Investigation of molecular factors associated with neurogenic bladder dysfunction by proteomic study

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Investigation of molecular factors associated with neurogenic bladder dysfunction by proteomic study

Directed by Professor Sang Won Han

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ABSTRACT

Investigation of molecular factors associated with neurogenic bladder dysfunction by proteomic study

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Neurogenic bladder is dysfunction of the urinary bladder caused by the central nervous system or peripheral nerves. The primary causes of neurogenic bladder in children are congenital neural tube defects including myelomeningocele, lipomeningocele, and sacral agenesis. Histopathological changes are vaguely elucidated. To reveal molecular factors associated with neurogenic bladder dysfunction, we investigated differences between neurogenic and healthy bladders.

The data of 7 patients with neurogenic bladder (M:F=4:3), a mean age of 9 years 3 months was compared to 6 patients with vesicoureteral reflux (VUR) serving as controls (M:F=4:2), a mean age of 10 months. Protein expressions were analyzed by 2D gel electrophoresis and Nanoflow LC-ESI-MS-MS analysis. Bladder smooth muscle cells (BSMCs) were cultured to investigate protein function and treated with siRNAs of TPI and Apo A-1 at various doses. After transfection, cell apoptosis was measured by flow cytometry.

Among 16 protein spots were relatively overexpressed, and 20 spots were underexpressed in neurogenic bladders compared to controls. Triosephosphate isomerase (TPI) and apolipoprotein A-1 (Apo A-1), which are associated with apoptosis, were selected. The expressions of TPI and Apo A-1 were decreased in neurogenic bladder, and confirmed to be valid after western blot and

masson's trichrome staining. After TPI and Apo A-1 gene inhibition by siRNA, TPI and Apo A-1 expressions were dose-dependently decreased, and apoptotic cells were increased in BSMCs.

Decreased TPI and Apo A-1 induce the apoptosis of myocytes in neurogenic bladder. The apoptosis of myocytes may cause bladder dysfunction. Therefore, these proteins would be potential targets for the treatment of or protection against the apoptosis of smooth muscle cells in neurogenic bladder.

Key words : Neurogenic bladder, Bladder Smooth Muscle Cell (BSMC), Proteomics, Triosephosphate isomerase (TPI), Apolipoprotein A-1 (Apo A-1)

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I. INTRODUCTION

The major functions of the urinary bladder are storage of urine and emptying through coordinated contraction of the bladder and relaxation of the urethra.[1-3] The bladder maintains low pressure and a relaxed state during the filling phase, whereas the detrusor smooth muscle contracts during the voiding phase.[1] Micturition is controlled by the nervous system at the levels of the forebrain, brainstem, and spinal cord.[2, 4] Urine storage depends on lumbosacral spinal reflexes. Micturition is mediated by spino-bulbo-spinal reflexes coordinated by the pontine micturition center (PMC) and occurs in response to afferent signals from the lower urinary tract.[2, 5, 6] In addition, signals generated in the urothelium and involving suburothelial nerves are important to both normal and diseased micturition[7]. Storage dysfunction may result in lower urinary tract symptoms (LUTS), such as urgency, frequency, urgency incontinence and components of overactive bladder syndrome (OAB).[8]

Neurogenic bladder is a dysfunction of the urinary bladder caused by the central nervous system or peripheral nerves. Nervous disorders that may result in neurogenic bladder include spina bifida, spinal cord injury (SCI), transverse myelitis, and cerebral palsy.[9-11] In SCI patients, voiding dysfunction can be caused by neurogenic detrusor over-activity and bladder urethral sphincter

dyssynergia.[12, 13] The coordination of detrusor activity and the urethral sphincter is reduced after SCI above the lumbosacral level.[14] Furthermore, detrusor over-activity results in reduced bladder storage capacity and frequent incontinence.[14-16]

The causes of neurogenic bladder in children are primary congenital neural tube defects including myelomeningocele (MMC), lipomeningocele, and sacral agenesis.[17] Neurogenic bladder is associated with significant risks for urinary tract infections (UTIs), renal scarring, renal failure, vesicoureteral reflux, and incontinence.[9] In congenital neural tube defects, voiding dysfunction typically starts immediately after birth.[10, 18] Recently, fetal closure of MMC has been performed but provided little positive impact on neonatal bladder function.[19-21] According to Clayton *et al*., patients who underwent fetal MMC closure still exhibited voiding dysfunctions such as OAB and detrusor sphincter dyssynergia.[19] However, the cause of the voiding dysfunction after fetal MMC closure is unclear. In addition, it is not clear whether congenital neurogenic bladder is caused by genetic effects.

The primary aim of pediatric neurogenic bladder treatment is the achievement of urinary continence and avoidance of deterioration of the upper urinary tract.[22] To protect against renal damage, such patients are treated with clean intermittent catheterization (CIC), anticholinergic agents, and botulinum toxin A injection, which convert high pressure bladders into low pressure reservoirs for the upper urinary tract.[18, 23] Despite comprehensive treatment strategies, voiding dysfunction often remains. Recent investigations have focused on stem cell therapy and bladder tissue engineering to treat neurogenic bladder. However, after transplantation of neural stem cells in SCI animal models, improvement of bladder function does not approach that of normal bladder, although motor and sensory functions are improved.[24, 25] The reason is that the histological change was not considered. Prior investigations detected muscle cell degeneration and fibrosis in neurogenic bladder.[26-29]

Voiding dysfunction can be caused by histological changes such as increased bladder wall thickness and changes in the smooth muscle/collagen ratio in the bladder.[22, 30] However, the mechanism of histological changes and apoptosis are still unclear.

To discover molecular factors that may cause histological changes and apoptosis in neurogenic bladder, we investigated differences between neurogenic and healthy bladders through a proteomics approach.

II. MATERIALS AND METHODS

1. Patients and Tissues

This study was compared 7 patients who underwent augmentation surgery for neurogenic bladder and 6 patients who underwent ureteroneocystostomy for vesicoureteral reflux (VUR) serving as controls between 2007 and 2009. The 7 patients with neurogenic bladder (M:F=4:3) had a mean age of 9 years 3 months (range: 4 yrs 3 mos - 15 yrs 3 mos) and the 6 control patients with VUR (M:F=4:2) had a mean age of 10 months (range: 9 mos - 2 yrs 1 mo). Frozen neurogenic bladder and control bladder tissues obtained during each operation were kept in liquid nitrogen at 70℃ until used for the proteomic analysis. The tissues were homogenized with a lysis buffer consisting of 7M urea (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 2M thiourea (Sigma Chemical Co., St. Louis, MO, USA), 2% CHAPS (Sigma Chemical Co., St. Louis, MO, USA), 0.5% Pharmalyte (pH 3-10; Sigma Chemical Co., St. Louis, MO, USA), 1% DL-dithiothreitol (DTT; Sigma Chemical Co., St. Louis, MO, USA) and 4% protease inhibitors in distilled water. The suspension was sonicated three times for 15s each time. To obtain tissue extracts for western blot, the specimens were homogenized with a pro-prep lysis buffer (Intron Biotechnology, INC., Seoul, Korea). The concentration of protein was determined using a Bio-Rad assay reagent (Bio-Rad, Inc., Hercules, CA, USA). Specimens obtained from these patients for immunohistochemistry were fixed in 10% buffered formalin, dehydrated in graded alcohol and processed for histological evaluation. Tissues were embedded in paraffin while maintaining the native orientation of the tissue section to be examined. Specimens were horizontally sectioned at 4μm. Paraffin sections were dewaxed and rehydrated through a decreasing alcohol series up to distilled water.

2. Two-dimensional gel electrophoresis

The amount of protein to be separated by 2D gel electrophoresis was 100μg of protein per immobilized pH gradient (IPG) strip (pH 3-10, 17cm; Bio-Rad, Hercules, CA, USA). IPG strips were rehydrated using a Protean IEF cell (Bio-Rad, Munich, Germany) for 12hs in a strip holder in 340μl of IPG buffer (7M urea, 2M thiourea, 2% CHAPS (w/v), 0.8% Pharmalyte (pH 3-10, v/v), 1% DTT, 0.002% bromophenol blue) containing 100μg of protein extract. The first-dimension isoelectric focusing (IEF) was carried out as follows: 500V/500VHr, 1,000V/1000VHr and 10,000V/40000VHr. After first-dimensional separation, the IPG gel was equilibrated in equilibration buffer I containing 50mM Tris–HCl (pH 8.8), 6M urea, 30% (v/v) glycerol, 2% (v/v) SDS and 1% DTT for 30min, then in equilibration buffer II containing 50mM Tris–HCl (pH 8.8), 6M urea, 30% (v/v) glycerol, 2% (v/v) SDS and 2.5% iodoacetamide for 30min. The equilibrated IPG gel was separated on 10% SDS-polyacrylamide gels. Proteins on the gel were visualized by silver staining before scanning, which allows for direct correlations of the intensity of the protein spots with the quantity of protein present.

3. Image analysis and quantification

The gels were scanned using a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and matched to analyze differentially expressed spots using PDQuest software version 8.0.1 (Bio-Rad, Hercules, CA, USA). The gel images were normalized using PDQuest software, to compensate for differences in staining intensities between gels. Each matched protein spot was assigned a unique sample spot protein (SSP) number in the PDQuest software. Statistical analysis was performed using Student's t-test with a 95% significance level to determine which proteins were differentially expressed between the neurogenic and control bladders. In order for proteins to be

considered up-regulated, a minimum change of 10.0-fold was established. Conversely, proteins considered down-regulated needed to exhibit a 0.1-fold change.

4. Nanoflow LC-ESI-MS-MS analysis

The excised gel pieces were resuspended in a solution of 10 mM dithiothreitol, and 0.1 M $NH4HCO_3$ at 56℃. After 30 min of incubation, the thiol group was alkylated with iodoacetamide at a total concentration of 20 mM for 30 min in the dark. For in-gel digestion with trypsin, the gel pieces were rehydrated in the digestion buffer containing 0.05 M $NH4HCO_3$ and 10 ng/μl of modified porcine trypsin (Promega, Madison, WI, USA) at 4°C for 45 min. The excess supernatant was removed and the gel pieces were covered with 0.05 M $NH4HCO_3$ buffer. The digestion was performed overnight at 37℃. The digested mixture was finally desalted using an Oasis HLB cartridge (Waters, Milford, MA, USA), dried, and resuspended in 2% CH_3CN in water for Nanoflow LC-MS-MS analysis.

The Nanoflow LC-ESI-MS-MS experiment was carried out using a CapLC equipped with a Q-TOF Ultima mass spectrometer (Waters) with a homemade pulled tip capillary column (75-μm i.d., 360-μm o.d., 15cm) and an end frit at the tip. For each sample, 5.0 μl of digested peptide mixture was injected via an autosampler to the trapping column. After loading, a binary RP gradient elution (mobile-phase composition of (A) 2% CH_3CN in water and (B) 98% CH_3CN in water, both containing 0.1% HCOOH) was pumped through the column and the effluent was fed into the mass spectrometer via the ESI method. The eluant was added in a gradient that began with 5% B (from 2% B at default) for 5 min and was increased to 12% for 25 min and then to 22% for 60 min. It was then ramped to 80% B over 3 min and was maintained at this level for 10 min, after which it was decreased to 5% B over 2 min and maintained at this level for at least 25 min for column reconditioning. The

flow rate during the gradient separation remained at 200 nL/min, and the eluted peptides were directly electrosprayed into the mass spectrometer with a spray voltage of 2.4 kV in the positive mode of ionization. Peptide ions were detected in the data-dependent analysis mode with an MS precursor scan (300-1700 amu) followed by three data-dependent MS-MS scans. For data analysis, the collected raw MS/MS spectra were analyzed with the Mascot Search program using NCBI databases. The mass tolerance used to accept was 100 ppm for both molar masses of the precursor peptide and peptide fragment ions. For screening the search data, only peptides yielding greater than a minimum Mascot score of 49 were accepted as extensive homology.

5. Western blotting

Proteins of interest among the proteins identified were selected from Q-TOF MS data. Antibodies for western blotting were mouse anti-human triosephosphate isomerase (Abcam, Cambridge, UK), mouse anti-human α-smooth muscle actin (α-SMA; Abcam, Cambridge, UK) and mouse anti-human apolipoprotein A-1 (Abcam, Cambridge, UK). The samples with equal concentrations of protein were mixed with a 5× sample buffer, heated at 100℃ for 8 min and separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto PVDF membranes (Amersham Life Science, Arlington Heights, IL, USA) with Novex Tris-Glycine Transfer buffer (Invitrogen Co., Carlsbad, CA, USA). The membranes were blocked for 1 h at room temperature with 5% nonfat milk in TBS-Tween20. The membranes were incubated at 4℃ overnight with specific antibodies, and they were then incubated with horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. After washing, the membranes were incubated with West-Q Chemiluminescent Substrate Plus Kit (GenDEPOT Inc., Barker, TX, USA). The intensity of the proteins was determined by the Multi Gauge software,

version 3.0 (Fuji Photo Film, Tokyo, Japan), and the relative density was expressed as a ratio of the control value.

6. Immunohistochemistry

Specimens were stained with Masson's trichrome (Sigma-Aldrich, St. Louis, MO, USA) to distinguish connective tissue, muscle and collagen fibers. Stained tissues show extracellular matrix, collagen and other connective tissue elements in blue and smooth muscle in red. To investigate apoptosis of the muscle layer, *in situ* detection of apoptosis was achieved with labeling of DNA strand breaks in tissue sections with the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique using an in situ cell death detection kit (Roche Diagnostics Corp., Laval, Quebec, Canada). All stained samples in NB and CB sections were captured under microscopy (×100 magnification) using an Olympus fluorescence inverted microscope.

7. Bladder smooth muscle cell primary culture

Human bladder smooth muscle cells (hBSMCs) were isolated from 5 patients who underwent ureteroneocystostomy without any evidence of bladder dysfunction and 5 patients who underwent augmentation surgery for neurogenic bladder. Briefly, 0.3- 0.5 cm sections of tissue arteries isolated from human umbilical cord were perfused with PBS to remove the remaining internal blood. After cutting, the tissue was incubated with 0.1% collagenase IV (Washington Biochemical Co., Lakewood, NJ, USA) at 37℃ in a humidified 5% CO_2 incubator for 1 hr. After neutralization with media and centrifuge, supernatants were passed through a 100μm cell strainer (BD Bioscience, Bedford, MA, USA) before plating. The pellet was washed in PBS and seeded in M199 media (Sigma Chemical, St. Louis, MO, USA) with 10 % fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/ml

penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

8. TPI and Apo a-1 inhibition

Transfection of BSMCs with siRNA was performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. In a T25 culture plate, 2×10^5 cells per plate were seeded and incubated until the cells were 50% confluent. To inhibit Apo A-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and TPI (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in BSMCs, siRNA were treated in a dose-dependent (20, 40, 60, 80 pmole) manner. After incubation for 5-6 hrs at 37℃ in a humidified 5% CO^2 incubator, transfection media was removed and replaced with normal growth media. The cells were observed in a time-dependent manner.

9. Cellular apoptotic evaluation

Apoptosis was measured using an annexin V-FITC/PI apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA) following the manufacturer's protocol. Briefly, cells were harvested at the indicated time periods, washed twice with cold PBS, then re-suspended in 100 μl of binding buffer (1×10^5 cells), followed by staining with 5 μl of annexin V-FITC and 10 μl of PI for 15 min at room temperature in the dark. The percentage of cell apoptosis was determined by flow cytometry.

10. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol reagent and then treated with DNase I. cDNA synthesis and gene amplification were performed as described by the protocol for the PCR kit. The mixture was incubated at 95℃ for 15 min before the first cycle and extended at 72℃ for 10 min after all the cycles were

completed. The primers for TPI and Apo A-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GAPDH control primer pair was obtained from BioSource International. The PCR products were electrophoresed in a 1% agarose gel in Tris–borate EDTA buffer and checked for size. The intensity was analyzed by densitometric scanning analysis, and the relative density was expressed as a ratio of the control value.

11. Immunocytochemistry

Cells were seeded in culture slides (BD Biosciences, Bedford, MA, USA) at 1×10^5 cells per plate and incubated until the cells were 50% confluent. To investigate morphology of the BSMCs, the BSMCs were fixed in 4% formaldehyde, and permeabilized with 0.2% Triton X-100, then stained with α-SMA antibody (1:200; Abcam, Cambridge, UK). Images of all stained samples in NBSMCs and CBSMCs were captured under microscopy (100× magnification) using an Olympus fluorescence inverted microscope.

III. RESULTS

1. Smooth muscle density and apoptosis in neurogenic bladder

To investigate the proportion of smooth muscle in neurogenic bladder compared to control bladder, Masson's trichrome staining was used to distinguish collagen from muscle in bladder tissue. The density of stained smooth muscle in neurogenic bladder was significantly lower than the control (Figure 1-A, B). To investigate causes of the decreased smooth muscle, TUNEL staining was performed to assess possible apoptosis of smooth muscle cells. The number of apoptotic cells was significantly greater in the smooth muscle layer of the neurogenic bladder compared to the control (Figure 1-C, D).

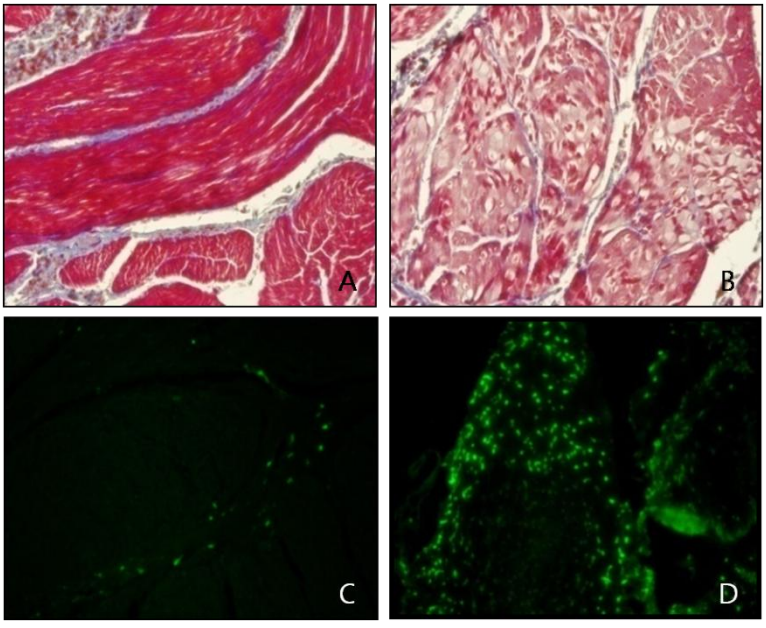

Figure 1. . Histological evaluation of smooth muscle density and apoptosis. The neurogenic and control bladders were stained by Masson's trichrome (A, B) and TUNEL (C, D). Collagen is stained in blue and muscle in red, reduced from x400. Apoptotic cells are stained in green, reduced from X200. (A), (C) control, (B),(D) neurogenic bladder.

2. Two-dimensional gel electrophoresis and Nanoflow LC-ESI-MS-MS analysis

Image analysis and quantification of 2D gel electrophoresis were performed by PDQuest software version 8.0.1. In analysis of the protein spots, over 100 spots were found to be common between neurogenic and control bladder (Figure 2-A). Statistically, 36 protein spots were differently expressed between the neurogenic and control bladders. Of those, 16 protein spots were relatively overexpressed and 20 spots were relatively underexpressed in the neurogenic bladder (Figure 2). Differentially expressed protein spots were identified through Nanoflow LC-ESI-MS-MS and a database search (Table 1). Proapolipoprotein, p24k-1, heat shock protein beta-1, peroxiredoxin 3 isoform a precursor and beta-fibrinogen precursor etc. were more highly expressed in neurogenic bladder than in control bladder. Conversely, apolipoprotein A-1 (Apo A-1), triosephosphate isomerase (TPI), smooth muscle protein and alpha-1-microglobulin etc. were more expressed in the control bladders (Table 1). Some protein spots were unknown or unnamed and not identified because of their low abundance. To investigate protein function in the bladder, several protein spots associated with histological change and apoptosis were selected. The matched sequences of TPI are K.IAVAAQNCYK.V (Ions score 67) and R.IIYGGSVTGATCK.E (Ions score 99), and Apo A-1 are -.DEPPQSPWDR.V (Ions score 45) and K.LLDNWDSVTSTFSK.R (Ions score 80) in Mascot Search Results.

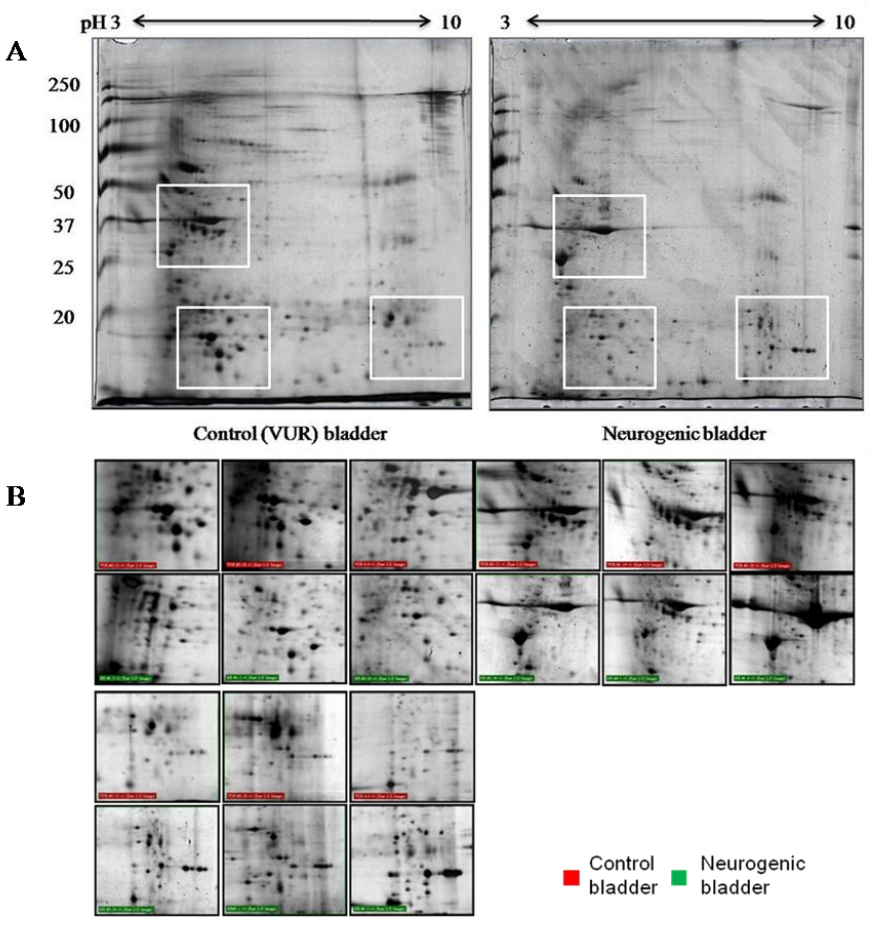

Figure 2. Analysis of protein spots and quantification in 2D gel images. (A) Image of 2D gel electrophoresis showing the protein expression profiles of neurogenic bladder tissue. Differential expressions of 36 protein spots were observed between neurogenic and control bladders. Of these, 16 protein spots were relatively overexpressed and 20 protein spots were underexpressed in neurogenic bladder. (B) Areas of the gels showing differential expression between neurogenic and control bladders.

Table 1. Differentially expressed protein spots from database searching and Nanoflow LC-ESI-MS-MS.

Spot no.	Protein ID	Accession no.	Score	MW (kDt) /PI	Sequence Coverage (%)
Underexpression					
1	Apolipoprotein A-1 (Apo A-1)	gi|253362	124	10.1/4.76	27
2	Triosephosphate isomerase	gi|136066	166	26.8/7.10	9
3	smooth muscle protein	gi|177175	235	22.5/8.56	21
4	alpha-1-microglobulin	gi|4502067	162	39.8/5.95	10
5	hypothetical protein	gi|51476390	322	71.3/5.88	8
6	apolipoprotein D (Apo D)	gi|619383	73	28.3/5.14	7
7	prostaglandin H2 D-isomerase	gi|32171249	49	21.2/7.66	8
8	Chain A, Human Serum Albumin In A Complex With Myristic Acid And Tri- Iodobenzoic Acid	gi|157830361	380	67.9/5.69	12
9	unnamed protein product	gi|194375974	282	20.2/8.90	29
10	unnamed protein product	gi|28590	313	71.2/5.92	11
Overexpression					
11	proapolipoprotein	gi|178775	166	28.9/5.45	13
12	cytokeratin 9	gi|435476	111	62.3/5.19	2
13	tyrosine 3/tryptophan 5 –monooxygenase activation protein, zeta polypeptide	gi|4507953	262	27.8/4.73	24
14	heat shock protein beta-1	gi|4504517	169	22.8/5.98	24
15	alpha-1 antitrypsin	gi|28637	72	22.8/6.11	13
16	p24k-1 (AA 1-91)	gi|35182	58	10.1/8.00	9
17	alpha-actin	gi|178027	81	42.4/5.23	5
18	cytokeratin 9	gi|435476	74	62.3/5.19	2
19	beta-fibrinogen precursor	gi|182430	42	55.5/8.31	1
20	WDR1 protein	gi|3420181	128	58.5/6.41	4
21	carbonic anhydrase I	gi|4502517	37	28.9/6.59	3
22	peroxiredoxin 3 isoform a precursor	gi|5802974	47	28.0/7.67	2
23	keratin	gi|1200072	107	51.4/5.41	4

3. The expressions of TPI, Apo A-1 and α-SMA in bladder tissue

The expressions of TPI and Apo A-1 proteins in the 2D gel were significantly decreased in neurogenic bladder (Figure 3-A, 4-A). To confirm results of LC-ESI-MS-MS, several protein spots, which are associated with histological change and apoptosis, were identified by western blot. The expression of α-SMA, TPI and Apo A-1 proteins were significantly decreased in neurogenic bladder compared to control bladder. These results coincide with the data from LC-ESI-MS-MS (Figure 3, 4).

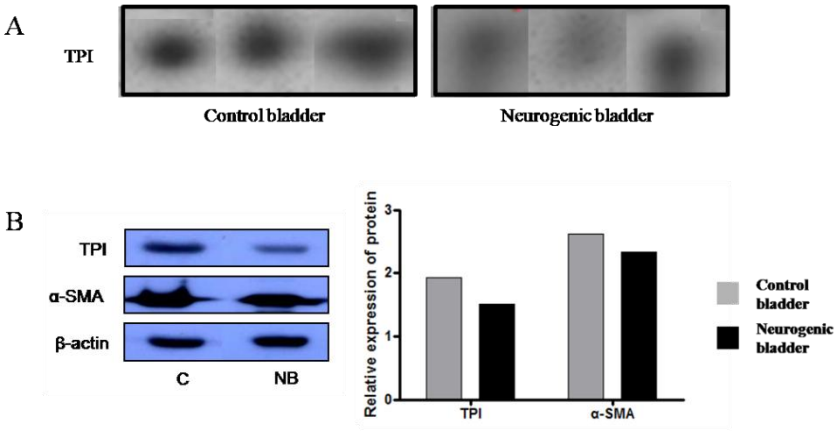

Figure 3. The expression of TPI and α-SMA proteins in neurogenic bladder. (A) The spots of TPI protein in the 2D gel were decreased in neurogenic bladder compared with control bladder. (B) In western blot analysis α-SMA and TPI proteins were significantly decreased in neurogenic bladder compared with control bladder.

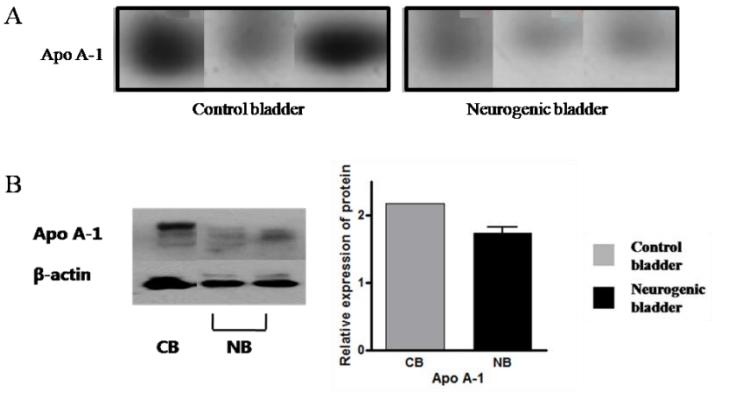

Figure 4. The expression of Apo A-1 proteins in neurogenic bladder. (A) The spots of Apo A-1 protein in the 2D gel were decreased in neurogenic bladder compared with control bladder. (B) In western blot analysis the Apo A-1 protein was significantly decreased in neurogenic bladder compared with control bladder.

4. Identification of bladder smooth muscle cells

To investigate the functions of selected proteins, BSMCs were cultured from patients who underwent ureteroneocystostomy without any evidence of bladder dysfunction. BSMCs exhibit spindle-like processes that are more rounded and tapering than characteristic SMCs when observed under an inverted microscope (Figure 5-A). The primary cultured cells were identified by immunocytochemistry with α-SMA antibody. The α-SMA positive cells were observed in primary culture cells (Figure 5-B).

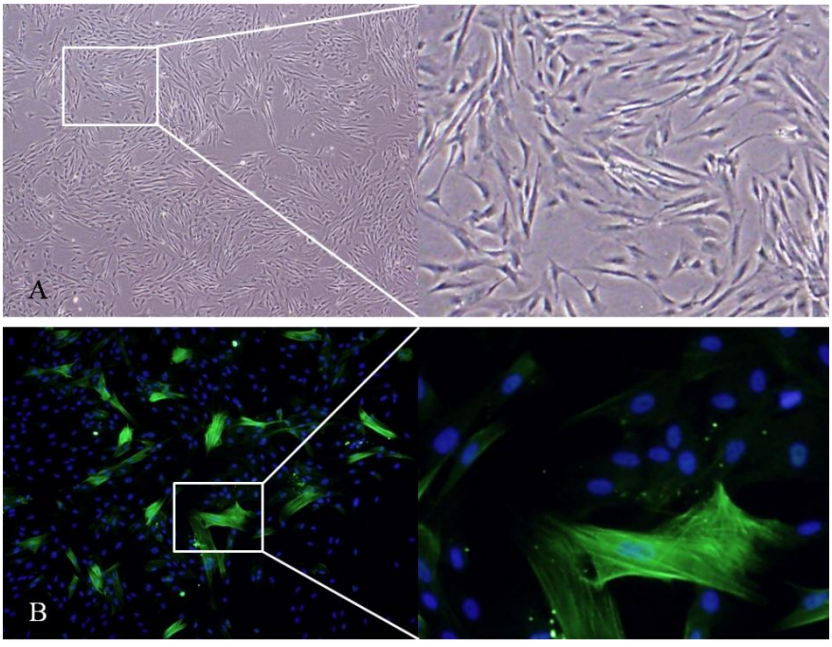

Figure 5. Identification of normal patient derived BSMCs. BSMCs were observed under an inverted microscope with immunofluorescence staining. (A) The morphology of BSMCs are rounded and tapering from the characteristic of SMCs, reduced from x100. (B) BSMCs are identified by immunofluorescence staining. α-SMA is stained in green, reduced from x200.

5. siRNA transfection of TPI and Apo Aa-1

To demonstrate the effect of decreased TPI and Apo A-1 in neurogenic bladder, siRNA against TPI and Apo A-1 were treated in a dose-dependent (20, 40, 60, 80 pmole) manner and observed at 12, 24, and 36hrs.

A. Inhibition of TPI

After transfection of siRNA, BSMCs were observed for 36 hrs (Figure 6). The number of BSMCs was decreased in a dose-dependent manner (20*, 40*, 60* pmol, *$P<0.05$) compared to control BSMCs (Figure 7-C). The down-regulation of the TPI gene was confirmed by RT-PCR. The gene expression of TPI was significantly decreased in inhibited BSMCs compared with control BSMCs (Figure 7-A, B). To demonstrate apoptosis of BSMCs after siRNA transfection, flow cytometry was performed using an annexin V-FITC/PI apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA) following the manufacturer's protocol. The apoptotic cells were increased in siRNA treated BSMCs in a dose-dependent manner compared to the non-treated BSMCs (Figure 8, Table 2).

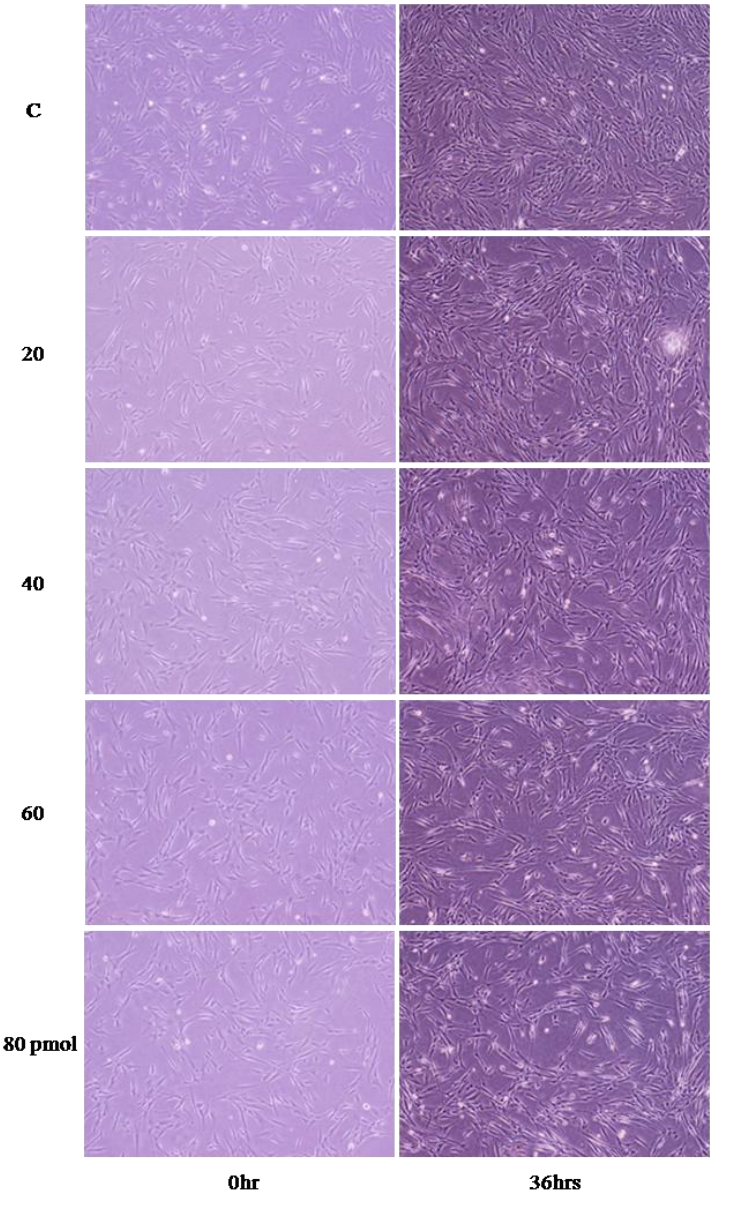

Figure 6. Cell growth after TPI inhibition. After transfection of TPI siRNA, the BSMCs were observed for 36 hrs. The number of BSMCs was decreased in a dose-dependent manner after TPI inhibition.

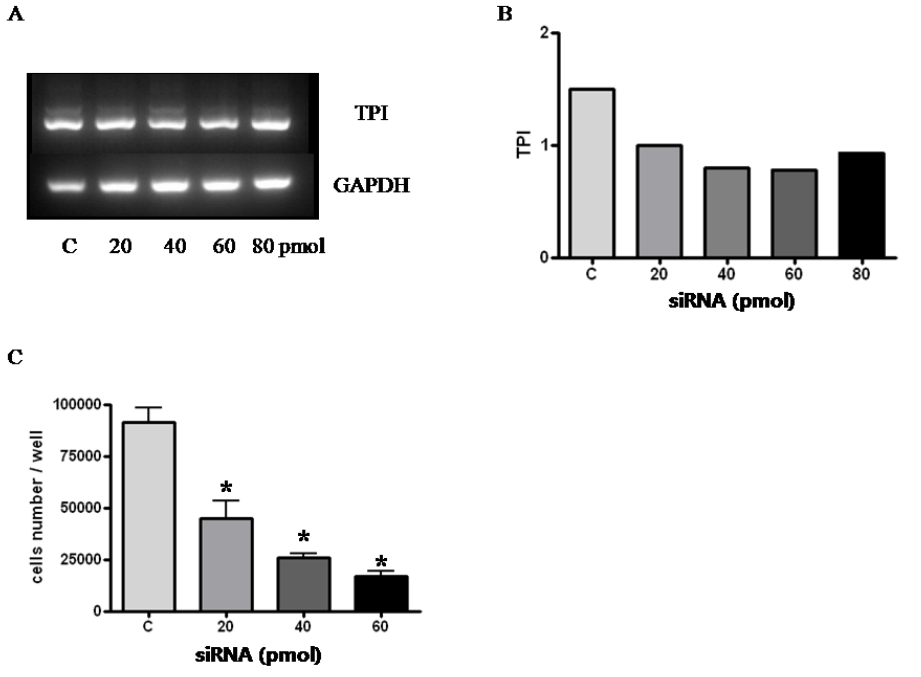

Figure 7. The gene expression of TPI and cell number after TPI inhibition. (A) The down-regulation of the TPI gene was confirmed by RT-PCR. (B) The expression of the TPI gene was measured, and was shown to be significantly decreased in inhibited BSMCs. (C) In manual cell counting, the number of inhibited BSMCs was decreased in a dose-dependent manner (20*, 40*, 60* pmol, *P<0.05).

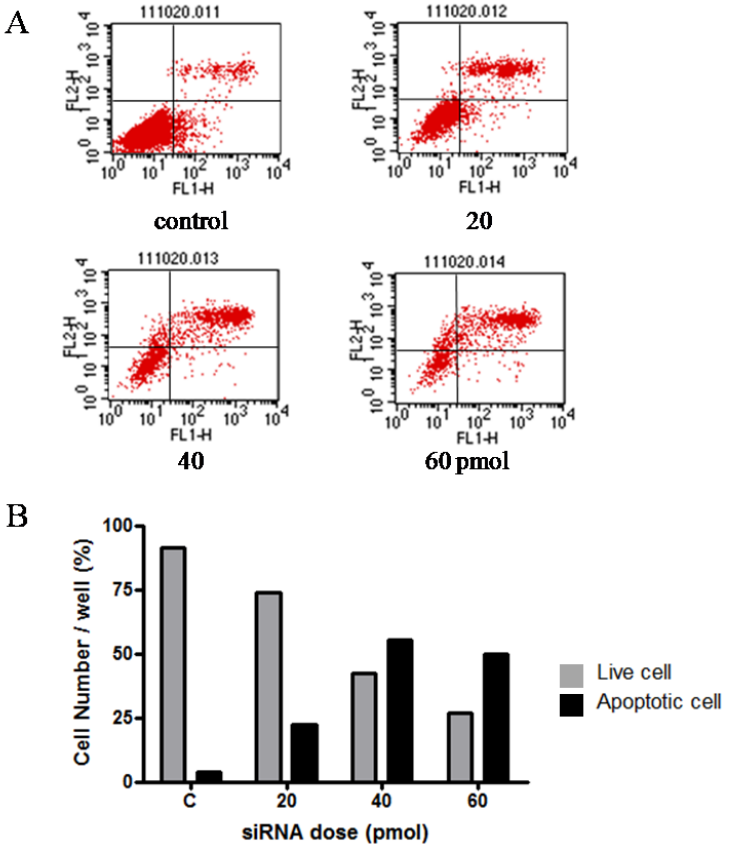

Figure 8. Apoptosis of BSMCs after TPI inhibition. (A) The analysis of apoptotic cells was performed by flow cytometry. (B) In flow cytometry, apoptotic cells increased in a dose-dependent manner in TPI inhibited BSMCs. On the contrary, live cells were significantly decreased after TPI inhibition.

B. Inhibition of Apo A-1

The transfection of Apo A-1 siRNA was performed under the same conditions as the TPI inhibition. The cell growth and number of BSMCs were significantly decreased in siRNA treated BSMCs (20*, 40*, 60* pmol, *P<0.05; Figure 9, 10-C). The gene expression of Apo A-1 was decreased in a dose-dependent manner in siRNA treated BSMCs compared to non-treated BSMCs (Figure 10-A, B). Flow cytometry showed that the apoptotic cells were particularly increased in siRNA treated BSMCs (Figure 11, Table 2).

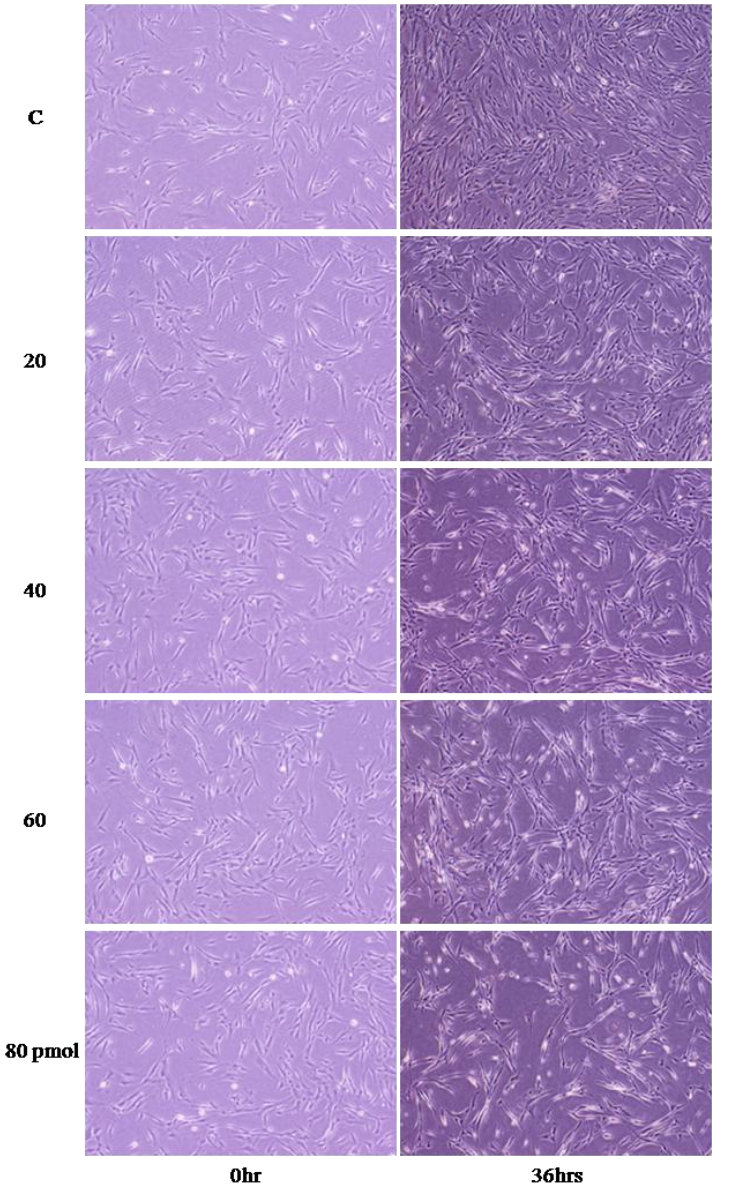

Figure 9. Cell growth after Apo A-1 inhibition. After transfection of Apo A-1 siRNA, the BSMCs were observed for 36 hrs. The number of BSMCs was decreased in a dose-dependent manner after Apo A-1 inhibition.

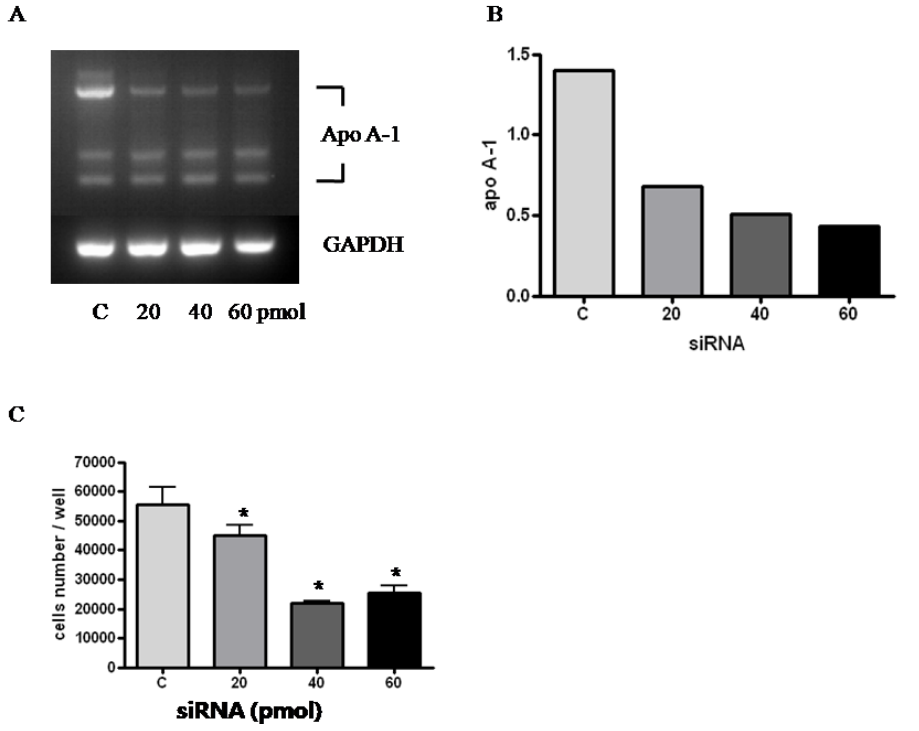

Figure 10. Gene expression of Apo A-1 and cell number after Apo A-1 inhibition. (A) The down-regulation of the Apo A-1 gene was confirmed by RT-PCR. (B) The expression of the Apo A-1 gene was significantly decreased in inhibited BSMCs. (C) In manual cell counting, the number of inhibited BSMCs was dose-dependently decreased (20*, 40*, 60* pmol, *P<0.05).

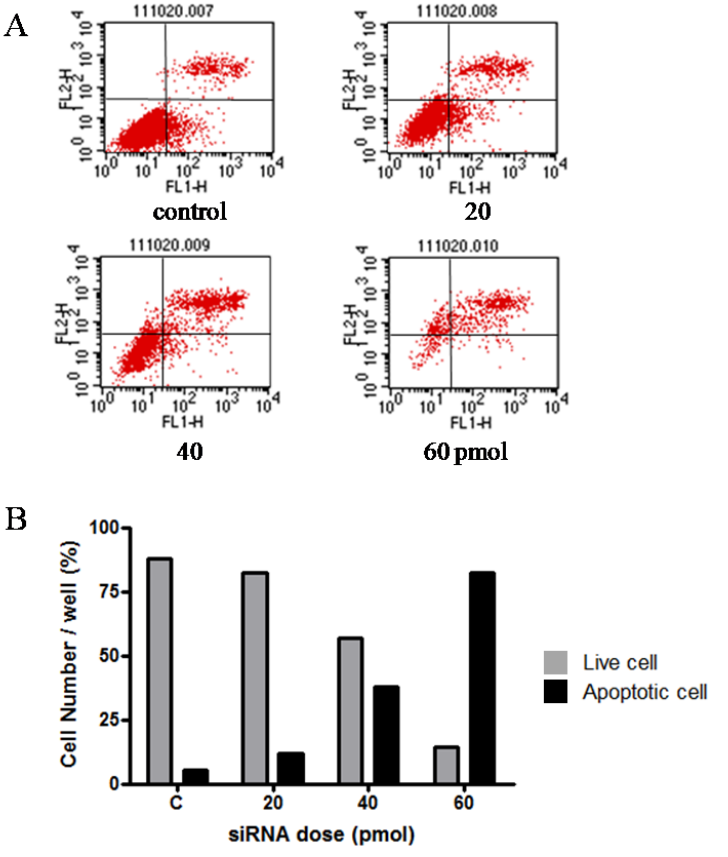

Figure 11. Apoptosis of BSMCs after Apo A-1 inhibition. (A) The analysis of apoptotic cells was performed by flow cytometry. (B) The apoptotic cells increased in a dose-dependent manner in APO A-1 inhibited BSMCs. On the contrary, live cells were significantly decreased after Apo A-1 inhibition.

Table 2. The percentage of apoptotic cells by flow cytometry.

		C	20	40	60 pmol
TPI Inhibition	live	91.17	73.60	42.40	26.94
	necrosis	5.02	3.91	2.32	3.33
	apoptosis	3.82	22.49	55.28	69.74
Apo A-1 Inhibition	live	87.74	82.15	57.01	14.15
	necrosis	6.83	6.25	5.17	3.70
	apoptosis	5.43	11.6	37.82	82.16

IV. DISCUSSION

Neurogenic bladder is a voiding dysfunction caused by neural defects such as spinal cord injury and spina bifida.[9-11, 22] Congenital neural tube defects include myelomeningocele, lipomeningocele, and sacral agenesis.[17] Neurogenic bladder patients suffer from voiding dysfunction, which can cause various problems such as urinary tract infections and renal failure.[28] Despite comprehensive treatment strategies such as CIC, anticholinergic treatment, and botulinum toxin A treatment, micturition dysfunction remains. In addition, the social implications and other quality of life issues are major problems for these patients.[25, 29] Therefore, recent investigations have been focused on stem cell therapy and bladder tissue engineering for the treatment of neurogenic bladder. Park *et al*. revealed that the bladder dysfunction after transplantation of human mesenchymal stem cells (hMSCs) was not sufficiently recovered in a spinal cord injury (SCI) model, although the motor and sensory functions were promoted.[25, 30] Temeltas *et al*., performed a urodynamic study after transplantation of bone marrow stromal cells (BSMCs) or neuronal /glial restricted precursor cells in the SCI rat model, but the improvement of bladder function was not complete compared to normal bladder.[24] Despite the improvement of motor and sensory function, the histopathologic changes were not regenerated in neurogenic bladder. Prior investigators have demonstrated that muscle cells are degenerated and fibrosis is induced in neurogenic bladder.[26, 27]

In this study, the density of smooth muscle in neurogenic bladder was significantly lower than in control bladder tissues (Figure 1-A, B). Furthermore, the number of apoptotic cells was significantly greater in the smooth muscle layer of the neurogenic bladder as compared to the control (Figure 1-C, D). Thus, the decrease in smooth muscle may be caused by apoptosis of bladder muscle. A proteomics approach was used to discover the molecular factors associated with apoptosis.

In analyses of protein spots, 36 protein spots were expressed differently between the neurogenic and control bladders. Proapolipoprotein, p24k-1, heat shock protein, beta-1 peroxiredoxin 3 isoform a precursor and beta-fibrinogen precursor were more highly expressed in neurogenic bladder than in control bladder. Conversely, Apo A-1, TPI, smooth muscle protein and c-myc binding protein were less expressed in the neurogenic bladder (Table 1). Several protein spots associated with histological change and apoptosis were selected for further analysis.

TPI is a glycolytic enzyme essential for efficient energy production and is expressed in all prokaryotic and eukaryotic organisms.[31] TPI regulates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) in the glycolysis pathway.[32] TPI deficiency causes progressive neurologic dysfunction, protein modification and oxidative damage due to the decreased adenosine triphosphate (ATP) levels in TPI deficient cells.[32] Our western blot and 2D electrophoresis results show that the expression of TPI was decreased in tissues of the neurogenic bladder (Figure 2, 3, 4, Table 1). To investigate TPI function, the TPI gene in the primary cultured BSMCs was inhibited with successively increasing siRNA levels. The number of BSMCs was decreased in siRNA treated BSMCs (20*, 40*, 60* pmol, *$P<0.05$) and the down-regulation of the TPI gene was confirmed by RT-PCR. Furthermore, the inhibition of TPI induced apoptosis of BSMCs (Figure 8, Table 2). ATP is released from urothelial cells and has an important role in chemical communication in the bladder.[33, 34] According to Birder *et al*., ATP is released during bladder distension and induces activation of several molecules and intracellular Ca^{2+} in afferent neurons.[33] However, the effects of ATP deficiency has not been reported in bladder smooth muscle. These data have revealed that TPI deficiency seen in neurogenic bladder probably causes apoptosis of smooth muscle cells and results in histological changes.

Apo A-1 plays a specific role in lipid metabolism and is the major protein

component of high-density lipoprotein (HDL) in plasma.[35-37] Apo A-1 deficiency or concentration change causes atherosclerosis and metabolic syndrome in the walls of arteries.[38, 39] The results of our proteomic analysis of neurogenic bladder tissues show that the expression of Apo A-1 was decreased, whereas pro-Apo expression was increased compared to control bladder tissues (Table 1). However, the function of Apo A-1 has not yet been reported in bladder smooth muscle. To reveal the function of Apo A-1 in BSMCs, siRNA treatment to inhibit Apo A-1 was performed as per the TPI experiment. After transfection of Apo A-1 siRNA, the number of BSMCs was decreased (20*, 40*, 60* pmol, *$P<0.05$). The gene expression of Apo A-1 was decreased in a dose-dependent manner in the siRNA treated BSMCs compared to non-treated BSMCs (Figure 10). Flow cytometry revealed that the apoptotic cells were particularly increased in siRNA treated BSMCs (Figure 11, Table 2). Lefterov *et al*. reported that Apo A-1 decreases amyloid β (Aβ) aggregation and toxicity in primary neurons and is protective against apoptosis caused by Aβ in vascular SMCs.[37] These data demonstrate that decreased Apo A-1 in neurogenic bladder may induce apoptosis of smooth muscle cells.

The present study revealed molecular factors and protein functions associated with the apoptosis in neurogenic bladder. However, these data are insufficient to demonstrate that bladder dysfunction is caused by genetic effects. Further investigations of BSMC derived from neurogenic bladder are necessary. However, these results show that the decreased TPI and Apo A-1 are associated with apoptosis of bladder smooth muscle cells and suggest potential targets for treatment of or protection from the apoptosis of smooth muscle cells in neurogenic bladder.

V. CONCLUSION

This study investigated molecular factors associated with neurogenic bladder dysfunction by a proteomics approach. A total of 36 protein spots were significantly differently expressed between neurogenic and healthy bladders. The decreased TPI and Apo A-1 levels induce apoptosis of myocytes in the smooth muscle layers of neurogenic bladders. The apoptosis of myocytes may cause bladder dysfunctions by histopathological changes, potentially affecting voiding dysfunction in neurogenic bladders. Therefore, these proteins would be potential targets for the treatment of or protection against the apoptosis of smooth muscle cells in neurogenic bladder.

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ABSTRACT (IN KOREAN)

신경탓 방광에서 배뇨장애에 영향을 미치는 단백질의 규명과 이를 이용한 치료가능성

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강 효 진

신경탓 방광은 방광기능에 관여하는 신경의 손상에 의해 야기되는 비정상적인 방광기능을 의미하며, 소아에서는 척수 수막류, 지방 수막류 등의 선천적 신경손상으로 야기되는 것이 대표적이다. 신경탓 방광에서의 배뇨장애는 방광의 조직학적 변화에 의해서 발생하지만 아직까지 그 기전이 명확하지 않다. 신경탓 방광의 배뇨기능 이상과 관계된 분자수준의 인자를 알아보기 위해 신경탓 방광 환자의 조직을 이용하여 단백질체(proteomics) 분석을 시행, 비교하여 그 기능을 관찰하였다.

신경탓 방광으로 진단된 7명의 환자(남:여=4:3)와 대조군으로서 방광요관역류로 진단된 6명의 환자 (남:여=4:2)의 방광 조직을 비교 분석하였다. 신경탓 방광 군의 평균나이는 9년 3개월이었고, 대조군은 10개월 이었다. 방광조직의 단백을 추출하여 2D 겔 전기영동법과 Nanoflow LC-ESI-MS-MS 분석을 이용하여 단백질 프로파일을 분석하였다. 조직변화에 관련된 단백질들을 우선적으로 선택하였으며, 방광 근육세포를 배양하여 그 기능 연구를 수행하였다. 신경탓 방광에서 염색된 평활근의 밀도는 대조군에 비해 현저하게

낮게 나타났으며, 자살세포수는 현저하게 증가하였다. 자살세포의 원인을 밝히기 위해 단백질체 연구기법을 이용하였다. 총 36개의 단백질들이 통계적으로 차이가 있는 것으로 밝혀졌으며, 이중 16개의 단백질들이 신경탓 방광에서 증가하였고 20개의 단백질들은 감소하였다. 36개의 단백질 중에서 Triosephosphate isomerase (TPI)와 apolipoprotein A-1 (Apo A-1)을 선정하였고 조직에서 그 변화를 재확인 하였다. 두 단백질의 기능을 연구하기 위해, siRNA를 이용하여 유전자 발현을 억제시켰다. TPI와 Apo A-1의 유전자 발현을 억제시킨 후, 세포수가 자살세포 기전에 의해 현저하게 감소하였음을 확인하였다.

그러므로, TPI와 Apo A-1의 감소가 신경탓 방광의 근육세포에서 자살세포 기전에 의해 세포사멸을 유도하는 것으로 보이며, 근육세포의 사멸이 신경탓 방광의 조직학적 변화를 초래하는 것으로 여겨진다. 잠재적으로 TPI와 Apo A-1은 신경탓 방광에서 조직학적인 변화를 방지할 수 있으며, 나아가 직접 또는 간접적인 치료제로서의 가능성을 제시할 수 있을 것이다.

핵심되는 말 : 신경탓 방광, 방광평활근 세포, 단백질체, TPI, Apo A-1