expression in both controls and patients with DO (Spearman's $r=-0.32$, $p=0.26$ and Spearman's $r=0.28$, $p=0.22$, respectively; Fig. 2).

4. Discussion

The results of this study revealed that although urinary NGF levels were higher in DO bladders, urothelium NGF expression was not increased in the DO bladders compared with the controls. It is possible that the elevated urinary NGF in the DO bladder is caused by excretion of NGF from smooth muscle cells or infiltrated inflammatory cells into the urine through the dysfunctional urothelium.

NGF is a small secreted protein that induces the differentiation and survival of particular target neurons. NGF can be produced from the urinary bladder through protein kinase C and protein kinase A-dependent intracellular pathways to play physiologic and pathophysiologic roles in the lower urinary tract [16]. Exogenous NGF may sensitize afferent nerves and induce bladder hyperactivity [1,2], and is associated with the mechanical stretch and reflex bladder activity [17]. Normally, NGF exists in the afferent nerves and ganglia of the bladder wall and is responsible for a normal sensation of bladder distention and conduction of abnormal sensory input [8].

In a recent study using a transgenic mouse model, chronic urothelial NGF overexpression led to neuronal proliferation, focal increases in urinary bladder mast cells, increased urinary bladder reflex activity, and pelvic hypersensitivity. Overexpression of NGF in mouse urothelium led to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function [18].

Visceral epithelia have been demonstrated to be a major source of NGF production, and NGF may regulate the function of adult visceral sensory and motor neurons [19]. Stretching of the urothelium during bladder distention might induce production of NGF in the

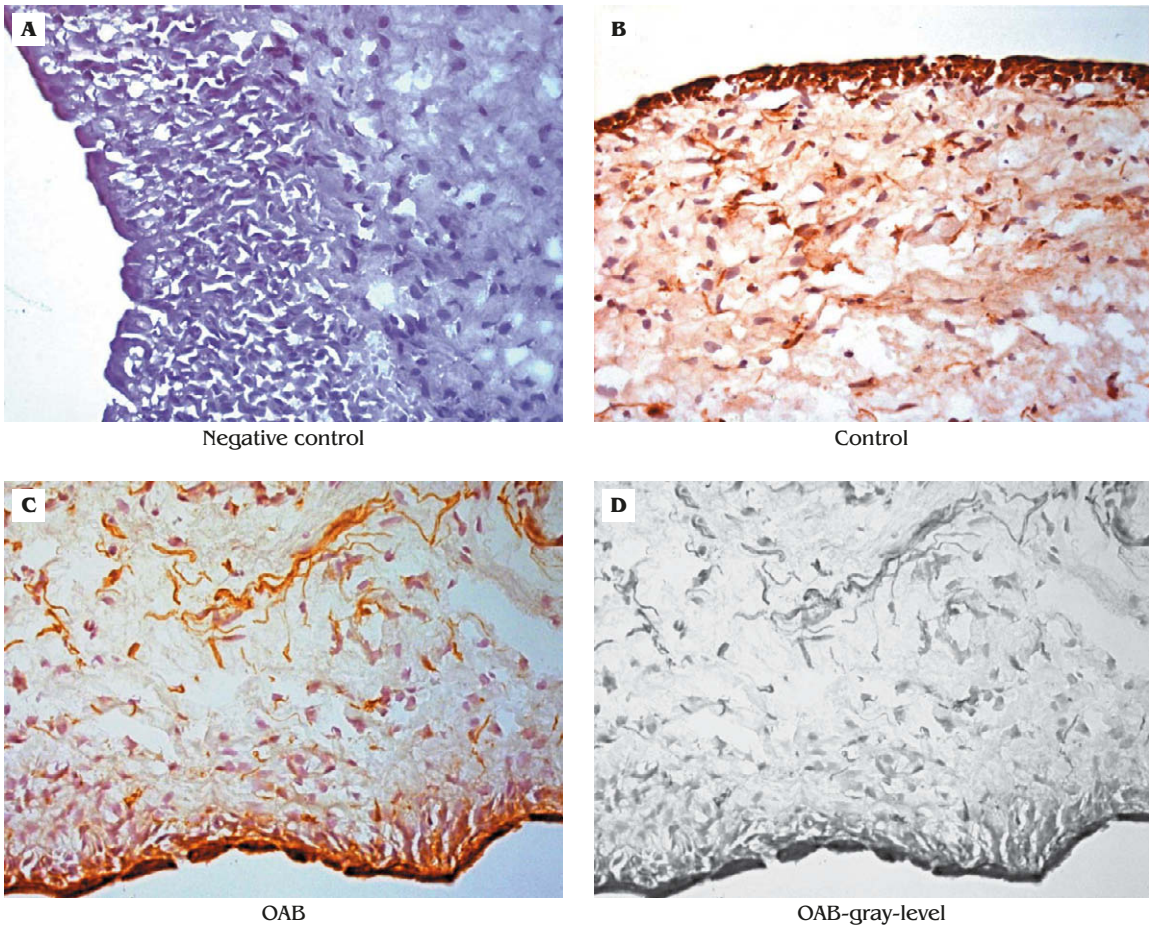

Fig. 1 — Immunohistochemical staining of nerve growth factor in the urothelium and suburothelium in the bladder tissues of patients with detrusor overactivity (overactive bladder) and controls. The density of nerve growth factor expression was calculated by Image J software.

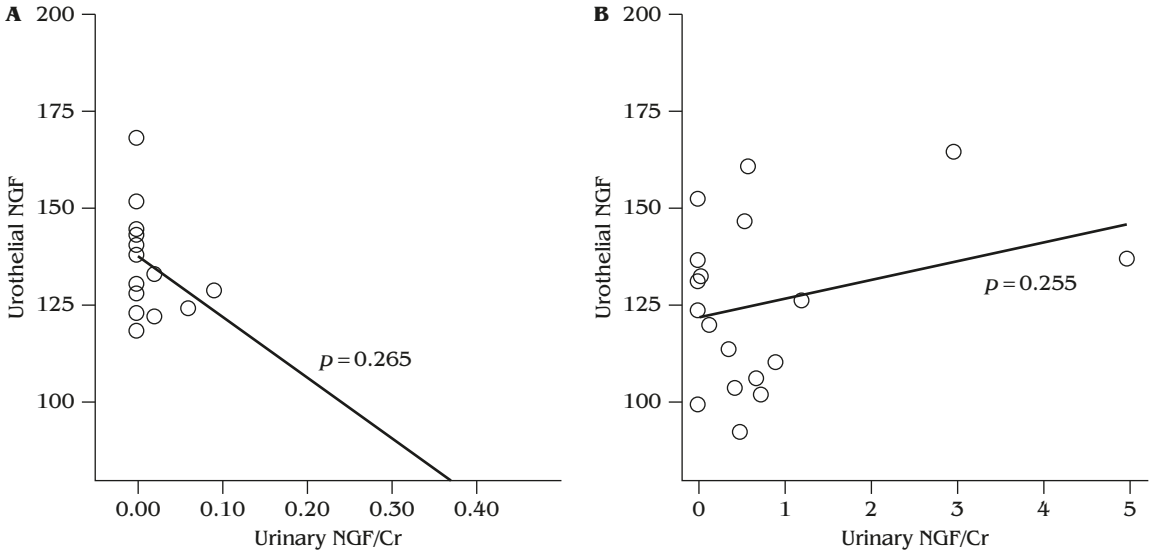

Fig. 2 — Correlation between urinary and bladder tissue nerve growth factor expression in: (A) control bladders; and (B) detrusor overactivity bladders.

bladder tissue and secretion into the urine. It is rational to hypothesize that NGF produced in the bladder wall can be secreted into the bladder lumen (17). Although the NGF levels in the bladder tissue and urine might not correlate well, an interaction between urinary NGF and sensory fibers, as well as an effect on detrusor hyperactivity, is likely.

Using immunohistochemistry study, basal level NGF expression in the urothelium was also noted in controls, indicating that a considerable amount of NGF is normally present in the urothelium. The higher urinary NGF in DO patients is possibly due to leakage from the bladder wall into the vesical lumen through a dysfunctional urothelium.

Increased levels of NGF have also been reported in the bladder urothelium and smooth muscle and urine of patients with IC/PBS, sensory urgency and DO (11,12,20). In patients with neurogenic DO, the NGF levels in bladder tissue increased and decreased after detrusor botulinum toxin A injection in association with a decrease in detrusor contractility (21). Previous studies of NGF in OAB or DO usually measured the bladder tissue levels. NGF bladder levels were elevated in patients with idiopathic DO, men with benign prostatic hyperplasia, and women with IC/PBS (8).

Our previous study has shown that the urinary NGF level may increase in not-full bladders of patients with DO but not in normal controls. In fully distended bladders, the urinary NGF levels were slightly increased in normal controls (22). This observation implies that the urothelium acts as a barrier to prevent NGF leaking out of the bladder wall in the resting state. In a fully distended bladder, however, the highly secreted NGF might leak into the visceral lumen, at higher levels in DO bladders and lower levels in control bladders. The results of this study showing highly expressed bladder tissue NGF and low urinary NGF levels in the controls further demonstrate that NGF is normally present in the bladder tissue and not leaked out into urine.

Although it seems rational to hypothetically link the elevated urinary NGF levels with urothelial dysfunction in DO bladders, the cause for the higher NGF expression in the control bladders remains unknown. Interestingly, our observation is compatible with that of Birder et al (12). Previous studies from animal models all showed elevated bladder tissue NGF expression in cystitis and BOO models (7,23,24). It is possible that part of the elevated NGF expression in those models originated from the detrusor. One study measuring human detrusor NGF concentrations also revealed that the mean NGF content was significantly higher in unstable tissues compared with that in the normal bladder (16). It is possible that detrusor NGF content in response to BOO or chronic inflammation might contribute to elevated bladder tissue NGF concentration.

Another possibility is that the increased NGF production in response to stimulation is rapidly taken up by sensory nerves and transported through the central nervous system in a retrograde fashion (25). The measured bladder tissue NGF, in fact, reflects the basic concentrations needed for physiological needs, both in controls and DO bladders. The source of increased urinary NGF might be produced from detrusor or infiltrated inflammatory cells. Urothelial dysfunction might exist in DO patients and cause NGF to leak from bladder tissue into urine. These speculations need further investigation.

This study was limited by a lack of protein measurements of NGF in bladder tissue. Although immunohistochemistry can reflect the NGF protein content in sensory nerve fibers, it cannot reflect the true NGF concentrations in the bladder wall. Using ELISA to measure NGF concentrations might be helpful to further clarify the relationship between bladder tissue and urine NGF levels. Further, we did not measure the detrusor NGF concentrations in this study. Whether the elevated urinary NGF levels are due to high detrusor NGF expression in DO bladders remains unknown. Because the calculation methods between NGF concentrations in the urothelium and suburothelium were different, it is not appropriate to compare the NGF concentrations between these two sites using numerical data.

This study has shown that DO patients have higher urinary NGF than controls. However, urothelial NGF level was no different between the two groups. This result suggests that increased urinary NGF in DO patients might not be due to higher urothelial NGF expression.

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