Basic fibroblast growth factor modulates proliferation and collagen expression in urinary bladder smooth muscle cells

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Am J Physiol Renal Physiol 293: F1007–F1017, 2007. First published July 18, 2007; doi:10.1152/ajprenal.00107.2007.—Bladder hypertrophy is a general consequence of bladder outlet obstruction (BOO) and a typical phenomenon observed in clinical urologic diseases such as benign prostate hyperplasia and neurogenic bladder. It is characterized by smooth muscle hyperplasia, altered extracellular matrix composition, and increased contractile function. Various growth factors are likely involved in hypertrophic pathophysiology, but their functions remain unknown. In this report, the role of basic fibroblast growth factor (bFGF) was investigated using a rat bladder smooth muscle cell (BSMC) culture system and an original animal model, in which bFGF was released from a gelatin hydrogel directly onto rat bladders. bFGF treatment promoted BSMC proliferation both in vitro and in vivo. In vitro, bFGF downregulated the expression of type I collagen, but upregulated type III collagen. ERK1/2, but not p38MAPK, was activated by bFGF, whereas inhibition of ERK1/2 by PD98059 reversed bFGF-induced BSMC proliferation, type I collagen downregulation, and type III collagen upregulation. In the in vivo release model, bFGF upregulated type III collagen and increased the contractile force of treated bladders. In parallel with these findings, hypertrophied rat bladder created by urethral constriction showed increased urothelial bFGF expression, BSMC proliferation, and increased type III collagen expression compared with sham-operated rats. These data suggest that bFGF from the urothelium could act as a paracrine signal that stimulates the proliferation and matrix production of BSMC, thereby contributing to the hypertrophic remodeling of the smooth muscle layer.

bFGF; bladder smooth muscle cell; type I collagen; type III collagen; ERK

URINARY BLADDER HYPERTROPHY is a general consequence of bladder outlet obstruction (BOO) and commonly observed in clinical urologic diseases such as benign prostate hyperplasia and neurogenic bladder (8, 48). It is a physiological response to compensate for outlet overload and is characterized by hyperplasia of smooth muscle cells, altered extracellular matrix composition, and increased contractile function (15, 24, 28, 33, 37). Additionally, altered collagen composition has been reported in the hypertrophic bladder (28, 33), with increased type III collagen expression or decreased type I/III collagen ratio particularly prevalent during the fibrotic stage of hypertrophy (13, 22). However, the mechanisms underlying such hypertrophic pathophysiology remain unclear.

Various growth factors, including basic fibroblast growth factor (bFGF), may be involved in the hypertrophic process (9, 17, 27, 40, 49). bFGF is a multifunctional growth factor with mitogenic activity in fibroblasts and endothelial cells, as well as neurotrophic and angiogenic properties (34). It has been shown to contribute to cardiac hypertrophy through its direct effect on cardiomyocytes (23). bFGF also has mitogenic activity in bladder smooth muscle cells (BSMC) in vitro (7, 11), and its mRNA levels increase in hypertrophied rat bladder tissue after obstruction (9). These findings suggest a possible BSMC-mediated regulatory role for bFGF in hypertrophic bladder.

However, the previous studies had several major drawbacks. First, the function of bFGF in BSMC was evaluated exclusively in terms of mitogenicity. In our previous study, we reported a potent inhibitory effect of bFGF on the differentiation of bone marrow stromal cells to BSMC but did not address the effects of bFGF on BSMC themselves (20). Given the multifunctional nature of bFGF, it is particularly important to investigate nonmitogenic roles in BSMC. Additionally, the precise intracellular signaling pathways downstream of bFGF have only been investigated in vitro (1, 2, 5), which may not always reflect in vivo effects, leaving the final biological consequence of bFGF signaling unknown.

In this report, the regulatory role of bFGF in bladder muscle was investigated using an in vitro BSMC culture system and two in vivo animal models. In the in vitro system, the function of bFGF was analyzed not only in terms of cell proliferation but also in the context of collagen production, as well as the downstream signaling pathway. Furthermore, to directly analyze in vivo effect of bFGF on bladder remodeling, a gelatin hydrogel incorporating bFGF was employed as a carrier for sustained release, since bFGF in solution form rapidly disappears from the application site. This gelatin hydrogel has been demonstrated to locally release bFGF over an extended time period, promoting accelerated tissue regeneration in various organs (18, 39, 42, 43). The findings of the in vitro study were corroborated in vivo, using analysis of protein expression and...
bladder contractile function to assess the physiological effects of bFGF release. Finally, a possible regulatory role for bFGF in bladder hypertrophy was explored in a rat BOO model.

MATERIALS AND METHODS

Reagents

Dispase was purchased from Godo Shusei (Tokyo, Japan), collagenase type IV and β-aminopropionitrile from Sigma (St. Louis, MO), PD98059 and SB203580 from Calbiochem (San Diego, CA), Immobilon-P membranes from Millipore (Bedford, MA), diaminobenzidine tetrahydrochloride from Dojindo Laboratories (Kumamoto, Japan), ascorbic acid and WST-8 reagent solutions from Nacalai Tesque (Kyoto, Japan), PCR primers from Proligo Japan (Kyoto, Japan), SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA), human type III collagen from Rockland (Gilbertsville, PA), and bovine insoluble collagen from MP Biomedicals (Solon, OH). Recombinant human bFGF was provided by Kaken Pharmaceutical (Tokyo, Japan).

The antibodies were purchased from the manufacturers described below: anti-α-smooth muscle actin (SMA), calponin, β-actin (Sigma), bFGF (Upstate, Lake Placid, NY), Ki67 (Dako, Glostrup, Denmark), phosho-ERK1/2, ERK1/2, phosho-p38MAPK, p38MAPK (Cell Signaling, Beverly, MA), type III collagen (GeneTex, San Antonio, TX), and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Pierce, Rockford, IL).

In addition, a Bio-Rad Protein Assay Kit was purchased from Bio-Rad Laboratories (Heracles, CA), SuperSignal West Pico Chemiluminescent Substrate from Pierce, Vectastain Elite ABC Kit (mouse IgG) from Vector Laboratories (Burlingame, CA), RNeasy Mini Kit from Qiagen (Valencia, CA), the SuperScript First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA), a Sircol Collagen Assay Kit from Biocolor (Newtownabbey, UK), and QuickCoomassie Brilliant Blue (CBB) Kit from Wako Pure Chemical (Osaka, Japan).

Cell Culture

BSCMC were isolated from 9-wk-old female Sprague-Dawley rats (Japan Slc, Shizuoka, Japan) using a combination of procedures described elsewhere (14, 20, 31, 38, 46). Briefly, dissected rat bladders were incubated on a silicone dish in 1,000 U/ml dispase for 12 h at 4°C. After the mucosa was peeled off, the remaining bladder tissue was minced with scissors and digested with 250 U/ml collagenase type IV for 30 min at 37°C. The cell suspension was sieved through a cell strainer, resuspended in DMEM with 10% FCS, and plated in 75-cm² cell culture flasks. The medium was changed 24 h later, and every other day thereafter. Cells at passage 3 were seeded at 2 × 10⁴ cells/ml in 6-well plates or 96-well plates and then serum deprived in DMEM containing 0.5% FCS for 48 h before each assay.

bFGF stimulation of rat BSCMC. Cells were treated with bFGF (0, 10, and 50 ng/ml, n = 3) or with 50 ng/ml bFGF plus 100 μg/ml anti-bFGF antibody (n = 3). For collagen expression experiments, 50 μg/ml ascorbic acid and 25 mM β-aminopropionitrile were added to the culture media. Cell proliferation was assessed by WST-8 assay at days 2 and 4. Total RNA was extracted after 6 and 24 h, and expression of type I and III collagen mRNA was evaluated by real-time PCR.

bFGF-induced signal pathway in rat BSCMC. Cells were treated with bFGF (0 and 50 ng/ml, n = 3) to evaluate phosphorylation of ERK1/2 and p38MAPK over the indicated time period. Next, cells were treated with PD98059 or SB203580 applied 60 min before bFGF treatment to inhibit ERK1/2 or p38MAPK activation (n = 3). For collagen expression experiments, 50 μg/ml ascorbic acid and 25 mM β-aminopropionitrile were added to the culture media. Cell proliferation and expression of type I collagen, type III collagen, and cyclin D1 mRNA were evaluated as described above.

Animals

7-wk-old female Sprague-Dawley rats, weighing 170 to 190 g, were purchased from Japan Slc. Animals were treated in accordance with National Institutes of Health animal care guidelines, and all animal experiments were approved by the Kyoto University Animal Experiment Committee.

In vivo effect of bFGF on rat bladder using gelatin hydrogels as release carriers. Gelatin hydrogel sheets were made as previously described (42). Sheets were freeze-dried, cut in rectangles (8 × 5 mm), and impregnated with an aqueous solution containing bFGF to obtain gelatin hydrogels incorporating bFGF. Rats were anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine, and bladders were exposed via midline incision. A gelatin hydrogel containing bFGF (0, 1, 5, and 10 μg/site bFGF, n = 8) was fixed over the ventral side of each bladder with four 8-0 nylon sutures. These sutures were utilized as marking sutures at specimen retrieval. Sham-operated rats (n = 8) did not receive the gelatin hydrogel treatment. Four rats in each group were killed after 7 days. The bladders were removed, and immunohistochemistry for Ki67 was performed to evaluate smooth muscle cell proliferation. The other four rats in each group were killed after 14 days. Total pepsin-solubilized collagen was extracted from the region of the bladders where the hydrogels were fixed. Muscle strips were prepared from the same bladder region of the bladders, and contractile responses of the muscle strips to potassium, carbachol, and electrical stimulation were measured.

Rat BOO model. Partial BOO was created in 12 rats using methods described elsewhere (44, 45). Briefly, the proximal urethra was freed from the vaginal wall. A longitudinal scission was made in the wall of 2-mm-long PE-200 polyethylene catheters (BD Intramedic, Sparks, MD). The open catheter was placed around the proximal urethra. Twelve sham-operated rats underwent similar procedures, but the catheter was removed before the abdominal incision was closed. After 4 wk, four rats in each group underwent filling cystometries through injection of saline at the rate of 0.06 ml/min under 900 mg/kg urethane anesthesia as described previously (21). Bladders were removed from all rats and weighed to confirm hypertrophy. Immunohistochemistry for Ki67 was performed for six bladders in each group. Western blot analysis and immunohistochemistry for bFGF were performed for the same six bladders in each group. Collagen was extracted from the other six bladders in each group.

Immunoblotting

Whole cell lysates from bladder tissue and cultured cells were lysed with RIPA buffer containing protease inhibitors. The protein content of the cell lysates was measured using the Bio-Rad Protein Assay Kit. Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane. The membranes were incubated with antibodies against bFGF (1:2,000), phosho-ERK1/2 (1:1,000), ERK1/2 (1:1,000), phosho-p38MAPK (1:1,000), p38MAPK (1:1,000), and β-actin (1:2,000) as an internal control. After incubation with horse-radish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies, immunoreactive proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate.

Immunohistochemistry

Bladder specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. The sections were heated in 0.01 M citrate buffer (pH 6.0) for 20 min and incubated with antibodies against bFGF (1:100), α-SMA (1:200), calponin (1:1,000), or Ki67 (1:25) for 12 h at 4°C. Negative control sections were incubated without primary antibodies. Antibody binding was detected using the Vectastain Elite ABC Kit (mouse IgG). The sections were visualized following incubation with diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. For in vivo proliferation analysis, the Ki67-positive cell ratio was determined in the
following manner. The cells in the hydrogel-treated muscle layer exhibited α-SMA and calponin immunoreactivity, indicating that they are smooth muscle cells. Four high-magnification (×400) fields in the same muscle layer were randomly selected for each specimen. Ki67-positive smooth muscle cells and total smooth muscle cells were counted in all four fields, and then the ratio of Ki67-positive smooth muscle cells to total smooth muscle cells was determined as the Ki67-positive ratio in each specimen.

**WST-8 Assay**

In vitro cell proliferation was assessed using the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay as described previously (19). After bFGF treatment, 10 μl of WST-8 reagent solution was added to the wells and incubated for 3 h at 37°C. The absorbance of the medium was measured at a wavelength of 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). A calibration curve was prepared by measuring absorbances with different cell densities at day 0.

**RNA Isolation and Real-Time PCR**

Total RNA extraction and cDNA synthesis were carried out using the RNeasy Mini Kit and SuperScript First-Strand Synthesis System for RT-PCR as described previously (19). Primers used for real-time PCR analysis were designed using Primer Express 2.0 software (Applied Biosystems) (Table 1). Quantitative real-time PCR was performed in 25-μl reactions containing the SYBR Green PCR Master Mix using the 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixtures were denatured at 95°C for 10 min, followed by 40 PCR cycles. Each cycle consisted of following three steps: 94°C for 15 s, 57°C for 15 s, and 72°C for 1 min. Each sample was normalized against an internal β-actin control.

**Collagen Analysis**

Bladder samples were weighed and cut into pieces. As a solvent, 0.5 M acetic acid including protease inhibitors and 10 mg/ml pepsin was prepared. Total pepsin-solubilized collagen was extracted from the samples by gently shaking them at 4°C for 2 days at a ratio of 10 volumes of solvent to tissue weight. After extraction, the suspension was centrifuged at 15,000 g for 60 min at 4°C, and the supernatant was retrieved. Collagen content in the supernatant was measured by a quantitative dye-binding method using the Sircol Collagen Assay Kit (Applied Biosystems) (Table 1). Quantitative real-time PCR was performed by 40 PCR cycles. Each cycle consisted of following three steps: 94°C for 15 s, 57°C for 15 s, and 72°C for 1 min. Each sample was normalized against an internal β-actin control.

**bFGF Promotes Rat BSMC Proliferation In Vitro and In Vivo**

In vitro proliferation of rat BSMC was assessed by the WST-8 assay. The number of rat BSMC was significantly increased 2 and 4 days after treatment with 10 and 50 ng/ml bFGF. This effect was blocked by addition of an anti-bFGF antibody (Fig. 1A).

In vivo proliferation of rat BSMC was evaluated by Ki67 immunohistochemistry of rat bladders treated with the gelatin hydrogels incorporating bFGF. After swelling with bFGF solution, freeze-dried gelatin hydrogels became translucent (Fig. 1B). Hydrogels incorporating bFGF were fixed tightly onto bladders with sutures (Fig. 1C). An increase in the number of Ki67-positive BSMC was observed for bladders treated with 5 and 10 μg/site bFGF compared with the sham-operated group (Fig. 1D). The ratio of Ki67-positive cells was significantly increased for bladders treated with 10 μg/site bFGF compared with the sham-operated group (Fig. 1E).

**bFGF Downregulates Type I Collagen Expression and Uregulates Type III Collagen Expression in Rat BSMC**

Real-time PCR revealed that type I collagen expression was significantly reduced when BSMC were treated with 50 ng/ml bFGF (Fig. 2A) but that type III collagen expression significantly increased under the same conditions (50 ng/ml bFGF, Fig. 2B). Treatment with the anti-bFGF antibody reversed both effects (Fig. 2, A and B).

After in vivo treatment with bFGF released from gelatin hydrogels, total pepsin-solubilized collagen was extracted from the rat bladders to assess the protein levels of type I and III collagen. The total pepsin-solubilized collagen per total collagen (pepsin-solubilized and insoluble) ratio was 89.8 ± 2.6%. These data indicate that the majority of collagen in the bladders of treated rats was pepsin-solubilized. There was no change in the

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<td>Type III collagen α1 chain</td>
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<td>Cyclin D1</td>
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<td>β-Actin</td>
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**Statistical Analysis**

All data are presented as means ± SD. Data were analyzed with an unpaired Student’s t-test using StatView version 5.0 for Windows (SAS Institute, Cary, NC). P < 0.05 was accepted as significant.
amount of total pepsin-solubilized collagen per bladder weight (Fig. 2C). However, Western blotting revealed that type III collagen (\(\alpha_3(III)\)) was markedly upregulated in bladders treated with 1, 5, and 10 \(\mu\)g/site bFGF compared with those without bFGF treatment. Treatment with the bFGF-neutralizing antibody (100 \(\mu\)g/ml) inhibited cell proliferation at 50 ng/ml bFGF. B and C: gelatin hydrogel used for in vivo bFGF stimulation. B: freeze-dried gelatin hydrogel (left) became translucent (right) after impregnation with an aqueous bFGF solution. C: gelatin hydrogel sheet was attached tightly to the bladder with sutures, which were also used to mark the field treated with bFGF. D: in vivo proliferation of BSMC as assessed by Ki67 immunostaining of bladder specimens. Bladders were treated with various amounts of bFGF released by gelatin hydrogels for 7 days (\(n=4\)). Paraffin-embedded bladder sections (5-\(\mu\)m thickness) were incubated with a primary antibody against Ki67 (1:25) and visualized with diaminobenzidine tetrahydrochloride (DAB). The brown color indicates Ki67 immunoreactivity. Scale bars = 20 \(\mu\)m. E: Ki67-positive cell ratio in the bladder specimens. The Ki67-positive cell ratio was determined by calculating the ratio of Ki67-positive BSMC to total BSMC in four randomly selected fields. The ratio for the bFGF-treated group (10 \(\mu\)g/site) was significantly higher than that of the sham-operated group. *Statistically significant difference (\(P<0.05\)).

**ERK1/2 Mediates bFGF-Induced Proliferation, Type I Collagen Downregulation, and Type III Collagen Upregulation in BSMC**

Incubation of rat BSMC with bFGF increased ERK1/2 phosphorylation compared with an untreated control group, whereas it did not affect p38MAPK phosphorylation (Fig. 3A).

To assess whether ERK1/2 is necessary to mediate the effects of bFGF in rat BSMC, ERK1/2 and p38MAPK signaling was pharmacologically blocked by specific inhibitors. The WST-8 assay revealed that upstream blockade of ERK1/2 with the MEK1 inhibitor PD98059 significantly suppressed cell proliferation in a dose-dependent manner (Fig. 3B) but blockade of p38MAPK with the specific inhibitor SB203580 had no effect. Regulation of collagen expression by ERK1/2 was assessed by incubation with 5 \(\mu\)M PD98059 based on the cell proliferation results. Real-time PCR revealed that blockade of ERK1/2 with PD98059 reversed both type I collagen downregulation (Fig. 3C) and type III collagen upregulation (Fig. 3D). However, blockade of p38MAPK with SB203580 did not affect type I and type III collagen levels. To assess whether cell proliferation and

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**Fig. 1.** Bladder smooth muscle cell (BSMC) proliferation under basic fibroblast growth factor (bFGF) stimulation. A: in vitro proliferation of rat BSMC. BSMC were treated with the indicated concentrations of bFGF (\(n=3\)). Cell counts were determined at the indicated time points by measuring the absorbance of the culture medium 3 h after addition of WST-8 reagent at 37°C. Cells treated with 10 and 50 ng/ml of bFGF showed significantly increased proliferation compared with those without bFGF treatment. Treatment with the bFGF-neutralizing antibody (100 \(\mu\)g/ml) inhibited cell proliferation at 50 ng/ml bFGF. B and C: gelatin hydrogel used for in vivo bFGF stimulation. B: freeze-dried gelatin hydrogel (left) became translucent (right) after impregnation with an aqueous bFGF solution. C: gelatin hydrogel sheet was attached tightly to the bladder with sutures, which were also used to mark the field treated with bFGF. D: in vivo proliferation of BSMC as assessed by Ki67 immunostaining of bladder specimens. Bladders were treated with various amounts of bFGF released by gelatin hydrogels for 7 days (\(n=4\)). Paraffin-embedded bladder sections (5-\(\mu\)m thickness) were incubated with a primary antibody against Ki67 (1:25) and visualized with diaminobenzidine tetrahydrochloride (DAB). The brown color indicates Ki67 immunoreactivity. Scale bars = 20 \(\mu\)m. E: Ki67-positive cell ratio in the bladder specimens. The Ki67-positive cell ratio was determined by calculating the ratio of Ki67-positive BSMC to total BSMC in four randomly selected fields. The ratio for the bFGF-treated group (10 \(\mu\)g/site) was significantly higher than that of the sham-operated group. *Statistically significant difference (\(P<0.05\)).

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**Fig. 2.** Collagen expression by bFGF in bladder smooth muscle. A: schematic diagram of the experimental design. BSMC were treated with the indicated concentrations of bFGF for 7 days. Cells were stained with pepsin/ethanol for total collagen and CBB for type I collagen, which were visualized with phase-contrast microscopy. C: schematic diagram of the in vivo experiment. Bladders were treated with various amounts of bFGF released by gelatin hydrogels for 7 days (\(n=4\)). Paraffin-embedded bladder sections (5-\(\mu\)m thickness) were incubated with a primary antibody against Ki67 (1:25) and visualized with diaminobenzidine tetrahydrochloride (DAB). The brown color indicates Ki67 immunoreactivity. Scale bars = 20 \(\mu\)m. D: in vivo proliferation of BSMC as assessed by Ki67 immunostaining of bladder specimens. Bladders were treated with various amounts of bFGF released by gelatin hydrogels for 7 days (\(n=4\)). Paraffin-embedded bladder sections (5-\(\mu\)m thickness) were incubated with a primary antibody against Ki67 (1:25) and visualized with diaminobenzidine tetrahydrochloride (DAB). The brown color indicates Ki67 immunoreactivity. Scale bars = 20 \(\mu\)m. E: Ki67-positive cell ratio in the bladder specimens. The Ki67-positive cell ratio was determined by calculating the ratio of Ki67-positive BSMC to total BSMC in four randomly selected fields. The ratio for the bFGF-treated group (10 \(\mu\)g/site) was significantly higher than that of the sham-operated group. *Statistically significant difference (\(P<0.05\)).

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**Fig. 3.** ERK1/2 signaling in BSMC. A: Western blot analysis of ERK1/2 and p38MAPK phosphorylation in BSMC treated with various amounts of bFGF for 15 min. B: WST-8 assay revealed that upstream blockade of ERK1/2 with the MEK1 inhibitor PD98059 significantly suppressed cell proliferation in a dose-dependent manner. C: real-time PCR revealed that blockade of ERK1/2 with PD98059 reversed both type I collagen downregulation and type III collagen upregulation. However, blockade of p38MAPK with SB203580 did not affect type I and type III collagen levels. To assess whether cell proliferation and
collagen expression were simultaneously regulated by bFGF, the mRNA expression of cyclin D1, a cell cycle protein, was assessed. Treatment with 50 ng/ml bFGF upregulated cyclin D1 expression, indicating that BSMC proliferation was enhanced. Blockade of ERK1/2 with PD98059 reversed the cyclin D1 upregulation, but blockade of p38MAPK with SB203580 did not affect expression (Fig. 3E).

**bFGF Increases Bladder Contractile Force**

The contractile force of rat bladder muscle strips treated with gelatin hydrogels incorporating bFGF was examined to assess the in vivo functional effects of bFGF. Under high concentration potassium induction, cholinergic stimulation, or electrical stimulation, the contractile force of bladder strips treated with 10 μg/site bFGF was significantly increased (Fig. 4, A–C).
Obstructed Bladders Display BSMC Proliferation, Type III Collagen Upregulation, and Increased Urothelial bFGF Expression

BOO rats had hypertrophied bladders with significantly increased bladder weight (Fig. 5A). BOO rats also showed significantly decreased micturition volume and increased residual urine (Table 2). Immunohistochemistry showed that more Ki67-positive BSMC could be observed in BOO rats compared with sham-operated rats (Fig. 5B). The total pepsin-solubilized collagen per total collagen (pepsin-solubilized and insoluble) ratio was 90.5 ± 2.3%. These data indicate that the majority of collagen in the bladders of treated rats was pepsin-solubilized. The expression of type I and III collagen in hypertrophic bladders was examined with respect to total pepsin-solubilized collagen levels. There was no difference in the amount of total pepsin-solubilized collagen per bladder weight between sham-operated and obstructed bladders (data not shown). Western blotting showed that type III collagen chain [α3(III)] expression was increased in obstructed bladders compared with sham-operated bladders but CBB staining showed that no change was observed for type I collagen chains (Fig. 5C). The bFGF level was upregulated in BOO rats compared with sham-operated rats, as demonstrated by immuno blotting (Fig. 5D). Immunohistochemistry showed that bFGF was more intensely expressed in the urothelial layer of obstructed bladders compared with sham-operated bladders (Fig. 5E).

DISCUSSION

In this study, we demonstrate that bFGF remodels the bladder smooth muscle layer through increased cell proliferation and altered collagen synthesis via the ERK1/2 pathway. We postulate that this process could underlie bladder hypertrophy after outlet obstruction based on the following evidence: 1) bFGF promoted rat BSMC proliferation in vitro and in vivo (Fig. 1); 2) bFGF induced type I collagen downregulation and type III collagen upregulation in rat BSMC (Fig. 2); 3) bFGF induced ERK1/2 phosphorylation in rat BSMC, and bFGF-induced rat BSMC proliferation and collagen regulation were mediated by ERK1/2 (Fig. 3); 4) bFGF increased the contractile force of rat bladders (Fig. 4); and 5) hypertrophic bladders increased urothelial bFGF expression concurrently with BSMC proliferation and type III collagen upregulation (Fig. 5). Taken together, these findings suggest that bFGF may function as a signal that adapts the bladder to outlet obstruction through modification of the smooth muscle layer.

A major finding of this study was that bFGF regulated not only BSMC proliferation but also collagen expression, with a notable contrast of type I collagen downregulation and type III collagen upregulation (Fig. 2, A and B). The increased prolif-

Fig. 4. Muscle strip contraction experiment. The contractile force of muscle strips covered with gelatin hydrogels was examined under high potassium (A), cholinergic (B), or electrical field stimulation (C); n = 4. In all experiments, the contractile force of muscles treated with 10 μg/site bFGF showed a significant increase compared with the sham-operated group. *Statistically significant difference (P < 0.05).

Fig. 3. Signaling pathway mediating bFGF-induced effects in BSMC. A: ERK1/2 and p38MAPK phosphorylation. Cell lysates were retrieved at the indicated time points from BSMC treated with or without bFGF (50 ng/ml; n = 3). Twenty micrograms of protein from each lysate was separated by SDS-PAGE and transferred to PVDF membranes. Densitometry was performed for ERK and phospho-ERK1/2. Phosphorylation of ERK1/2 was sustained until 8 h on bFGF treatment, whereas without bFGF, it returned to basal levels within 2 h. Phosphorylation of p38MAPK returned to basal levels within 2 h irrespective of bFGF treatment. These data were consistently replicated in other experiments. B: inhibition of bFGF-induced proliferation in BSMC. BSMC were treated with 50 ng/ml bFGF in the presence or absence of the indicated doses of PD98059 (ERK1/2 inhibitor) or SB203580 (p38MAPK inhibitor; n = 3). PD98059 significantly inhibited bFGF-induced proliferation in a dose-dependent manner, whereas SB203580 did not inhibit proliferation. C–E: inhibition of bFGF-induced collagen expression in BSMC. BSMC were treated as in the proliferation study (n = 3). PD98059, but not SB203580, significantly inhibited the bFGF-induced decrease in type I collagen expression (C) and increase in type III collagen expression (D). PD98059, but not SB203580, also significantly and simultaneously inhibited the bFGF-induced increase in cyclin D1 expression (E). *Statistically significant difference (P < 0.05).
Table 2. Voiding function of rat BOO model

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<td>0.20 ± 0.092*</td>
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<td>0.21 ± 0.029*</td>
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<td>Bladder capacity, ml</td>
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<td>Maximum voiding pressure, mmHg</td>
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<tr>
<td>Compliance, ml/mmHg</td>
<td>0.0377 ± 0.0110</td>
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Values are means ± SD. BOO, bladder outlet obstruction. *Statistically significant difference (P < 0.05).

It should be noted that the pattern of expression for collagen protein in vivo and mRNA in vitro was somewhat discrepant. In vivo bFGF treatment upregulated type III collagen protein but did not affect type I collagen, whereas in vitro treatment upregulated type III mRNA and downregulated type I (Fig. 2, A, B, and D). However, it has been shown that protein measurements detect both previously existing and newly formed collagen (35), whereas in vitro mRNA data only reflect the regulation of de novo synthesis. In the present study, it is possible that in vivo downregulation of type I collagen synthesis was balanced by a downregulation of degradation, or simply that reduced type I collagen levels could not be detected in our experimental context and time frame.

In terms of intracellular signaling pathways, bFGF activated ERK, but not p38MAPK, in BSMC (Fig. 3A). Since bFGF activates both ERK and p38MAPK pathways in vascular smooth muscle cells to induce cell proliferation (16), it was unexpected that bFGF-induced effects in BSMC would be mediated exclusively by ERK. Smooth muscle cells play a central role in the contraction of many organs including blood vessels, intestine, and bladder, but they vary in their response to different external stresses such as stretch (2). Physiologically, vascular smooth muscle cells respond to rapid cyclic stretch, whereas BSMC respond to noncyclic and static stretch. Such a different biological context may have led smooth muscle cells to different organ-specific subtypes. It has been additionally reported that such stretch activates ERK in BSMC and that ERK is more highly activated in BSMC stretched statically than cyclically (2). Different signal activation pathways between vascular smooth muscle cells and BSMC have also been reported for PDGF. In vascular smooth muscle cells, PDGF activates PI3/Akt, p38MAPK, and ERK (16), but in BSMC, PDGF activates only PI3/Akt and p38MAPK (1).

bFGF-induced effects on BSMC such as cell proliferation, type III collagen upregulation, and increased contractile force reflect some characteristics of BOO. BOO is divided into three stages: 1) hypertrophy, 2) compensation and 3) decompensation (2). Bladders in the first hypertrophic stage show increased contractile force (9, 25, 37). bFGF is also upregulated during hypertrophy but decreases to normal levels during decompensation (4, 9). In conjunction with the present study, these findings suggest that bFGF treatment induces a bladder condition similar to the first hypertrophic stage of BOO.

Uprogation of bFGF in pathological bladder conditions has been reported in several studies (5, 7, 27). These studies investigated whole bladder tissue, making it difficult to determine whether bFGF affects BSMC in an autocrine or paracrine manner. However, it should be noted that bFGF has been reported to be a potent BSMC mitogen in vitro (7, 11). Therefore, the differential expression of type I and III collagen is an unreported phenomenon that may play a major role in bladder smooth muscle remodeling.

Most of the bladder extracellular matrix consists of type I and III collagen. Unique characteristics of type III collagen include a high degree of proline hydroxylation and a higher glycine content, which causes rapid intermolecular cross-linking (12). As a result, type III collagen predominates during periods of rapid growth such as wound healing and fetal bladder development (6, 29). However, whether type III collagen upregulation leads to bladder dysfunction remains controversial. Some studies have reported type III collagen upregulation and a decrease in the type I/III ratio in non-compliant, fibrotic bladders (13, 22). Similar findings have also been reported in cardiac muscle, where the decreased collagen I/III ratio may increase heart stiffness, leading to cardiomyopathy and cardiac hypertrophy (26, 32, 47). On the other hand, type III collagen-mediated enhancement of bladder function has also been observed. Chang et al. (10) have reported that type III collagen has a coil conformation that allows it to extend and accommodate large changes in bladder volume. In the present study, bladders treated in vivo with bFGF showed some type III collagen upregulation but no change in total pepsin-solubilized collagen content (Fig. 2C), indicating that bFGF does not render bladders fibrotic. Moreover, bladders treated with bFGF showed a higher contractile response to stimulation (Fig. 4, A–C). Thus excessive type III collagen upregulation may cause bladder deformation and dysfunction, but moderate upregulation could result in increased contractility with preserved elasticity as shown in our study. This possibility is further supported by a recent finding that bladders of type III collagen-deficient mice showed reduced contractility compared with those of wild-type mice (41).
A natural adaptation against outlet overload, and the inhibition of this adaptive pathway could lead to decompensation or urinary retention. Thus the clinical application of bFGF-ERK inhibition might be limited to specific conditions where muscle hypertrophy must be absolutely avoided, such as extremely noncompliant bladder observed in patients with spinal dysraphism (13). On the other hand, our findings also suggest that bFGF could be a potent therapeutic agent to treat conditions such as an underactive detrusor, where decompensated bladders fail to void urine. For clinical application of this concept, controlled release systems for bFGF would be indispensable. The gelatin hydrogel system employed in the present study could be a promising candidate method for that purpose. It can be manufactured into transurethrally injectable micropellets (3, 30). This strategy could also be applied to conditions commonly observed in patients after pelvic surgery or with diabetes mellitus (50).

In summary, our data suggest that bFGF could be a pivotal signal in adapting the bladder to obstructive overload through BSMC proliferation and altered collagen production via the ERK pathway. Blockade of the bFGF-ERK pathway could be a potential method to treat the extremely overactive bladder, whereas bFGF itself could be a promising therapeutic agent for the treatment of an underactive detrusor.

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