Cyr61 and CTGF are molecular markers of bladder wall remodeling after outlet obstruction

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The most prominent histopathologic finding in obstructed bladders is a rapid and marked remodeling of the bladder wall, including compartment-specific cellular hypertrophy, hyperplasia of the detrusor muscle, and changes in the structural relationship between connective tissue and smooth muscle elements (9, 50, 54). Muscle hypertrophy gives rise to trabeculae and folds, and microscopically it is associated with hyperplasia of all cell layers. Functionally, hypertrophy/hyperplasia enable the bladder, during the early stages of the obstruction, to compensate transiently for the extra effort required for it to empty against increased urethral resistance. Such a compensatory hypertrophy commonly occurs, for instance, in the kidney as a result of unilateral nephrectomy or in the heart in response to regular vigorous exercise (16). However, as a result of a sustained increased urethral resistance, an excessive and eccentric hypertrophy arises and results in physiological insufficiency and, ultimately, organ failure.

Many lines of evidence have suggested that overexpression of growth factors is a major factor in the development of the remodeling of the bladder wall after urethral obstruction. Baskin and colleagues (3, 4) have reported an increase in transforming growth factor (TGF)-β2, TGF-β3, and TGF-α in bladders subjected to partial urethral outlet obstruction and correlate such increases with excessive deposition of extracellular matrix proteins. Studies in various animal models and in humans showed that hypertrophied obstructed bladders contain significantly more insulin-like growth factor I (IGF-I) and cyclooxygenase-2 than normal bladders (1, 11, 43). Other studies emphasized the role of the renin-angiotensin system and inducible nitric oxide synthase in the early responses of the bladder to the outlet obstruction (5, 12). However, it is not certain which of these factors is important in the setting of bladder obstructive disease development. Additionally, pathological remodeling of bladder smooth muscle is determined by complex interactions of many factors, including growth factors; mechanical cues such as hy-
drowave static pressure, tissue stretch, or distension; and the physicochemical environment.

Evidence has emerged that the effects of numerous physical and chemical stimuli are mediated by immediate early growth factors, such as the cysteine-rich protein 61 (Cyr61/CCN1) and connective tissue growth factor (CTGF/CCN2) (32, 39). Physical and chemical stimuli are thought to activate the expression of Cyr61 and CTGF to augment their own activities and/or to carry out additional functions that may be required to render their biological effects.

The Cyr61 and CTGF are members of the CCN family of proteins, which also consists of nephroblastoma overexpressed (Nov/CCN3), Elm-1/WISP-1/CCN4, Cop-1/WISP-2/CCN5, and WISP-3/CCN6 (32, 45). The Cyr61 and CTGF proteins, the most extensively studied proteins in this family, are characterized by a high degree of sequence homology. They are organized into conserved modular domains that share similarities with insulin-like growth factor-binding proteins, von Willebrand factor type C repeats, thrombospondin type I repeats, and growth factor cysteine knots. However, despite their highly conserved structure, these proteins display nonredundant biological functions. Both Cyr61 and CTGF are co-induced in fibroblasts by growth factors involved in tissue repair and in extracellular matrix remodeling, i.e., platelet-derived growth factor, bovine fibroblast growth factor, and TGF-β1. Purified Cyr61 mediates cell adhesion and chemotaxis for fibroblasts and enhances growth factor-stimulated DNA synthesis (28). Cyr61 also up-regulates the expression of matrix metalloproteinases 1 and 3 and promotes biological processes such as wound healing, angiogenesis, homeostasis, and thrombosis (22, 44). The CTGF was reported to promote proliferation of fibroblasts and mediate TGF-β-induced extracellular matrix production (21). Overall, on the basis of their expression pattern in development and diseases, it has been suggested that Cyr61 and CTGF are involved in the development of fibrotic pathologies (renal sclerosis, systemic scleroderma, hepatic fibrosis, biliary atresia) and in the pathogenesis of cancer (8, 32, 57).

Relatively little is known about the expression pattern and functional significance of Cyr61 and CTGF gene expression in smooth muscle under normal and pathological conditions. We have recently demonstrated that mechanical forces strongly induced the expression of the Cyr61 gene in cultured bladder smooth muscle cells (52). Both the Cyr61 gene expression pattern and its regulatory mechanism seemed to be similar to those of prohypertrophic molecules, i.e., serum response factor or α-actin (35, 51). Another study has shown that overexpression of CTGF promotes vascular smooth muscle cells to express more extracellular matrix protein collagen I and fibronectin (14). Therefore, we hypothesized that Cyr61 and CTGF are potential early molecular signals of smooth muscle cell phenotype remodeling, involved in the activation of smooth muscle hypertrophic response in the obstructed bladder.

To address this hypothesis, we investigated the temporospatial expression of both Cyr61 and CTGF in rats with hypertrophied bladder as a result of outlet obstruction. In vitro studies were also performed to determine whether agonists of smooth muscle hypertrophy, such as angiotensin II, endothelin-1, and parathyroid hormone-related peptide (PTHrP), regulate the expression of Cyr61 and CTGF genes as well.

**MATERIALS AND METHODS**

**Surgical induction of partial outlet obstruction.** Eight-month-old Sprague-Dawley male rats (Charles River Laboratories, Wilmington, MA) were used in these studies. Twenty-four rats were separated into four groups of six rats each. Each rat was sedated with ketamine-xylazine (80–10 mg/kg ip), and surgical anesthesia was maintained with pentobarbital (Nembutal, 25 mg/kg). The bladder was exposed through a midline incision, and a partial outlet obstruction was created by tying a 2-0 silk ligature loosely around a length of PE-20 tubing placed on the urethra (above the pubis). The incision was closed with 3-0 vicryl sutures. In the sham rats, the bladder and urethra were exposed, but no ligature was applied.

**Tissue handling.** At the end of the period of obstruction, each rat was anesthetized as just described, and the bladder was exposed through a midline incision and excised rapidly. For immunohistochemical analyses, a full-thickness strip of each bladder was fixed in 10% neutral buffered formalin for 4–8 h, routinely processed, and embedded in paraffin block to provide a cross-sectional view of bladder wall after microtome sectioning. Five-micrometer-thick sections were cut from each block and mounted on positively charged slides. The remainder of the bladder was frozen in liquid nitrogen and stored at −70°C for RNA and protein analysis.

**RNase protection assay.** Total RNA extraction was performed as described by Chomczynski and Sacchi (13). Cyr61 mRNA levels were measured by RNase protection assay relative to those of the GAPDH mRNA. Accordingly, two rat riboprobes were used. One probe is complementary to Cyr61 mRNA and protects a 451-nucleotide fragment. A second probe is complementary to GAPDH RNA and protects a 181-nucleotide fragment. The riboprobes were generated by reverse transcription and PCR amplification by use of the following primers: for Cyr61, 5′-caacccaatgtaaacatc3′ (GenBank accession no. NM023964). The PCR product was 181-nucleotide fragment. The riboprobes were generated by reverse transcription and PCR amplification by use of the following primers: for Cyr61, 5′-caacccaatgtaaacatc3′ (GenBank accession no. NM0313327); for GAPDH, 5′-cagctggtcgaacatgctgca-3′ and 5′-tcagctggaacatgctgca-3′ (GenBank accession no. NM023964). The PCR products were cloned into the cloning vector pCRII from Invitrogen and further sequenced to verify their orientation and identity. For the RNase protection assay, the plasmids were linearized and served as a template for in vitro transcription by either SP6 or T7 RNA polymerase to generate [32P]UTP-labeled RNA probes with an in vitro transcription kit (Promega). Total RNA (12 μg) was resuspended in hybridization buffer containing 80% formamide, 1 mM EDTA, 40 mM piperazine-N, N′-bis(2-hexanesulfonic acid), pH 6.4, and 0.2 M sodium acetate, 1 × 106 cpm riboprobe, and denatured at 85°C for 5 min. After 24 h of incubation at 45°C, nonhybridized RNAs were digested with 40 μg/ml ribonuclease A and 100 U/ml ribonuclease T1. The protected hybrids were then precipitated and separated on a 4% polyacrylamide/urea denaturing sequencing gel followed by autoradiography. The protected bands were quantitated by use of a Molecular Dynamics phosphorimager.

**Northern blot analysis.** Samples of total RNA (10 μg) were dissolved in 50% formamide, 2.2 M formaldehyde, 0.1 M...
MOPS (pH 7.0), 40 mM sodium acetate, and 5 mM EDTA (pH 8) and were denatured by heating at 65°C for 10 min. RNA was then fractionated by electrophoresis in 1% agarose/formaldehyde gel for 4 h at 70 V and transferred overnight by capillary blotting in 20× SSC (1× SSC = 0.15 M NaCl, and 0.015 M sodium citrate, pH 7) to a zeta-probe nylon filter. The nylon filter was then prehybridized for 24 h at 42°C in 50% formamide, 7% SDS, 0.25 M Na2HPO4 (pH 7.2), and 0.25 M NaCl. Specific DNA probes were labeled with α-[32P]dCTP with a random priming DNA labeling kit. After hybridization at 42°C for 24 h, the filter was washed twice in 2× SSC-0.1% SDS at room temperature and twice in 0.1× SSC-0.1% SDS at 50°C, and exposed to a phosphorimager screen. The hybridization signals were quantitated and normalized to GAPDH mRNA used as a control gene. A rat CTGF DNA probe was prepared by RT-PCR by use of the primers 5′aggagtggtgtgatgtag3′ and 5′caacagcttagaaccag3′ (GenBank accession no. NM022266). The PCR product was purified, cloned into the expression vector pCRII (Invitrogen, Carlsbad, CA), and sequenced before its utilization in Northern blot analysis. A DNA probe for α2(I) collagen has been described previously (37).

Immunohistochemistry and immunoblotting. For Western blot analysis, tissue lysates were prepared by harvesting bladder tissue samples in 0.1% Triton X-100 lysis buffer. Protein concentration was determined by using the Bradford protein assay (Bio-Rad). Protein samples (30 μg) were separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane, and Western blot analysis was performed using either anti-rat Cyr61 antibody (Santa Cruz Biotech, San Diego, CA) or anti-human CTGF antibody (generous gift from Dr. B. Perbal; see Ref. 46). The CTGF antibody was raised against a 28-synthetic amino acid peptide corresponding to human CTGF positions 232–259 (GenBank accession no. XM037056). The latter peptide shares 100% sequence homology with the deduced amino acid sequence of rat CTGF (GenBank accession no. NM022266). Immunodetection was performed using enhanced chemiluminescence (Amersham). For histochemical analyses, 5-μm-thick cryostat sections from obstructed and nonobstructed bladders were mounted on albumin-coated slides, air-dried, and fixed for 5 min in 0.4% formaldehyde-PBS. For qualitative assessments, bladder cross sections were stained with hematoxylineosin. Other sections were incubated for 24 h with either anti-Cyr61 antibody at 1:200 dilution or anti-CTGF antibody (1:120 dilution). Immunodetection was performed with an anti-IgG-rhodamine conjugate (1:300 dilution). Sections were washed several times in PBS between incubations. The immunostaining was visualized by confocal microscopy.

Cell culture and molecular analyses. Primary cultures of smooth muscle cells were prepared from fetal bovine bladders obtained from mid- to late-gestational fetal calves. Our previous studies have shown that cells from fetal bovine bladder maintain several of their differentiated properties in culture even after multiple passages (up to 8–10 passages) (10, 52). By contrast, Kropp et al. (30) showed that both cultured human and rat bladder smooth muscle cells are susceptible to variable loss of phenotypic markers of cell differentiation, such as smooth muscle α-actin, smooth muscle myosin, and desmin. However, primary cultures from the rat maintained a more differentiated profile than human cells (30). Cells were maintained in modified medium 199 from GIBCO (Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO2 in air at 37°C. Cells between their third and eighth population doubling level were used. Cells were treated with exogenous growth factors, as indicated in the text. Total RNA was then extracted from each sample and processed for Northern blot hybridization (as described in Northern blot analysis). DNA probes for either Cyr61 or CTGF have been previously described (52).

Statistical analysis. A paired two-sample t-test was used to compare the differences between control and test groups. Values are means ± SE. P < 0.05 was considered significant.

RESULTS

In agreement with previously published data, partial obstruction of the rat bladder outlet initiated tissue hypertrophy and induced a progressive increase of bladder wet weight from 121 ± 42 to 152 ± 15 mg within 1 day, to 239 ± 25 mg within 7 days, and to 249 ± 31 mg within 14 days after the outlet obstruction.

We examined a potential temporal correlation between bladder hypertrophy and Cyr61 and/or CTGF gene expression by measurements of their respective mRNA levels throughout the time course of the outlet obstruction. The Cyr61 mRNA levels in the control and the partially obstructed bladders were determined using the solution hybridization/RNase protection assay, a representative experiment of which is shown in Fig. 1A. Hybridization of total RNA to Cyr61 and GAPDH riboprobes yielded the predicted 451- and 181-bp protected RNA bands, respectively, after RNase digestion. No protected bands were present when tRNA was used instead of RNA from tissue samples (data not shown). Phosphorimager analyses of the Cyr61 hybridization signals indicate that Cyr61 mRNA, although undetectable in the nonobstructed controls, was markedly increased in the obstructed bladders. Cyr61 mRNA levels were significantly elevated (3- to 6-fold) after 1 day postobstruction and remained increased to a relatively similar extent throughout the time period of the outlet obstruction (P < 0.01).

The mRNA levels of CTGF were measured in control and obstructed bladders by Northern blot hybridization (Fig. 1B). When normalized to GAPDH mRNA, the CTGF mRNA levels were not significantly altered after 1 and 7 days postobstruction compared with controls. In contrast, in the 14-day-obstructed bladders, there was a two- to fourfold increase of CTGF mRNA (P < 0.05).

To determine whether obstruction-induced changes in Cyr61 and CTGF mRNAs were accompanied by corresponding changes in their protein levels, we analyzed total proteins from tissue homogenates by Western blot with specific antibodies raised against Cyr61 and CTGF. Immunodetection was performed by enhanced chemiluminescence. The anti-Cyr61 antibody recognized an ~42-kDa protein band in tissue homogenates from control and partially obstructed bladders (Fig. 2A). As determined by densitometric scanning of the protein band, there was a nearly 3.5-fold increase of Cyr61 band intensity throughout the time period of the partial outlet obstruction. Meanwhile, the ~43-kDa CTGF protein band, although barely detectable
Fig. 1. Cysteine-rich protein 61 (Cyr61) and connective tissue growth factor (CTGF) gene expression patterns in rat bladder after variable time periods of partial outlet obstruction. A: Cyr61 mRNA profile as determined by ribonuclease protection assay. Total RNA (12 μg) was co-hybridized to Cyr61 and GAPDH 32P-labeled riboprobes and subjected to RNase digestion. The protected hybrids are 451 and 181 bases long for Cyr61 and GAPDH, respectively. The formed hybrids were separated by electrophoresis in a polyacrylamide denaturing gel and were visualized by autoradiography. The hybridization signal for Cyr61 mRNA in each sample was normalized to that of GAPDH. B: CTGF mRNA profile as determined by Northern blot hybridization. Total RNA was fractionated by electrophoresis, transferred to a nylon membrane, and probed with a CTGF radiolabeled DNA probe. The hybridization signal for Cyr61 was normalized to that of GAPDH. Normal and sham-operated animal bladders served as controls. Values are means ± SE; N, no. of animals. ***P < 0.01; **P < 0.05.

Fig. 2. Western blot analysis of Cyr61 (A) and CTGF (B) protein in unobstructed bladders (lanes 1, 2) and after 1 (3, 4), 7 (5, 6), and 14 (7, 8) days of partial outlet obstruction (representative samples from 2 animals at each obstruction time point are shown). Thirty micrograms of total proteins from tissue homogenates were fractionated by SDS-PAGE, transferred to a nylon membrane, and incubated with either Cyr61 (1:200 dilution) or CTGF (1:120 dilution) antibodies. Immunodetection was performed by enhanced chemiluminescence.

CTGF protein levels. This underlines the well known individual heterogeneity of the rat model of outlet obstruction.

Transverse histological sections of the bladder showed evidence of bladder wall hypertrophy and a striking thickening of the detrusor muscle layer after outlet obstruction (Fig. 3, A and B). Previously reported infiltration of detrusor muscle by extracellular matrix proteins can also be seen in the 14-day-obstructed bladder (Fig. 3C). Localization of Cyr61 and CTGF proteins was performed by immunohistochemistry. Transmural sections of the bladder wall were incubated with either anti-rat Cyr61 or anti-human CTGF antibodies, and immunodetection was performed with an anti-IgG-Rhodamine conjugate. There was minimal or no detectable immunostaining for Cyr61 in the control nonobstructed bladders (Fig. 3D). Conversely, in the 7- and 14-day-obstructed bladders, Cyr61 localized prominently in the detrusor muscle within and surrounding the smooth muscle fascicles (Fig. 3, E and F). The Cyr61 immunoreactivity was undetectable in the urothelium lining the lumen of the bladder, and only a minimal immunostaining was seen within the lamina propria. This immunostaining pattern was noted consistently throughout the duration of the obstruction and was confirmed with another Cyr61 antibody that was directed against a human epitope of Cyr61 (data not shown) (52). In the 14-day-postob-
structed bladders, Cyr61 immunoreactivity was observed around the arteries as well (data not shown). Meanwhile, as shown in Fig. 3, G-I, immunostaining for CTGF was not seen in control nonobstructed bladders. No detectable immunoreactivity was seen for CTGF after 1-day postobstruction either (data not shown). At day 7, two of four obstructed bladders showed immunostaining to CTGF within the detrusor muscle, the lamina propria, and urothelial cells lining the lumen of the bladder. At day 14, there was a heterogeneous or patchy immunostaining to CTGF, with pockets of fairly intense staining interspersed with regions of less intense staining within both detrusor and lamina propria layers. The areas of intense staining colocalized with regions between the muscle bundles heavily infiltrated by extracellular matrix proteins. The CTGF protein changes that had been quantitated by Western blotting corroborate those seen by immunofluorescence staining. The staining attributed to either Cyr61 or CTGF was absent when their respec-
tive antibodies were omitted from the staining protocol (data not shown).

Numerous lines of evidence indicate that CTGF is an important player in fibrosis. In various fibrotic disorders, upregulation of CTGF gene strongly correlated with that of TGF-β1, the principal growth factor promoter of connective tissue synthesis, and with enhanced extracellular matrix protein synthesis such as type I collagen. Therefore, to examine a potential correlation between CTGF gene expression and that of extracellular matrix proteins in the bladder wall, we determined the expression profile of type I collagen, the most abundant extracellular matrix protein in the bladder wall, during the course of partial outlet obstruction. As shown in Fig. 4, the steady-state levels of type I collagen mRNA were significantly increased in 7- and 14-day-obstructed bladders compared with control nonobstructed ones. Type I collagen mRNA pattern seems similar to that of CTGF in the obstructed bladders. By contrast, we did not observe notable changes in TGF-β1 mRNA in the bladder wall throughout the time period of the outlet obstruction (data not shown).

Next, we sought to determine whether the changes in Cyr61 and CTGF gene expression could be initiated as a result of release of trophic factors, such as angiotensin II, endothelin-1, and PTHrP. The latter are known to be produced during the early stages of the obstruction and may be of variable importance as inducers of smooth muscle hypertrophy. For this purpose, primary cultures of bladder smooth muscle cells were initiated, as described in MATERIALS AND METHODS. Cells were then treated in serum-free medium with exogenous angiotensin II, endothelin-1, or PTHrP. Expression of Cyr61 and CTGF genes was assessed at the

Fig. 4. Expression profile of α2(I) collagen gene in rat bladder after variable time periods of partial outlet obstruction as determined by Northern blot hybridization. Total RNA was fractionated by electrophoresis, transferred to a nylon membrane, and probed with α2(I) DNA probe. The hybridization signal was normalized to that of GAPDH. A: representative autoradiogram of hybridization signals; B: statistical analysis of densitometric measurements. Values are means ± SE; N, no. of animals. ***P < 0.01; **P < 0.05.

Fig. 5. Expression of Cyr61 and CTGF genes in cultures of bladder smooth muscle cells treated with angiotensin II (0.5 μM), endothelin-1 (0.1 μM), or parathyroid hormone-related peptide (PTHrP, 0.1 μM). Total RNA was extracted at indicated time periods of incubation and analyzed by Northern blot hybridization sequentially with specific DNA probes for Cyr61 and CTGF. The blot was stripped from the label between 2 consecutive hybridizations. To control for equal RNA loading, the blot was probed with GAPDH DNA probe.
mRNA levels. As shown in Fig. 5, treatment of the cells with angiotensin II, endothelin-1, or PTHrP strongly induced the expression of both Cyr61 and CTGF. Cyr61 and CTGF mRNA levels rapidly increased after 30 min, peaked at 1 h, and decreased progressively but in a time-dependent manner. These results indicate that Cyr61 and CTGF are immediate early target genes of prohypertrophic factors.

**DISCUSSION**

In the rat model, partial outlet obstruction of the urinary bladder results in a significant increase in bladder mass followed by progressive bladder dysfunction associated with alterations in bladder contractility and detrusor muscle instability. Various etiologic factors have been implicated in initiating such a pathological remodeling, including increase in tension and/or strain on the wall of such a hollow organ and reduced blood flow to the bladder musculature (18, 48, 53). Several molecular changes take place in the obstructed bladder, including increase in overall protein synthesis and expression of extracellular matrix protein genes and embryonic genes, i.e., nonmuscle myosin heavy-chain B, and reorganization of the actin cytoskeleton.

Our present study implicates Cyr61 and CTGF as potential key molecules in bladder wall remodeling and dysfunction. We showed that bladder hypertrophy secondary to partial outlet obstruction induced the expression of Cyr61 and CTGF at both the mRNA and the protein levels. The expression of Cyr61 gene occurred with the onset of the obstruction (within 1 day) and remained elevated throughout the time period of the obstruction. The expression pattern of Cyr61 gene is reminiscent of that of gene 33, an immediate early gene that was shown to be expressed at constant levels in rapidly growing and in chronic stress conditions, such as incipient diabetic renal failure (36). In contrast, the steady-state mRNA levels of CTGF gene seemed to peak only after either 7 or 14 days of partial obstruction. In fact, various gene expression patterns for CTGF have been reported mainly in fibrogenic lesions. For instance, Sedlaczek et al. (49) reported increases in CTGF mRNA levels in the rat liver model of acute fibrogenesis only 3 days after a single administration of CCl4. In biliary fibrosis (chronic fibrogenesis), a prominent accumulation of CTGF occurred after 6 wk. Similarly, Lasky et al. (31) showed that CTGF mRNA levels in the lung of bleomycin-sensitive mice were consistently upregulated at 16 and 30 days after bleomycin administration. Taken together, these studies clearly indicate that CTGF gene expression does not occur immediately after either chemical or physical insults and, rather, mimics that of delayed responsive genes, such as those of growth factors. Obstruction-induced increases in Cyr61 and CTGF mRNA could be a result of both increased transcription and/or increased mRNA stability. However, it has been shown that the bulk of the regulation of Cyr61 and CTGF mRNA levels is at the transcriptional level (14, 47).

On the basis of their gene expression pattern in the obstructed bladder, Cyr61 and CTGF genes appear to be differentially regulated. The early, comparatively selective upregulation of Cyr61 gene may confer specificity in the cellular response. Indeed, our immunohistochemical analyses have indicated that Cyr61 protein was mainly confined to the detrusor muscle layer of the obstructed bladders. Therefore, Cyr61 protein expression seems to be associated with hypertrophic and rapidly proliferating smooth muscle cells, as 50–90% of the cells in the obstructed bladders consist of hypertrophic/hyperplastic smooth muscle cells (1, 55). However, in vitro studies have shown that the purified Cyr61 does not have intrinsic mitogenic activity but enhances growth factor-induced cell proliferation in both fibroblast and endothelial cells (29). It was proposed that Cyr61 action becomes most relevant when the concentration of growth factors in the immediate cell microenvironment is limiting. Cyr61 acts by displacement of growth factors bound to the extracellular matrix, thus increasing their accessibility to their cell receptors. Thus enhanced Cyr61 levels in the obstructed bladder potentially promote smooth muscle cell proliferation in cooperation with other locally produced growth factors.

Meanwhile, we (52) and others (44) have previously demonstrated that Cyr61 gene expression is strictly regulated through signaling pathways that have been implicated in the progression to hypertrophy. In particular, phosphatidylinositol 3-kinase and/or Rho GTPase activation strongly activates the expression of Cyr61 gene in bladder smooth muscle cells. These signaling pathways converge downstream to modulate the actin cytoskeleton, whose rearrangement can solely modulate Cyr61 gene expression. These signaling cascades have been shown to be critical mechanisms for the regulation of smooth muscle cell differentiation and to control the gene expression of several prohypertrophic molecules, i.e., serum response factor, α-actin (2, 34, 51). Therefore, Cyr61 is a potential early molecular signal of smooth muscle cell differentiation and potentially activates specific features of the cells' hypertrophic response to pathological conditions.

Immunolocalization analyses of CTGF protein in the obstructed bladder showed a heterogeneous and patchy immunostaining either within or between the muscle bundles, as well as in the lamina propria. No such change was seen in the control, nonobstructed bladders. Additionally, overexpression of CTGF correlates well with the upregulation of α2(I) collagen gene in the obstructed bladders, suggesting that CTGF potentially promotes extracellular matrix gene expression and deposition. In fact, previous in vivo studies have associated overexpression of CTGF with fibrotic disorders such as keloids, systemic sclerosis, and renal and lung fibrosis (19, 25, 32, 40). CTGF is expressed at very high levels in atherosclerotic human vessels and localizes predominantly in areas with excessive deposition of extracellular matrix proteins (41). This likely reflects, as shown by others (42), the affinity of this growth factor to abundant matrix proteins such as collagens and fibronectin. Additionally, our finding...
that two of four 7-day-obstructed bladders showed immunoreactivity within urothelial cells, although unexpected, parallels the observation by Sedlacek et al. (49) of a prominent expression of CTGF in proliferating bile duct epithelial cells. However, the biological significance of CTGF production in epithelial cells in vivo is unknown and will require further investigation. Overall, our findings seem to indicate that overexpression of Cyr61 in obstructed bladder is associated with the initial stage of hypertrophy/hyperplasia of the detrusor muscle, whereas that of the CTGF occurred at times that correlate with the onset of extracellular matrix remodeling.

Our study does not identify the chemical and/or physical factors that trigger Cyr61 and CTGF gene induction in the obstructed bladder. However, many lines of evidence suggest that the increased mechanical stress on the cellular component of the bladder wall during the obstruction contributes to changes in Cyr61 and CTGF gene expression and to the pathogenesis of bladder hypertrophy. Indeed, as a result of the anatomic obstruction, both the intravesical pressure and the volume work of the bladder increase, and the cellular components of the bladder wall receive mechanical “cues” that are different or beyond a normally acceptable range. This increased mechanical stress then causes a cascade of cellular and molecular events that promote structural and functional changes in the bladder wall. The importance of the mechanical factors, such as stretch and pressure on Cyr61 and CTGF gene expression, is supported by our previous data showing that mechanical strain alone strongly upregulates the expression of Cyr61 gene in cultured bladder smooth muscle cells (52). Similarly, Hishikawa et al. (23) reported that CTGF gene expression was strongly increased by high static pressure in mesangial cells and contributes to the remodeling of mesangium and glomerulosclerosis (23).

Meanwhile, the local hemodynamic and metabolic changes that accompany the changes in mechanical stimulation induce, in turn, the expression and release of molecules involved in the regulation of muscle tone and contraction, i.e., angiotensin II, endothelin-1, and PTHrP (12, 27, 41, 58). The rapid release of these factors is thought to be a general physiological response of tissues like the bladder wall that undergo continuous or periodic stretching and adjustment of muscle tonicity. Using in vitro primary cultures of bladder smooth muscle cells, we showed that angiotensin II, endothelin-1, or PTHrP strongly induced the expression of both Cyr61 and CTGF genes. Thus Cyr61 and CTGF are downstream effectors of angiotensin II, endothelin-1, and PTHrP.

Numerous in vivo and in vitro studies have now provided an abundance of evidence that angiotensin II, endothelin-1, or PTHrP acts as a trophic factor that causes smooth muscle cell hypertrophy/hyperplasia and increases collagen production (17, 24). Angiotensin II and endothelin-1 are potent prohypertrophic agonists in the hypertensive heart, the diabetic kidney, and the hypertrophic bladder (12, 24). Locally generated angiotensin II plays autocrine and paracrine regulatory roles, contributing to the development of tissue hypertrophy via its growth-promoting effects as well as indirectly by inducing proliferation of fibroblasts and deposition of extracellular matrix proteins. Angiotensin II also stimulates synthesis of endothelin-1, which, in turn, induces hypertrophy and fibrosis. Blockade of either angiotensin II or endothelin-1 signaling pathways prevents the progression of hypertrophy and decreases tissue remodeling (12, 26, 56). Similarly, PTHrP is expressed in a myriad of tissues, including bladder smooth muscle, and is upregulated by mechanical stretch, by hypertension, by vasoconstrictors such as angiotensin II, and by mechanical injury such as angioplasty (15, 38). PTHrP, in addition to being a smooth muscle vasodilator, is a factor that regulates the growth, development, and/or differentiation in virtually every tissue in which it has been examined. At present, we do not know whether these trophic factors have an actual role in the modulation of bladder wall remodeling through Cyr61 and CTGF. Our in vitro studies showed that angiotensin II, endothelin-1, and PTHrP all upregulate the gene expression of both Cyr61 and CTGF, whereas in the obstructed bladder, upregulation of Cyr61 gene was not concomitant with that of CTGF. Therefore, Cyr61 and CTGF are likely differentially regulated in vivo, possibly because of other factors that negatively regulate CTGF gene expression, hindering or delaying its co-induction with Cyr61 gene at the onset of the obstruction. The variety of stimulatory and inhibitory factors that regulate Cyr61 and CTGF gene expression underscores the complexity of their regulation in vivo and their potential functional role.

In conclusion, the data for the expression profile of Cyr61 and CTGF genes in the obstructed bladder and the analysis of the effects of trophic factors on their gene expression in vitro provide insights into the functions that Cyr61 and CTGF may exert in the hypertrophic bladder. Cyr61 is likely to have a role in the control of smooth muscle cell growth and differentiated phenotype, whereas CTGF is likely to affect bladder cell synthetic phenotype, as well as the extracellular matrix remodeling of the whole bladder wall. Therefore, both Cyr61 and CTGF may serve as specific targets for selective intervention and pharmacological modulation in processes involving smooth muscle hypertrophy and extracellular matrix accumulation, such as bladder obstructive disorders. Further analyses of the potential functions of Cyr61 and CTGF in smooth muscle will be facilitated by the development, in progress, of mice in which the expression of either Cyr61 or CTGF is targeted to smooth muscle tissues.

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