Null mutation in macrophage migration inhibitory factor prevents muscle cell loss and fibrosis in partial bladder outlet obstruction

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Taylor, John A., Qing Zhu, Brian Irwin, Yazeed Maghaydah, John Tsimikas, Carol Pilbeam, Lin Leng, Richard Bucala, and George A. Kuchel. Null mutation in macrophage migration inhibitory factor prevents muscle cell loss and fibrosis in partial bladder outlet obstruction. Am J Physiol Renal Physiol 291: F1343–F1353, 2006.—Idiopathic detrusor underactivity (DU) and detrusor decompensation which develops following partial bladder outlet obstruction (pBOO) are both associated with smooth muscle degeneration and fibrosis. Macrophage migration inhibitory factor (MIF), an important mediator of bladder inflammation, has been shown to promote fibroblast survival and muscle death in other tissues. We evaluated the hypothesis that MIF has similar actions in the bladder by studying detrusor responses to pBOO or sham surgery in anesthetized female mice rendered null for the mif gene (MIF KO) and in wild-type (WT) controls, all killed 3 wk after surgery. WT mice revealed intense MIF immunoreactivity in urothelial cells which decreased, without change in overall mif mRNA levels. Stereologically sound quantitative morphometric measurements were performed in the midsudetor region of each bladder. MIF KO bladders were normal in appearance, yet were 30–40% heavier, with increased midsudetor collagen and muscle, compared with WT controls. In WT mice, pBOO increased the collagen-to-muscle ratio 1.9-fold and midsudetor collagen 1.8-fold, while nucleated muscle counts were 22% lower. In MIF KO mice, by contrast, pBOO had no significant effect on any of these parameters. In primary bladder muscle cultures, treatment with rMIF protein increased TUNEL staining, raising the proportion of early and late apoptotic cells on flow cytometry. Our studies implicate MIF in the sequence of events leading to detrusor muscle loss and fibrosis. They raise the possibility that strategies designed to antagonize MIF synthesis, release, or biological activity could prevent or delay DU and urinary retention.

Aging; apoptosis; incontinence; urinary retention; inflammation

DETRUSOR UNDERACTIVITY (DU) represents a common and challenging clinical problem in older adults (75). DU has been defined as a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or a failure to achieve complete bladder emptying within a normal time span (1). This condition develops in some men with bladder outlet obstruction (BOO) due to benign prostatic hyperplasia (BPH) (77). Its presence greatly complicates BPH management, placing the effectiveness of well-established surgical approaches to BPH into question (77). DU is also very common in elderly women and men, particularly those who are frail (75). While most such individuals show no evidence of BOO (66), DU-related urinary retention with bladder distension could further accelerate the progression of DU by mechanisms similar to those which take place in BOO (75).

In fact, a study of incontinent nursing home residents demonstrated that 59% have evidence of DU without any BOO on urodynamic studies (66). Approximately half of such individuals have evidence of both detrusor overactivity and DU, a condition referred to by some investigators as detrusor hyperactivity with impaired contractility (DHIC) (65, 66). Its management remains especially unsatisfactory as antispasmodic anticholinergic medications for overactivity may worsen retention, while using bethanechol to enhance detrusor contractility is ineffective (75). Biopsy studies have established a strong relationship between urodynamic evidence of DU and structural changes including detrusor fibrosis, muscle degeneration, and axonal degeneration (12, 29). Moreover, the development of these changes in the setting of BOO correlates with symptom severity (50, 73) and may herald the development of DU (13).

Animal studies have provided a wealth of information regarding cellular events that are associated with detrusor muscle hypertrophy that occurs as part of the compensatory response to BOO. Nevertheless, a complicating factor in being able to directly implicate specific molecules in these compensatory events has been the fact that mouse deletions of some of the most promising candidate genes [e.g., TGF-β (24), TGF-β-R (59), cyr61 (53), CTGF (21), and FGF-10 (72)] are lethal. An alternative approach is one which seeks to define mechanisms involved in the development of detrusor decompensation (9, 22, 30, 41, 82). Detrusor muscle loss and collagen deposition represent two morphological features which are common and central to both detrusor decompensation in BOO (13) and idiopathic DU (12, 29). Such structural changes can be measured precisely and in humans may in fact precede the development of urodynamically evident DU (26).

Macrophage migration inhibitory factor (MIF) is a potent proinflammatory cytokine which has been shown to be a key mediator of bladder inflammation (47). This protein is abundantly expressed in urothelial cells (47) and is known to be released by varied inflammatory stimuli (79). MIF was discovered as a potent inhibitor of random macrophage migration which enhances macrophage retention and tissue inflammation (23, 42). While MIF enhances fibroblast survival (14, 52) and fibrosis (70), it also promotes cardiomyocyte death in vivo (20). With these considerations in mind, we formulated a


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hypothesis that MIF plays a role as a mediator of smooth muscle death and collagen deposition in the obstructed bladder. Mice genetically deficient in the mif gene (MIF KO) provide an optimal model to examine this hypothesis since they are viable, fertile, and exhibit no apparent basal phenotype (11). We adapted existing mouse protocols for creating partial BOO (31, 38) and identified a time point at which two of the key morphological features of detrusor decompensation (detrusor muscle cell loss and collagen deposition) become evident in a reproducible fashion in our obstructed wild-type (WT) mice, thus offering a unique opportunity for testing our hypothesis.

MATERIALS AND METHODS

Animal care. Mice lacking MIF were generated by homologous recombination (11) and were then backcrossed into a pure C57BL/6 background. Animals were bred and maintained at the University of Connecticut Health Center for Laboratory Animal Care under National Institutes of Health guidelines. All procedures were approved by an institutional animal care committee. All surgeries were performed using 2-mo-old female WT and mif−/− (KO) mice. Mice had ad libitum access to water and food (Teklad 2918; Harlan, Indianapolis, IN).

Animal surgery. Partial bladder outlet obstruction (pBOO) surgery was created as described by Lemack et al. (38) and by Felsen et al. (31). Anesthesia was maintained using inhaled Isoflurane delivered at 1.5–2% in pure oxygen flow (250 ml/min). A 22-gauge angiocatheter was introduced into the bladder through the urethra under sterile conditions. A 1-cm lower midline incision was made. Blunt dissection allowed for identification of the urethra with angiocatheter in place. After minimal dissection of surrounding tissues, a curved clamp was passed posteriorly and a 4–0 silk tie was secured snuggly but not tightly at the bladder neck. If too much force was needed to remove the catheter (too tight) or if there was no movement of periurethral tissue (too loose), the suture was replaced. Sham-operated mice underwent an identical procedure, but the tie was not placed. Animals were observed for signs of discomfort for the duration of the study. Buprenorphine (0.1 mg/kg) was injected subcutaneously for postoperative pain control. All pBOO animals were killed 3 wk (31, 39) following surgery.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from murine bladder tissues with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer’s protocol. Samples were treated with DNase I (DNA-free kit, Ambion, Austin, TX) to remove contaminating DNA from RNA preparations following the manufacturer’s protocol. Total RNA was converted to cDNA by ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol. Real-time PCR was performed for different gene expressions in separate wells (singleplex assay) of 96-well plates, in a reaction volume of 20 μl. GAPDH was used as endogenous control. Three replicates of each sample were amplified using Assays-on-Demand Gene Expression assay (Assay Mm00478374_m1 for COX2, Mm00443258_m1, Mm01604696_g1 for MIF, and Mm99999915_g1 for GAPDH) which contained predesigned unlabeled gene-specific PCR primers and TaqMan MGB FAM dye-labeled probe. The PCR reaction mixture (including 2x TaqMan Universal PCR Master Mix, 20x Assays-on-Demand Gene Expression Assay Mix, 50 ng of cDNA) was run in Applied Biosystems ABI Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. All primers were tested for equal efficiency over a range of target gene concentrations. The relative quantification of target gene expression in a test sample to a reference calibrator sample (ΔΔCt method) was used for data analysis.

Tissue preparation for trichrome staining and immunohistochemistry. Following death, bladders were harvested and bivalved in a reproducible manner across the detrusor midsection. Specimens were then fixed in 4% paraformaldehyde-PBS and embedded in paraffin wax with the detrusor midsection facing and lying parallel to the micromtne blade. Twenty coronal sections (5-μm thickness) were obtained from each detrusor midsection and mounted on slides. This consisted of four sets of sequential sections with each set separated by 50 μm. A single section from each set was randomly chosen for Masson’s trichrome staining (15) and subsequent quantitative analysis. After dewaxing and hydration, slides first underwent treatment in Bouin solution (Poly Scientific, Bay Shore, NY) for 1 h at 56°C. They were then serially stained in Weigert hematoxylin and Gomori trichrome stain (Poly Scientific) and differentiated in 0.5% acetic acid before going through dehydration and mounting with cytoseal. This protocol stains muscle cell cytoplasm red, collagen blue, and nuclei purple (15).

Quantitative morphometric analysis. As described above, four sections randomly selected from different regions of bladder midsection underwent quantitative morphometric analysis. Slides were viewed in a blinded fashion. Digital micrographs were taken using a Zeiss Axioship 2 Plus microscope. Images were captured and separated into urothelial, suburothelial, and muscle compartments. Images from the muscle compartment were then digitized into red and blue signal representing muscle and collagen, respectively, and were then quantified using Adobe and Image J software (http://rsb.info.nih.gov/ij/). An eye objective-mounted grid was placed over 10 distinct regions of each section to count numbers of grid intercepts overlying detrusor tissue, muscle, and muscle nuclei. Results were then analyzed as 1) the collagen-to-muscle ratio, 2) amount of signal representing muscle per bladder midsection, 3) amount of signal representing collagen per bladder midsection, and 4) numbers of nucleated muscle cells per bladder midsection.

Immunohistochemistry. Mounted slides were prepared and deparaffinized as noted above. Endogenous peroxidase was quenched with 3% H2O2. Nonspecific staining was blocked by incubation with a Power Block Universal Blocking Agent (Biogenex, San Ramon, CA). Tissue sections were incubated with a polyclonal anti-MIF antibody (CPCM00, Cell Sciences, Canton, MA) at 4°C overnight. This was followed by washes, incubations with an appropriate biotinylated secondary antibody, DAB development (Vector Laboratories, Burlingame, CA), a Methyl Green counterstain, dehyration, and mounting. Cultured cells were stained using a α-smooth muscle actin mouse monoclonal (A2547; Sigma, St. Louis, MO) and a pan-cadherin rabbit polyclonal (C 3678; Sigma, St. Louis) antibody. The monoclonal antibody was visualized with CY3-tagged goat anti-mouse IgG (H+L; Jackson Immunoresearch Laboratories, West Grove, PA), while the polyclonal antibody was visualized with Alexa 488-tagged goat anti-rabbit F(ab) immunoglobulin G (H+L; A11070; Molecular Probes, Carlsbad, CA). Cells were viewed and confocal images were obtained using a Zeiss LSM 410 confocal microscope.

Tissue culture. Primary bladder muscle cultures were derived from newborn Sprague-Daley rats (Charles River, Wilmington, MA). The bladders were harvested, minced, and added to 3 ml dissociation solution containing 0.5 mg/ml collagenase, 0.5 mg/ml elastase, 2 μg/ml DNase (Worthington, Lakewood, NJ), and 1 mg/ml soybean trypsin inhibitor (GIBCO, Carlsbad, CA). The tissue was triturated at 15-min intervals for 45 min using fire-polished Pasteur pipets. The cell solution was filtered through a 40-μm cell strainer and spun at 1,000 rpm for 5 min. The supernatant was aspirated and cells were resuspended in DMEM-F/12 medium containing 1% FBS, 1% N-2 supplement, and 1% penicillin/streptomycin (GIBCO). The cell number was determined and cells were plated at a density of 25,000 cells/cm2 in poly-L-lysine-coated (P-6282, Sigma) 12-well plates (3513, Corning, Corning, NY) for biochemical and flow cytometry studies and on plastic eight-well chamber slides (354108, BD Bioscience, San Jose, CA) for immunohistochemistry or TUNEL staining. Cells were main-
tained at 37°C and 5% CO₂ in a humidified chamber. The presence of 1% FBS was required for the success of initial plating. However, 24 h after plating cells were maintained in serum-free defined medium to promote the presence of the fully differentiated contractile phenotype (44, 60). Cell labeling studies established that essentially all of our cultured cells expressed smooth muscle markers, with no apparent contamination by urothelial cells (see Fig. 4). Treatments took place on day 5 after plating when in our system most cultured cells express smooth muscle differentiation markers, with many making cadherin-positive cell-cell contact (see Fig. 5).

**Tissue culture characterization.** Flow cytometry was used to evaluate the expression of both bladder muscle and urothelial differentiation markers. Floating and trypsinized adherent cells were separated into equal aliquots and were labeled with monoclonal antibodies for MLCK (M7905, Sigma), calponin (C2687, Sigma), MHC (SC-6956, Santa Cruz Biotechnology, Santa Cruz, CA), or cytokeratin-17 (M7046, Dako, Carpinteria, CA). Following incubation with a PE-conjugated goat anti-mouse antibody, cell aliquots were analyzed on flow cytometry as described below.

**MIF ELISA.** A sandwich ELISA utilizing a monoclonal IgG1 and a purified polyclonal IgG has been extensively validated (8, 17). MIF concentrations were calculated by extrapolation from a quadratic standard curve using human rMIF (range: 0–12 ng/ml, sensitivity: 10 fg/ml). Recombinant mouse MIF was produced in our laboratory and purified free from endotoxin as described previously (8). Following treatment with rMIF, cells were analyzed using a kit (PF032, Calbiochem, San Diego, CA) to separate apoptotic and necrotic cells on flow cytometry. Both floating and trypsinized adherent cells were collected and washed in PBS. Binding buffer (0.5 ml), 1.25 l media binding reagent, and 10 l propidium iodide. Cells were analyzed using a Becton Dickenson FACS caliber flow cytometer with collection and analysis of data performed using Becton Dickinson CELLQuest software. TUNEL staining was detected using a kit (Roche) with DAB microscopy of cells grown 5–7 days in eight-well chamber slides. To quantify the rate of apoptosis (TUNEL-stained cells/all visible cells), we counted cells in four randomly chosen fields in each of four wells per group. All studies were performed in the presence of cycloheximide (1 μg/ml; C4859, Sigma) in all of the treatment and control wells (80). This inhibitor of protein synthesis has been shown to enhance the toxicity of TNF-α in human saphenous vein smooth muscle cells (80), yet at these concentrations cycloheximide alone does not induce apoptosis or necrosis either in vascular (80) or in bladder (data not shown) smooth muscle cells.

**Statistical analysis.** Statistical comparisons were performed using SigmaStat (San Rafael, CA) and SPSS (Chicago, IL) software.

**RESULTS**

**MIF expression.** In sham-operated WT mice, intense cytoplasmic, and occasionally nuclear, MIF immunoreactivity was detected throughout basal and apical layers of the urothelium (Fig. 1A). After 3 wk of pBOO, MIF immunoreactivity was markedly decreased in all urothelial cells (Fig. 1B), but increased in very few muscle or mononuclear inflammatory cells (not shown). In MIF KO mice, MIF immunoreactivity was completely absent (not shown). At this 3-wk time point following pBOO, overall mif mRNA levels remained similar in pBOO and sham-operated WT bladders (Fig. 2). However, as expected from results of earlier studies (63), pBOO increased cox-2 mRNA levels more than twofold (63).

**Body and bladder weight.** Body weights of sham-operated WT and MIF KO mice were similar, with no change following pBOO (Table 1). Whole bladder weights were 36.7% higher in

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sham-operated MIF KO mice and 50.8% higher in obstructed MIF KO mice compared with sham-operated WT controls (Table 1). While pBOO surgery appeared to increase bladder weights in some WT and some MIF KO mice, overall these differences did not reach statistical significance. Expressing bladder weights per body weight did not change these relationships (Table 1).

**Detrusor morphology.** Using Masson’s trichrome stain, muscle cytoplasm is red, collagen blue, and nuclei purple. At a low magnification, bladders from MIF KO sham-operated mice (Fig. 3C) appeared somewhat larger than those from sham-operated WT (Fig. 3A) mice, yet their gross appearance was similar. Following 3 wk of pBOO, WT bladders seemed slightly larger (Fig. 3B) with a greater degree of fibrosis (blue stain) at a higher magnification in pBOO (Fig. 3b) compared with sham-operated (Fig. 3, A and a) mice. In contrast, pBOO surgery did not appear to increase the size of bladders in MIF KO mice (Fig. 3D), with similar degrees of fibrosis in pBOO (Fig. 3d) and sham-operated (Fig. 3c) MIF KO mice.

**Detrusor quantitative morphometry.** All of the following quantitative morphometric measurements were performed in a total of four sections randomly selected from the mid detrusor region of each bladder (Table 2). A two-way repeated-measures ANOVA model was used for statistical analysis. A log transformation was applied to the collagen, as well as the collagen-to-muscle ratio variables to correct for skewness and nonconstant variance. No transformation was deemed appropriate for the muscle and the nucleated muscle cell count variables. The estimated between mice and within mice (due to sections) variances were: \( \text{Log (collagen-to-muscle ratio)}: Var_{mice} = 0.06124, Var_{section} = 0.07076; \) \( \text{Log (collagen)}: Var_{mice} = 0.03981, Var_{section} = 0.06490; \) muscle: \( Var_{mice} = 6.1277 \times 10^8, Var_{section} = 10.099 \times 10^8; \) nucleated muscle cell count: \( Var_{mice} = 3.823, Var_{section} = 244,991. \)

A statistically significant interaction between the KO (vs. WT) factor and the pBOO (vs. sham) factor was detected for the collagen-to-muscle ratio variable \( (P = 0.0167) \), the collagen variable \( (P = 0.0216) \), and the nucleated muscle cell count variable \( (P = 0.0013) \). No similar statistically significant interactions were detected for the muscle variable \( (P = 0.4283) \). However, the MIF WT vs. MIF KO comparison was statistically significant for muscle only \( (P = 0.0001) \). All the reported \( P \) values and 95% confidence intervals (Table 2) were adjusted for multiple comparisons using the Tukey-Kramer method.

No statistically significant effect on the mid detrusor collagen-to-muscle ratio was observed when rendering mice null for the mif gene \( (CI: 0.93–1.81; P = 0.17) \). As noted above (Table 1), MIF KO bladders were nearly 40% heavier than WT bladders. In MIF KO mice median mid detrusor collagen was 1.6-fold \( (CI: 1.17–2.05; P = 0.001) \) higher and mean mid detrusor muscle was 0.173 mm\(^2\) greater \( (CI: 0.012–0.333; P = 0.0305; \) an observed 19.9% relative increase) compared with WT animals.

The median of the mid detrusor collagen-to-muscle ratio was 1.9-fold \( (CI \text{ for the ratio of medians: } 1.37–2.65; P < 0.0001) \).
Table 2. Impact of pBOO and MIF on middetrusor quantitative morphometric measurements

<table>
<thead>
<tr>
<th></th>
<th>WT Sham (11)</th>
<th>WT pBOO (10)</th>
<th>MIF KO Sham (10)</th>
<th>MIF KO pBOO (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-to-muscle ratio</td>
<td>0.245</td>
<td>0.466*</td>
<td>0.317†</td>
<td>0.386*</td>
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<tr>
<td>Collagen, mm²</td>
<td>[0.206–0.291]</td>
<td>[0.389–0.558]</td>
<td>[0.265–0.380]</td>
<td>[0.319–0.467]</td>
</tr>
<tr>
<td>Muscle, mm²</td>
<td>0.208</td>
<td>0.373*</td>
<td>0.323</td>
<td>0.402*</td>
</tr>
<tr>
<td>Muscle cell count</td>
<td>0.868</td>
<td>0.816</td>
<td>1.040†</td>
<td>1.059†</td>
</tr>
<tr>
<td></td>
<td>[0.784–0.951]</td>
<td>[0.729–0.904]</td>
<td>[0.953–1.128]</td>
<td>[0.966–1.151]</td>
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Middetrusor sections underwent trichrome staining, with 4 sections then randomly selected for quantitative morphometric analysis as described in MATERIALS AND METHODS. WT and MIF KO female mice were studied 3 wk after sham or pBOO surgery, with 9–11 animals in each experimental group. The middetrusor collagen-to-muscle ratio and collagen are reported as mean plus 95% confidence intervals since a log transformation was used in the analysis for these measures. In contrast, middetrusor midsection muscle and nucleated muscle cell counts are reported as mean plus 95% confidence intervals. The no. of animals is in parentheses. Statistical comparisons were adjusted for multiple comparisons. Statistically significant differences compared with WT sham animals (*) or compared with WT pBOO animals (†) are indicated in the table. The experiment-wise type I error rate was set at α = 0.05.

higher in obstructed compared with sham-operated WT animals (Table 2). In contrast, in mice rendered null for the mif gene, pBOO surgery had no impact on the collagen-to-muscle ratio (CI: 0.86–1.72; P = 0.44). The median middetrusor collagen was 1.8-fold (CI: 1.35–2.37; P < 0.0001) higher in pBOO compared with sham-operated WT mice (Table 2). In contrast to its effect in WT animals, pBOO had no statistically significant effect on middetrusor collagen in MIF KO mice (CI: 0.93–1.67; P = 0.20). A mif gene deletion increased mean middetrusor muscle by 0.242 mm² (0.073–0.411; P = 0.0025; an observed 29.7% relative increase) in pBOO mice. Following pBOO, the mean middetrusor nucleated muscle cell count decreased by 356.04 (CI: 11.87–700.22; P = 0.04; an observed 21.9% relative decrease) in WT mice, whereas in the absence of MIF, the mean middetrusor nucleated muscle cell count in obstructed mice was not statistically significantly different than that of sham-operated controls (CI: −73.53–665.15; P = 0.155). Finally, the observed mean middetrusor nucleated muscle cell count for obstructed MIF KO mice was higher by 593.39 than the mean for obstructed WT mice (CI: 224.04–871.36; P = 0.0006; an observed 46.7% relative increase).

**MIF effects on detrusor muscle cells in vitro.** All tissue culture studies were performed 5 days after plating. Immunolabeling with antibodies for smooth muscle cell and urothelial markers followed by flow cytometry (Fig. 4A) revealed that nearly all (99.7 ± 0.1%) of cells express α-smooth muscle actin, 80.0 ± 0.6% express myosin light chain kinase (MLCK), 26.0 ± 3% express myosin heavy chain (MHC), and none express urothelial markers such as cytokeratin-17. Moreover, images obtained using confocal microscopy revealed that many cultured cells expressing α-smooth muscle actin make cadherin-positive cell-cell contact (Fig. 5) similar to the “zipper”-like areas of contact described between epithelial cells (78). Treatment of these cultures with TNF-α (50 ng/ml) for 24 h resulted in an ~30% increase in MIF protein levels detectable in the supernatant (P < 0.01; Fig. 4B). Treatment with rMIF protein (100 ng/ml) over 3 h increased the proportion of TUNEL-positive bladder muscle cells in our cultures more than 2.5-fold (7.8 vs. 20.1%; P < 0.05; Fig. 6). The addition of rMIF protein (100 ng/ml for 24 h) decreased the proportion of healthy cells, while increasing the proportion of cells demonstrating evidence of early apoptosis or late apoptosis/necrosis (Fig. 7).

**DISCUSSION**

**MIF.** Macrophage MIF is abundantly expressed in urothelial cells (47) and our tissue culture studies indicate that bladder

![Fig. 4. Cellular phenotype of cultured bladder cells. A: following trypsinization, rat bladder muscle culture cells were immunolabeled using 3 smooth muscle (α-smooth muscle actin, myosin light chain kinase, and myosin heavy chain) and one epithelial (cytokeratin-17) marker. They were then resolved using flow cytometry. Nearly all (99.7%) of cultured cells expressed α-smooth muscle actin. Other smooth muscle markers were also common, with most (80%) cells expressing myosin light chain kinase and 26% expressing myosin heavy chain. Cytokeratin-17, a urothelial marker (10), was not detected in our cultures. B: these well-differentiated bladder muscle cells released MIF protein after being exposed to TNF-α (50 ng/ml) over 24 h. MIF was measured using ELISA in supernatants obtained from 3 separate cultures (*P < 0.01).
smooth muscle cells release MIF which is known to be released by varied inflammatory stimuli (79). MIF has been shown to enhance fibroblast survival (52). We formulated a hypothesis proposing that MIF could play a physiological role as a mediator of smooth muscle death and collagen deposition in the obstructed bladder. Mice rendered null for the mif gene (MIF KO) provide an optimal model to examine this hypothesis since they are viable, fertile, and exhibit no apparent phenotype under basal conditions (11). Our interest was also prompted by the recent description of functional polymorphisms in the human MIF gene, which are being shown to be associated with different chronic inflammatory conditions (34).

Using existing protocols for creating pBOO in mice (31, 38), we identified a time point at which two of the key morphological features of DU (detrusor muscle cell loss and collagen deposition) become evident in a reproducible fashion in our obstructed WT mice. To perform stereologically sound quantitative morphometric measurements in a three-dimensional structure such as the bladder, we adapted approaches developed by scientists in other fields (55).

Obstruction studies in mice. The use of genetically modified mice offers a unique opportunity to implicate a specific gene in the sequence of events by which partial bladder obstruction results in specific categories of detrusor responses. Several investigators pioneered the development of female (31, 38, 62, 71) and male (4) mouse models of pBOO.

Unfortunately, the impact of pBOO on detrusor structure has not always been consistent. For example, Lemack et al. (39) studied young (4 – 6 mo old) ICR strain female mice and reported that 3 wk after surgery the collagen-to-muscle ratio decreased by nearly a half from 1.38 in sham-operated to 0.72 in pBOO mice. In contrast, Felsen et al. (31) used older middle-aged (8 – 12 mo old) C57BL/6 female mice and reported a significant increase at the same time point, with the collagen-to-muscle ratio more than doubling from 0.72 in sham-operated to 1.5 in pBOO animals. Impact on bladder weights has also been variable. For example, bladder weights of our obstructed WT mice underwent only minimal (23%) and statistically nonsignificant increases (Table 2), resembling a similar (28%) increase described at this time point by Lemack et al. (39). In contrast, other investigators reported a doubling in mouse bladder weights at 1 wk (71) and a nearly threefold increase at 5 wk (62) after obstruction. It remains to be seen to what extent differences in surgical technique, animal age, genetic factors, the selection of specific inbred mouse strains, and/or “tightness” of the pBOO lesion are responsible for differences between these two studies (31, 39). Our obstructed mice demonstrated evidence of elevated collagen-to-muscle ratio (Table 2) and raised COX-2 mRNA level (Fig. 2A), with a remarkably low degree of variability between individual mice in each of the obstructed groups. Although none of these considerations detract from the validity and importance of our

Fig. 5. Primary rat bladder muscle cultures express α-smooth muscle actin and make contact via cadherin-positive junctions. A: most cells in our primary bladder muscle cultures expressed α-smooth muscle actin (red). B: confocal microscopy revealed areas of cadherin-positive contact (green-yellow) with a “zipper-like” appearance previously described with epithelial cells (78).

Fig. 6. MIF effect on TUNEL staining. Primary rat detrusor muscle cultures were processed for TUNEL staining after a 3-h incubation in the presence (B) or absence of (A) rMIF protein (100 ng/ml). A minimum of 100 cells were counted in each of 6 separate slides. The percentage of TUNEL-positive slides increased from 7.8 ± 2.2 to 20.0 ± 3.9 (*P < 0.05).
findings, it will be important for bladder researchers to develop tools to better standardize the “tightness” of pBOO lesions between different surgeons and laboratories.

Insights from other animal models. Studies evaluating detrusor responses to obstruction in rabbits have been much more numerous. For example, investigators have described hypertrophic changes involving muscle cells, followed by myocyte and axonal degeneration plus collagen deposition (33). [3H]thymidine incorporation studies have demonstrated consistently increased incorporation of this label into urothelial and connective tissue compartments, but not into the muscularis layer (54). Based on these considerations, it has been proposed that most of the initial increase in overall bladder mass following obstruction can likely be attributed to growth involving individual muscle cells (muscle hypertrophy), with muscle cell death playing a likely role in the subsequent detrusor decompensation. Nevertheless, it has been very difficult to obtain in vivo evidence of apoptosis in pBOO, with published evidence limited to rabbit bladders “regressing” after removal of obstruction (68) and to fetal ovine bladders obstructed in utero (76). Moreover, to date, no published study has examined the impact of pBOO on numbers of nucleated detrusor muscle cells.

Unfortunately, quantitative morphometry is both time and labor intensive. Another obstacle to further progress has been a failure to adopt the type of stereologically sound morphometric approaches used in fields such as neuroscience (55). Unlike other tissues (e.g., gut, bronchial airways), mammalian bladder smooth muscle cells lack a predictable organizational pattern (25, 27, 45). The obstructed detrusor also undergoes extensive structural plasticity (16, 27, 45) and any quantitative morphometry protocol must respect basic stereological principles to avoid possible biases (27, 45, 55). The traditional collagen-to-muscle ratio provides only limited information since it does not establish the magnitude, or even the direction, of change involving either compartment. Moreover, in the absence of formal cell counting, no conclusions can be drawn about whether a change in overall muscle bulk results from differences in numbers of individual muscle cells and/or their size. Finally, basing quantitative morphometric measurements on a single tissue section presents substantial risks in terms of confounding experimental results with stereological biases (55).

MIF: a urothelium-derived mediator of bladder inflammation. Our studies indicate that, as shown by others (47, 79), urothelium represents a rich source of MIF protein (Fig. 1A). As expected (63, 64), cox-2 mRNA levels are upregulated following obstruction (Fig. 2A). Nevertheless, overall bladder mif mRNA levels remain unchanged (Fig. 2B), while MIF immunoreactivity is significantly decreased (Fig. 1B). Based on these findings, as well as studies examining the role of inflammatory signals in mediating MIF protein release from urothelial cells (46, 49), macrophages (3), and uterine epithelial cells (69), we believe that during pBOO preformed MIF protein undergoes release from urothelial cells and that regulation of this release represents a principal locus of regulation of MIF bioactivity in this system.

Impact of mif gene deletion on the mouse detrusor. As expected, rendering the mice null for the mif gene did not change body weight (Table 1). However, bladders from MIF KO female mice were nearly 40% heavier than those from age-matched WT animals (Table 1). Based on our quantitative morphometric measurements (Table 2), increases in both muscle and connective tissue compartments in the middestrusor regions appear to be contributing to this increase in bladder mass. We believe that this represents the first evidence demonstrating an impact of a mif gene deletion on any animal phenotype under basal conditions.

Impact of partial obstruction on detrusor structure in WT mice. In our model system, 3 wk of pBOO resulted in a 1.9-fold increase in the collagen-to-muscle ratio in the middestrusor region (Fig. 3 and Table 2). This was accompanied by a 1.8-fold increase in collagen and no difference in muscle, but a 21.9% decrease in numbers of nucleated muscle cells in the middestrusor region (Table 2). We selected this time point for our studies since we believe that these findings are indicative of early cellular events ultimately leading to DU. Our observation of decreased numbers of nucleated muscle cells together with no change in overall muscle mass most likely indicates the loss of individual muscle cells which is accompanied by a compen-
satory hypertrophy involving remaining muscle cells. Our preliminary electron microscope studies (not shown) support these observations with evidence of muscle and axonal degeneration (29), fibrosis (28, 29), as well as enlarged hypertrophic muscle cells (28) in WT mice studied 3 wk after pBOO surgery.

Impact of mif gene deletion on detrusor responses to partial obstruction. Rendering mice null for the mif gene abolished increases in the collagen-to-muscle ratio and overall mid detrusor collagen which had been demonstrated in WT mice after obstruction (Fig. 3 and Table 2). Moreover, the absence of MIF also abolished declines in mid detrusor nucleated muscle counts which took place in WT mice after pBOO (Table 2). Mid detrusor nucleated muscle counts were nearly 50% higher when obstructed MIF KO mice are compared with obstructed WT mice (Table 2). In summary, our studies indicate that MIF must play an in vivo role in contributing to both detrusor fibrosis and muscle cell loss following bladder outlet obstruction.

MIF: a mediator of bladder fibrosis and muscle death in vitro. Published in vitro studies demonstrated MIFs antiapoptotic properties in macrophages (51), neutrophils (6), and fibroblasts (52). While the impact of MIF on muscle survival or apoptosis has remained unknown, the addition of rMIF to differentiated L6 rat skeletal myotubes resulted in an unexpected increase in glycolysis with lactate accumulation (7). More recently, early MIF neutralization was shown to decrease endotoxin-mediated cardiac muscle cell death (20) and to also stabilize atherosclerotic plaque formation (70). With these considerations in mind, we explored the impact of adding a physiological concentration of rMIF protein on parameters reflecting apoptosis in primary rodent bladder muscle cultures. In view of our hypothesis that in contrast to its antiapoptotic effect in fibroblasts (52), MIF promotes apoptosis in bladder smooth muscle cells, our cultures were grown under serum-free conditions to ensure that cultured cells were more representative of the “contractile” phenotype typical of bladder muscle cells in vivo, as opposed to the “synthetic” fibroblast-like phenotype which is promoted by the addition of serum (18, 44, 60). As shown in Fig. 4, in our system nearly all (99.7%) cultured cells expressed α-smooth muscle actin, a classic but relatively stable marker of smooth muscle differentiation (32, 60), with no cells expressing the epithelial marker cytokeratin-17 which has been used to define urothelial cell cultures (10). MLCK and MHC represent markers of smooth muscle differentiation which are expressed in later stages of smooth muscle differentiation and appear to be much less stable or more sensitive to dedifferentiation stimuli than is the case for α-smooth muscle actin (37, 60). Nevertheless, ~80% of cultured cells expressed MLCK, whereas 26% expressed MHC. Recent studies have shown that the cellular phenotype within populations of cultured human vascular smooth muscle cells determines whether TNF-α induces a proliferative or a proapoptotic response (80). Serum-based bladder muscle culture protocols have proven remarkably useful in bladder research. Nevertheless, given the fact that bladder smooth muscle cells can undergo significant changes in cellular phenotype with development (25), aging (29), obstruction (19, 81), and menopause (67, 83), future studies will need to explore the influence of bladder muscle differentiation on cellular responses to relevant cytokines.

Mechanism of MIF-mediated smooth muscle toxicity. MIF concentrations of 50–100 ng/ml have been shown to result in a sustained induction of the extracellular-signal regulated kinase 1/2 (ERK 1/2) MAPK (52). This effect has been shown to be dependent on MIF interacting with the membrane protein CD74 (40). Activation of cell surface CD74 may require an interaction with CD44, an adhesion molecule also known to function as a receptor for hyaluronan (HA) and osteopontin (56). Both CD74 and CD44 have been shown to be expressed in rat urothelium (48), while CD44 mRNA and protein are also expressed in rat bladder muscle cells (2). Substance P-induced bladder inflammation has been shown to upregulate both CD74 and CD44 protein expression (48). CD44 expression is increased in urothelium and interstitial bladder space following pBOO (61). It remains to be seen whether differences in CD74 and CD44 receptor expression or in downstream signaling pathways could account for the opposing effect of MIF on fibroblast and muscle cell survival. It has been shown that higher MIF concentrations (e.g., MIF > 100 ng/ml) as could be achieved during inflammatory states inhibit the JNK/AP-1 pathway through JAB1 activation (35). Recent studies indicate that a more transient and rapid ERK MAPK activation requires Src kinase activity (43), while Rho kinase appears to be involved in the pathways downstream from more sustained ERK MAPK activation (74). It remains to be seen whether MIF-mediated increases in Rho kinase activity could also contribute to dysfunction in detrusor performance due to the effects of this pathway on myosin light chain phosphorylation (9).

Future directions. In summary, we present evidence of bladder muscle cell loss and fibrosis in the partially obstructed mouse bladder. Applying stereologically sound quantitative morphometric approaches to partially obstructed bladders from mice rendered null for the mif gene we are able to directly implicate, for the first time, a specific molecule in the cellular events leading to structural changes which define the presence of idiopathic DU, as well as DU in the setting of partial obstruction. In the only comparable study, a deletion of the inducible nitric oxide gene abolished only one-half of the increase in the collagen-to-muscle ratio (31) and given the nature of the morphometric studies performed no conclusion could be drawn regarding the impact of this gene deletion on muscle cell numbers (31). Our data implicating MIF in obstruction-mediated muscle loss and fibrosis are supported by in vitro experiments which demonstrate that, contrary to MIFs established antiapoptotic effects in fibroblasts, this cytokine is capable of proapoptotic effects in bladder smooth muscle cells. We believe that our results illustrate the power of combining the use of genetically modified mice with careful morphometric measurements, yet future challenges remain. Human biopsy studies have clearly established the presence of muscle loss, fibrosis, and axonal degeneration as highly reliable and early markers of DU (12, 13, 29). In fact, in some individuals such ultrastructural changes may precede the presence of urodynamically detectable DU (26). Nevertheless, given advances in our ability to conduct reliable urodynamics in mice (62), it will be important to similarly link these structural changes to altered detrusor performance in mice. Future studies will also be needed to define signaling pathways involved in mediating MIFs proapoptotic effects in bladder muscle cells, particularly in terms of differentiating these from MIFs antiapoptotic ef-
fects in fibroblasts and macrophages. It will also be important to examine the effects of MIF on the extracellular matrix since MIF has been shown to induce MMP-9 in vascular smooth muscle cells and macrophages (36), MMP-9 and -13 in osteo-
brasts (58), as well as MMP-1 and MMP-3 in fibroblasts (57).

Induction of such enzymes may destabilize human atheroscle-
rotic plaques (36) and could contribute to some of the degen-
erative changes involving both cells and extracellular matrix in DU.

Finally, our findings also raise the specter of novel diagno-
tic and therapeutic approaches in the future. The human mif
gene promoter expresses several functional polymorphisms which have been shown to correlate with MIF protein expres-
sion and disease severity in several inflammatory conditions (5).

It has also been proposed that strategies designed to
antagonize MIF synthesis, release, or biological activity could infu-
ence the progression of common inflammatory conditions.

It remains to be seen whether mif functional polymorphisms
and/or MIF protein levels correlate with the clinical progres-
sion of DU and whether interventions designed to interfere
with MIF could alter clinical course of individuals at risk for
DU and urinary retention.

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