Structural and functional characterization of bladder smooth muscle in fetal rats with retinoic acid-induced myelomeningocele

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Danzer E, Kiddoo DA, Redden RA, Robinson L, Radu A, Zderic SA, Doolin EJ, Adzick NS, Flake AW. Structural and functional characterization of bladder smooth muscle in fetal rats with retinoic acid-induced myelomeningocele. Am J Physiol Renal Physiol 292: F197–F206, 2007. First published August 29, 2006; doi:10.1152/ajprenal.00001.2006.—Myelomeningocele (MMC) is the most common cause of neurogenic bladder dysfunction (NBD). We recently developed a novel retinoic acid (RA)-induced MMC model in fetal rats. The objective of this study was to use this model to assess functional and structural characteristics of the detrusor muscle in MMC-associated NBD. Time-dated pregnant Sprague-Dawley rats were gavage fed 60 mg/kg RA dissolved in olive oil or olive oil alone [embryonic day 10 (E10)]. Bladder specimens from olive oil-exposed fetuses (OIL; n = 71), MMC (n = 79), and RA-exposed-no MMC (RA, n = 62) were randomly assigned for functional and histopathological evaluation and protein analysis. Contractility responses to field and agonist-mediated stimulation (KCl and bethanechol) were analyzed. The expression patterns of α-smooth muscle actin, myosin, desmin, vimentin, and collagen III and I were analyzed by immunohistochemistry and Western blotting. Spatial and temporal distribution of nerve fibers within the detrusor muscle was monitored by neurotubulin-β-III throughout gestation. Neither OIL, MMC, nor RA detrusor responded to field stimulation. MMC bladder strips showed a significant decrease in contractility after KCI and bethanechol stimulation compared with OIL and RA bladders. Detrusor morphology and expression patterns of smooth muscle markers were similar between groups. Detrusor muscles in OIL and RA fetuses were densely innervated, possessing abundant intramural ganglia and nerve trunks that branch to supply smooth muscle bundles. In MMC bladders, neurotubulin-β-III-positive nerve fibers were markedly decreased with advancing gestational age and were almost completely absent at term (E22). We conclude that the biomechanical properties of fetal rat MMC bladders are analogous to that seen in humans with MMC-associated NBD. Decreased nerve density indicates loss of peripheral neural innervation throughout gestation. The early observation of decreased innervation and decreased contractility in the absence of morphologic abnormalities in muscle structure or extracellular matrix supports a pathophysiologic hypothesis that denervation is the primary insult preceding the observed alterations in bladder muscle structure and function.

all-trans retinoic acid; contractility

MYELOMENINGOCELE (MMC) is a nonlethal neural tube defect with sequelae that affect both the central and peripheral nervous system. It is a severe form of open spina bifida affecting ~1 of every 2,000 children born worldwide. The lesion is characterized by protrusion of the meninges and spinal cord through open vertebral arches, leading to lifelong physical disabilities, including paraplegia, skeletal deformations, neurogenic bowel and bladder dysfunction, and the Arnold-Chiari II malformation with secondary hydrocephalus (12). The spectrum of neurogenic bladder dysfunction (NBD) associated with MMC ranges from an atomic poorly emptying bladder to a noncompliant hyperreflexic bladder with or without detrusor sphincter dyssynergia resulting in urinary incontinence and, in selected circumstances, hydropnephrosis, pylonephritis, and deterioration in renal function.

Morphometric studies of human fetal MMC specimens have demonstrated that as early as 23 wk gestation innervation of the lower genitourinary tract is markedly decreased and that this might be associated with disorganization or arrest of normal smooth muscle development in the fetal bladder (42, 50). These findings may explain the observed clinical abnormalities of the lower genitourinary tract as well as the poorly contractile and noncompliant neurogenic bladder reported in children and adolescents with MMC.

MMC is postulated to cause neurological deficits in the fetus in stages (by-hit hypothesis). First, defective neurulation results in a neural tube defect with associated myelodysplasia. Second, subsequent exposure to the amniotic fluid, direct trauma, hydrodynamic pressure, or a combination of these factors causes secondary injury to the exposed spinal cord. Recently, encouraging results have been obtained in various experimental and preliminary clinical studies of midgestational closure of MMC indicating that fetal intervention may prevent progression of secondary destruction of the exposed neural tissue, reduce hindbrain herniation associated with the Arnold-Chiari II malformation, reduce the incidence of shunt-dependent hydrocephalus, and improve fetal head and brain biometry (1, 8, 11, 30, 43, 56). However, it remains unclear whether fetal surgical repair with coverage of the exposed spinal cord will reduce the extent of deficit to the lower genitourinary tract and improve postnatal bladder function. In a preliminary research study of 23 patients who had undergone fetal MMC repair, no significant improvements in urologic function could be appreciated compared with patients treated with standard postnatal care (26). However, these data should be interpreted with caution, because fetal surgical intervention was performed late in pregnancy (between 25 to 30 wk of gestation) and irreversible damage to the developing genitourinary tract may have already occurred. In addition, this patient population was

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studied at an average age of 6 mo when urologic testing is relatively unreliable, and the study was confounded by the lack of an appropriate age- and level-matched control population. Thus, research in analogous animal models is needed to better understand the developmental biology and pathophysiology of NBD associated with MMC and to optimize its prenatal and/or postnatal treatment.

We recently developed a highly efficient new animal model of MMC in fetal rats by maternal administration of all-trans retinoic acid (RA) (13). To better understand the pathophysiology of NBD in fetuses with MMC, we evaluated the morphologic, molecular, and functional changes of bladder specimens in our rat model of RA-induced MMC.

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children’s Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Animal preparation and RA exposure. The procedures for creating MMC-like defects in fetal rats have been described elsewhere (13). Time-dated primigravida Sprague-Dawley rats weighing 210 to 300 g (Charles River Laboratories, Wilmington, MA) were used for this study. Animals were allowed a minimum of 24 h to adapt to housing conditions before any manipulation. Animals were housed one per cage with a standard 12:12-h light-dark schedule (with light from 7 PM to 7 AM; 22°C) with food and water available ad libitum. After a brief exposure to isoflurane (Abbott Lab, North Chicago, IL), dams were gavage fed 60 mg/kg body wt (BW) of all-trans RA (Sigma, St. Louis, MO) dissolved in 2 ml olive oil at embryonic day 10 (E10). This dosage has been previously shown to induce isolated MMC-like defects in more than 80% of the offspring (13). Control animals were fed 2 ml olive oil.

Collection of fetuses and bladder tissue. Fetuses were collected at E16, E18, E20, and at term (E22). Pregnant rats were anesthetized by isoflurane and euthanized by cervical dislocation. Fetuses were delivered by cesarean section under a dissecting microscope. Only fetuses with isolated MMC or no defects after RA exposure were used for analyses. At term, each fetus was blotted dry, and body weight was recorded. In fetuses with MMC, the lesion was photographed and the craniocaudal and transverse diameter of the defect was measured. The superior extent of the spina bifida defect was determined by counting the entire vertebral complement, from the craniocaudal junction on sagittal section in a subset of MMC fetuses. The fetal abdomen was oriented to section in the coronal, horizontal, or sagittal plane. After processing using a Leica TP 1050 tissue processor (Leica, Heidelberg, Germany), fetuses were embedded in paraffin, sectioned at 4- to 6-μm thickness using a RM 2035 microtome (Leica), and collected on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA).

For general morphology, sections were deparaffinized, rehydrated to distilled water, and stained with hematoxylin and eosin or Mason’s trichrome according to standard protocols. Sections assigned for immunohistochemistry were air-dried overnight in a 50°C incubator, deparaffinized, and rehydrated to distilled water. The sections were then immersed in antigen unmasking solution (Vector Laboratories, Burlingame, CA) and microwaved (Ted Pella, Redding, CA) for 4 min on high power, cooled at room temperature for 30 to 40 min, washed in distilled water, and transferred to PBS containing 0.1% Triton X-100 (pH 7.4). To block endogenous peroxidase, slides were incubated for 30 min with peroxidase blocking reagent (Dako Cytomation, Glostrup, Denmark). The following primary antibodies were used in this study: anti-α-smooth muscle actin (α-SMA; DAKO Cytomation, 1:100), rabbit-anti-myosin (Boimed Technology, Stoughton, MA, 1:100), mouse-anti-α-sarcinemic actin (Abcam, Cambridge, MA, 1:100), mouse-anti-Desmin (Zymed Laboratories, San Francisco, CA, 1:200), mouse-anti-vimentin (DAKO Cytomation, 1:100), and mouse anti-neurotubulin-β-III (Abcam, 1:100).

Primary antibodies were diluted using ready-to-use antibody diluent with background reducing components (Dako Cytomation), and slides were incubated overnight at 4°C. On the following day, the sections were washed three times with PBS containing 0.1% Triton X-100 (pH 7.4) and then incubated with the secondary antibody [polyclonal anti-rabbit-horseradish peroxidase (HRP) DAKO, Envision system HRP rabbit] or Polymer anti-mouse-HRP (DAKO, Envision system HRP mouse) for 30 min at room temperature. Finally, the sections were washed one time with PBS containing 0.1% Triton X-100 (pH 7.4), visualized using 3,3′-diaminobenzidine (DAB) substrate-Chromogen (Sigma), counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher Scientific). Immunoreactivity for α-SMA and neurotubulin-β-III was detected using fluorescein isothiocyanate-conjugated secondary antibody (FITC-antimouse, Vector Lab, Burlingame, CA, 1:100 in PBS, 30-min room temperature) and counterstained and mounted using DAPI (Molecular Probes, Eugene, OR).

Protein preparation and Western blot analysis. Thirty-six fetal rat bladders from each condition (OIL, MMC, and RA) were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until use. For protein preparation, three to four fetal bladders per lane and per condition were pooled and sonicated in ddH2O containing protease inhibitors (Complete, Roche, Mannheim, Germany). Total protein concentration was determined by protein microassay (DC Protein Assay, Bio-Rad, Hercules, CA). Protein extracts (50 μg) were heated at 90°C for 10 min and size fractionated on Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA). The proteins were transferred to nitrocellulose membranes (Duralose UV, Stratagene, Cedar Creek, TX), blocked with 5% fat-free milk in Tris-buffered saline (1 h, room temperature) and incubated overnight at 4°C in primary antibody. The following day, the membranes were incubated in secondary antibody (goat anti-rabbit, or goat anti-mouse HRP conjugate, respectively; Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. The membranes were washed and developed using a chemilu-
inescent substrate kit (SuperSignal West Pico, Pierce, Rockford, IL). To confirm equal protein loading between samples, membranes were stripped (Restore Western Blot Stripping Buffer, Pierce) and reprobed with mouse anti-actin antibody (Abcam, Cambridge, MA). For Western blot analysis, densitometric values were analyzed using Scion Image 4.02 for Windows (Scion, Frederick, MD).

Statistical analysis. All numerical data are presented as means ± SE. Significance was defined as \( P \leq 0.05 \). All analyses were performed using the Statistical Package for Social Science for Windows version 11.5 (SPSS, Chicago, IL).

RESULTS

General observation. All MMC lesions were confined to the lumbosacral area of the fetus and, similar to observed pathological changes in human fetuses with MMC, the defect was covered by a thin fibrous membrane (Fig. 1, A-C). Microscopic analysis clearly demonstrated the morphological changes of the vertebrae, the leptomeningeal membranes, and the spinal cord; including loss of neural tissue, disruption of neural bundles, and areas of cord necrosis and hemorrhage in the exposed segments (Fig. 1, D and E). Clinically, fetuses with MMC showed bilateral clubfoot deformity of their hindlimbs and had lower-extremity paralysis and loss of sensation (Danzer E, unpublished observations). The mean cranio-caudal and transverse diameter of the MMC lesion at term was 6.8 ± 4.9 mm (range 2 to 13) and 4.2 ± 2 mm (range 1 to 9), respectively. The superior level of the defect assigned in 34 MMC fetuses ranged from L1 through L6 [L1 \( (n = 9) \), L2 \( (n = 8) \), L3 \( (n = 5) \), L4 \( (n = 7) \), L5 \( (n = 4) \), L6 \( (n = 1) \)].

Body weight of fetuses with MMC (4.87 ± 0.3 g) was significantly reduced \( (P < 0.0001) \) compared with OIL (5.75 ± 0.3 g) and RA fetuses (5.52 ± 0.2 g). Fetal body weights tended to be lower in the RA group compared with OIL, although this did not reach statistical significance \( (P = 0.06) \). The bladder-to-body weight ratio was significantly reduced \( (P < 0.001) \) in MMC fetuses \( (0.08 ± 0.02) \) compared with OIL \( (0.12 ± 0.02) \) and RA fetuses \( (0.11 ± 0.01) \).

Smooth muscle physiology. We also compared the contractile responses of fetal bladder strips of OIL, MMC, and RA detrusor muscles. As revealed by previous studies (31, 38, 57), spontaneous activity was not detected and none of the bladders responded to electrical field stimulation (data not shown). The contractility profiles produced by detrusor muscle strips from the OIL, MMC, and RA bladders in response to 125 mM KCl are depicted in Fig. 2A. The strips from OIL- and RA-exposed fetal bladders developed a transient contractile force in response to KCl, followed by a partial relaxation and a second rise in tension, as expected (25). The tension decreased slowly and reached the basal level within 6 min. Muscle strips from MMC fetuses did not show the initial transient force, and the maximum force generated was significantly less \( (P = 0.002) \) compared with OIL and RA muscle strips. The mean maximum active force produced by OIL, MMC, and RA bladder strips after KCl stimulation was 37.3 ± 17.1, 11.4 ± 9.6, and 34.0 ± 11.4 g/mm², respectively (Fig. 2C). The addition of bethanechol, a cholinergic agonist, at various concentrations induced large tonic contractions of bladder strips from OIL and RA animals. After reaching the maximum, this force was maintained throughout the 6-min measurement period (Fig. 2B). Similar to the KCl stimulation, the maximum force generated from MMC bladder strips was significantly reduced \( (P < 0.001) \), or completely absent. The mean maximum active force produced by OIL, MMC, and RA bladder strips by bethanechol treatment was 51.7 ± 17.7, 17.1 ± 14.6, and 53.8 ± 26.1 g/mm², respectively (Fig. 2D). These results

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Fig. 1. Representative photographs of retinoic acid (RA)-induced lumbosacral myelomeningocele (MMC) lesion in fetal rats at birth (A, B, C). The spinal cord remnants rest on the dorsal aspect of a fluid-filled cystic sac (arrowheads). Similar to human MMC defects, the severely altered spinal cord tissue appears to split into 2 separate parts (arrows). D: transverse section through the center of the MMC defects. The neural elements are exposed on the surface and covered by a hemorrhagic membrane. Despite massive destruction of the dorsal part of the spinal cord, white (arrow) and gray (arrowhead) matter are distinguishable. E: sagittal section through the defect demonstrates severe alterations of the normal cytoarchitecture. Arrowheads mark the skin lesion. F: spinal cord remnants lying exposed on the dorsal aspect of the cystic sac, which in vivo was filled with cerebrospinal fluid. The spinal cord demonstrates extreme flatting and erosion of the dorsal portion. Magnification: ×10 for A, B, and C; ×50 for D, E, and F.
suggest a marked reduction of phasic and tonic characteristics of the contractility forces generated in neurogenic MMC bladders.

**Bladder morphology and immunohistochemistry.** Sections of the bladder body wall revealed many bundles of smooth muscle cells composing the detrusor. Two layers of these bundles were discernable: an outer layer composed primarily of longitudinal sections of smooth muscle cells (running longitudinal from the bladder dome to the base) and an inner layer composed primarily of cross sections of smooth muscle cells (circumferential to the bladder cavity). Similar to OIL and RA bladders, well-organized smooth muscle bundles were apparent within the MMC bladder (Fig. 3, A-C). However, stereological measurements of the bladder wall thickness were not taken because this variable is dependent on bladder distension during fixation that could not be standardized. In contrast to previous studies with human myelodysplastic bladders (49), Masson’s trichrome staining did not reveal an increased bladder wall trabeculation and no obvious sign of increased amounts of intrafascicular and interfascicular connective tissue could be found in the fetal rat bladder (Fig. 3, D-F).

Immunoreactivity for α-SMA and myosin was observed within the bladder wall along the longitudinal and circular muscle layer. Staining intensity, as shown in the representative section in Fig. 3, G-I, for α-SMA and Fig. 3, P-R, for SM-myosin did not vary between OIL, MMC, and RA bladders. Immunolabeling with α-sarcomeric actin, a marker for skeletal muscle, was performed to analyze whether MMC fetal bladders undergo “transdifferentiation” into skeletal muscle (50). As shown in Fig. 3J, none of the detrusor myocytes of OIL, MMC, and RA bladders were positive for α-sarcomeric actin. Desmin immunoreactivity revealed a dense network of varicose smooth fibers within the circular and longitudinal detrusor muscle layers (Fig. 3, M-O). Vimentin-positive filaments were expressed throughout the lamina propria and connective tissue between the smooth muscle bundles (Fig. 3, S and T). The

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**Fig. 2.** A: representative examples of isometric force tracings from MMC, olive oil-exposed fetuses (OIL), and RA whole bladders in response to KCl or bethanecol. B: graphic representation of mean maximal response after KCl stimulation (125 mM) was significantly reduced \( (P = 0.002) \). C: mean maximal response after bethanecol stimulation (250 μM). D: similar to the KCl, bethanecol treatment resulted in a significant reduction of contractility in MMC bladders \( (P < 0.001) \). Compared with OIL bladders, RA alone did not affect the contractile response after KCl \( (P = 0.65) \) or bethanecol \( (P = 0.85) \) stimulation. *Significant differences between mean values of MMC bladders to OIL and RA bladder controls.

**Fig. 3.** Histological presentation of bladder smooth muscle preparation from OIL, MMC, and RA fetuses. The thickness of the bladder wall varies with the degree of bladder distention during fixation. Stretching the urothelium to a thickness of two cells in the most distended areas. The lamina propria and muscularis are also thin, with the latter reduced to a few thin parallel fascicles. A-C: hematoxylin-eosin staining demonstrates normal arrangement of the outer and inner layer of smooth muscle composing the bladder detrusor. D-F: full-thickness section of the bladder detrusor stained with Masson’s trichrome staining. Smooth muscle organization and differentiation appear to be normal. No increase in extracellular matrix protein deposition was observed in bladders of OIL, MMC, and RA fetuses. G-I: intense α-smooth muscle actin (SMA) immunofluorescein staining is present within the outer longitudinal and inner circular smooth muscle layer of the bladder wall indicating normal detrusor muscle development in fetal rats with MMC. J-L: absent reactivity for α-sarcomeric actin indicates that the detrusor smooth muscle in MMC does not contain skeletal muscle fibers. J-L, insets: strong reactivity of the rectus abdominis muscle for α-sarcomeric actin. M-O: bladder specimens from MMC, OIL, and RA fetuses show similar immunophenotypic pattern for desmin immunohistochemistry. Desmin-positive filaments are packed according to the typical orientation of the smooth muscle within the detrusor wall. P-R: myosin immunolabeling demonstrates no difference in expression pattern within the longitudinal and circular muscle layer of OIL, MMC, and RA bladders. S-U: similar to OIL and RA, in MMC bladders vimentin-positive filaments were found throughout the bladder wall, at the edge of smooth muscle bundles and in the spaces between the bundles. Magnification: \( \times 10 \) for A-L and P-U; \( \times 20 \) for M-O.
absence of α-sarcomeric actin muscle fibers and the appearance of SM-myosin, desmin, and vimentin in differentiating smooth muscle cells confirm normal ontogeny of bladder smooth muscle in MMC fetuses.

Double immunofluorescence staining for α-SMA and neurotubulin-β-III at E16, E18, E20, and E22 was done to examine the regional and spatial distribution of smooth muscles and nerves within the muscle layer of the fetal bladder throughout gestation (Fig. 4). At 16 days of gestation α-SMA-expressing smooth muscle cells were detected in the periphery of the detrusor muscle immediately subjacent to the serosa in OIL, MMC, and RA bladders (Fig. 4, A–C). The organization and maturation of the smooth muscle, as noted by individual fascicles, progressively increased in all three groups throughout gestation and no differences between OIL, MMC, and RA bladder smooth muscle development were found (Fig. 4, D–O). Neurotubulin-β-III was first detected at 18 days of gestation and numerous neurotubulin-β-III-labeled thick nerve bundles and fine nerve fibers were seen in the muscle layer of OIL, MMC, and RA bladders (Fig. 4, D–F). While an increase of this intensive well-defined pattern of neurotubulin-β-III-positive nerve varicose fibers and fibers was observed within the circular and longitudinal muscle layer in OIL (Fig. 4, G, J, M) and RA (Fig. 4, I, L, O) bladders with increasing gestational age, the density of neurotubulin-β-III-positive nerve fibers within the detrusor muscle of MMC bladders was markedly reduced at E20 (Fig. 4H) and almost completely absent at term (Fig. 4, K and N), suggesting progressive loss of peripheral innervation throughout gestation in fetal MMC bladders.

Control slides for α-SMA, α-sarcomeric actin, desmin, myosin, vimentin, and neurotubulin-β-III where the primary antibody was omitted were completely negative for immunostaining (data not shown).

Western blot analysis. Western blot analysis specific for α-SMA (Fig. 5A), desmin (Fig. 5B), and myosin (Fig. 5C) was done to quantify the protein expression of these smooth muscle phenotypic markers. No difference in α-SMA (OIL 230.1 ± 1.0, MMC 223.8 ± 11.3, RA 233.8 ± 6.1), desmin (OIL 107.9 ± 8.9, MMC 111.4 ± 21.0, RA 100.9 ± 0.9), and myosin (OIL 247.4 ± 5.5, MMC 247.5 ± 6.9, RA 248.5 ± 7.2) was observed. To quantify extracellular matrix components, Western blot analysis was performed for collagen III and I. While no difference in the protein expression for collagen III (Fig. 5D) was found between myelodysplastic and control bladders (OIL 226.7 ± 7.7, MMC 224.3 ± 10.3, RA 229.1 ± 14.1), collagen I protein expression (Fig. 5E) was significantly decreased in the MMC bladders compared with the control bladders (OIL 92.4 ± 23.2, MMC 41.7 ± 11.4, RA 100.6 ± 31.4).

DISCUSSION

MMC is the most common cause of NBD in children, and more than 95% of babies born with MMC have abnormal function of their urinary bladder (48). NBD is accompanied by a number of well-described changes in normal bladder function: loss of voluntary control of micturition, bladder hyperactivity, bladder/spincter dyssynergia, increased vesical pressure, and bladder hypertrophy (2, 7, 10, 35, 47, 60). The natural history of NBD, however, remains controversial and has not been formally assessed. This lack of understanding hinders the development of more appropriate treatment strategies for MMC-associated NBD. We previously developed and extensively characterized the rat model of MMC used in this study. Maternal administration of RA at the time of fetal neural tube closure results in pathology that has striking morphological and clinical similarities to human MMC (13). In humans, the defect is typically found in the lumbosacral area and the protruding, plate-like remnants of the spinal cord lie exposed on the dorsal aspect of a cystic sac. We find the same characteristics in our model. Clinical complications often associated with human MMC, such as hindbrain herniation, clubfoot deformity, and paraplegia, are also present. The primary objective of this study was to investigate whether known functional and morphological abnormalities in myelodysplastic bladder in human patients with MMC can be reproduced in the fetal rat model of RA-induced MMC.

For the smooth muscle physiology, we utilized three forms of stimulations. Bethanecol stimulates contraction through direct activation of muscarinic receptors. KCl stimulates contraction through direct depolarization of the smooth muscle cell membrane and requires the presence of cells that have excitable membranes and functional contractile filaments. Field stimulation activates the embedded motor nerve endings and causes a frequency-dependent contraction via neurohumoral transmitters.

With respect to exogenously applied agonists (bethanecol and KCl), our results confirm that the biomechanical properties of the bladders in the fetal rat with MMC are analogous to those seen in humans with MMC-associated NBD (17–19). In the current study, ex vivo bladder contractility was remarkably reduced in fetal rats with MMC. These findings are consistent with those of German and associates (17) who demonstrated that the maximal response after direct and indirect stimulation of isolated detrusor muscle strips from human patients with MMC-associated NBD was significantly lower than that obtained from normal controls. As the response of bladder smooth muscle to bethanecol and KCl measures muscarinic cholinoergic and nonspecific contractile properties, respectively,
our results suggest that both properties might be primarily affected in MMC-associated NBD. The significant decrease in contractility could be responsible for some forms of bladder dysfunction observed in MMC, such as high residual volume and marked difficulties in efficient bladder emptying.

Based on the morphological studies (Fig. 4), one might have expected that field stimulation of MMC muscle strips would show a reduced sensitivity and a reduced overall contractile response compared with OIL and RA bladders. In this study, however, the responses to direct nerve stimulation in fetal OIL, MMC, and RA muscle strips were virtually nonexistent. The nonresponse of control bladder strips is consistent with a number of other reports evaluating the maturation of micturition and voiding pathways in neonatal rats which demonstrated that the contractile response to field stimulation in normal newborn rat bladders develops within the first week of life and is not present at term (40). Also, micturition in newborn rats is not primarily mediated by the spinobulbospinal pathway in which bladder distension above a threshold level causes bladder contraction and complete emptying, but rather by a somato vesical spinal reflex pathway, which is activated when the mother licks the perineum of the neonate (9, 55, 57). During the postnatal maturation of central nervous system and bladder, this primitive reflex is replaced by a supraspinal bladder-to-bladder reflex that induces voluntary voiding in response to bladder distension. Postnatal evaluation of field stimulation-induced contractility responses of the bladder smooth muscle would be helpful to further characterize the origin and progression of bladder dysfunction in the present model. However, due to high infection rates related to the MMC defect, the somato vesical reflex, and the fact that the maternal rat cannibalizes affected newborns, we are currently unable to keep MMC rat fetuses alive for an adequate period of time after birth.

Interestingly, other reports concerning contractile responses of neurogenic bladders in humans have varied from “little change” to even an increased contractility response (33, 46). However, some of the reported discrepancies on the effect of MMC on bladder smooth muscle contraction may be attributed to lack of appropriate normalization of the contractile force. In the present study, all force data were normalized to the overall size of the muscle strips for a more accurate comparison. Furthermore, in the current study, an additional control group consisting of bladders obtained from fetuses exposed to RA without the development of MMC was investigated, because of known primary effects of RA on genitourinary tract development (5, 6). Due to the lack of normal control bladders in human studies, physiological behavior of bladder specimens from patients with MMC-associated NBD is frequently compared with bladder specimens obtained from patients with nonneurogenic bladder disease (e.g., bladder outlet obstruction, primary vesicoureteric reflux, or genuine stress incontinence). However, these “control” bladder specimens are also affected by abnormal innervation, detrusor sphincter dysynergia, and reduced bladder contractility, which have been well-documented by several investigators (23, 51, 52). Thus, an animal preparation, such as the one presented in this study, might be very useful to further characterize early changes in bladder function in MMC as unlike to human specimens, other confounding factors, including lack of appropriate controls, or secondary NBD-associated complications can be excluded.

Previous studies in humans showed that NBD in MMC is associated with abnormal bladder innervation. Shapiro and associates (50) reported that fetuses with MMC have only scant neural elements present within the bladder wall. Gup et al. (20) showed that muscarinic cholinergic receptor density is significantly reduced in myelodysplastic bladders. German and associates (17) demonstrated denervation of detrusor muscle bundles in patients with MMC-associated NBD, using acetylcholine esterase staining. Finally, Haferkamp and co-workers (20, 21) recently evaluated ultrastructural changes of detrusor innervation in myelodysplastic bladders in adolescents. They showed that NBD is not only associated with axonal degeneration and depletion of terminal organelles (e.g., vesicles and mitochondria) but also with an increased width of the neuro muscular junctions, indicating that long-standing loss of peripheral innervation, as seen in MMC-associated NBD, may lead to a universal intrinsic neurodevelopmental defect of the detrusor muscle.

We examined the spatial and temporal distribution of neurotubulin-β-III within the detrusor muscle throughout gestation in OIL, MMC, and RA fetuses. Early in gestation at E16, neurotubulin-β-III-positive nerve fibers could not be detected. At E18, the immunohistochemistry for neurotubulin-β-III showed numerous nerve fibers within the smooth muscle layers in OIL, MMC, and RA bladders. While the density of the neuronal network further increased in OIL and RA bladders as component of normal maturation and development, MMC bladders at E20 showed only few scattered neurotubulin-β-III-labeled cells. At term, the detrusor muscle of OIL and RA fetuses was densely innervated, possessing abundant intramural ganglia and nerve trunks that branch to supply smooth muscle bundles. In contrast, bladder specimens from MMC fetuses demonstrated a marked decrease or complete absence of neurotubulin-β-III-positive nerve fibers within the detrusor muscle. Our data clearly demonstrate that prenatally acquired loss of peripheral innervation is one of the key components in the development of MMC-associated NBD.

Intact innervation is required for normal development and function of genitourinary structures (41, 58, 59). An early disturbance in cell migration, cell-cell adhesion, or colonization by neural crest precursors (34), as seen in MMC, may
directly modulate bladder smooth muscle development. There is a growing body of evidence that the neurological deficits associated with MMC are related to progressive damage to the exposed spinal cord (12, 24, 36). Therefore, it seems reasonable to speculate that abnormal development and dysfunction of the bladder smooth muscle in MMC are caused by progressive autonomic nerve injury. Indeed, several investigators demonstrated that the function of the myelodysplastic bladder deteriorates over time (7, 18). As revealed by morphological studies of human MMC bladder specimens, functional changes are accompanied by an increased paucity of muscle bundles, decreased size of the muscle bundles, and disruption of muscle bundles by increased connective tissue deposition (16, 49, 50). Shapiro and associates (49, 50) suggested that in MMC, prolonged abnormal innervation of the bladder might result in disorganization or arrest of normal smooth muscle development and transdifferentiation into skeletal muscle. Thus we examined several smooth muscle markers essential for normal development but no differences of smooth muscle expression patterns between groups could be found by immunohistochemistry and protein analysis. Nevertheless, our finding of a decreased bladder weight-to-body weight ratio in MMC fetuses suggests that abnormal innervation may be associated with abnormalities of bladder smooth muscle maturation that are not detected by our limited analysis.

Several investigators have examined the effect of NBD on extracellular matrix protein components within the detrusor smooth muscle bundle in patients with MMC. The noncompliant, low-capacity bladder is histologically characterized by striking changes in the quantity, location, and type of the extracellular matrix protein components (14–16, 29, 32, 39, 45, 49). We did not observe any significant changes in the relative volume of extracellular matrix protein or the connective tissue-to-smooth muscle ratio by histopathology. Neither was the collagen III protein expression different between groups. Moreover, we found a significant downregulation of collagen I protein expression in bladders obtained from fetal rats with MMC.

The apparent discrepancies between our findings and previously reported studies in humans may be explained by the fact that the rat fetuses at term (E22) developmentally correspond to an early second trimester human fetus. Morphological studies in humans with MMC, however, were performed on terminated midgestational fetuses, stillborn neonates, or on specimens obtained from adolescent patients with MMC at the time of reconstructive surgery later in life. Especially in the latter group recurrent infections, catheterization, and other complications may have already occurred and the abnormal smooth muscle structure and increased deposition of extracellular matrix proteins in human MMC bladder could be explained, at least in part, by changes within the bladder wall secondary to these complications. The relative developmental immaturity of the term rat bladder relative to human bladders is also supported by the physiology of micturation of the newborn rat described above, as well as by the documented absence of contraction of the term rat bladder on field stimulation.

Despite the normal smooth muscle development in fetal rats with MMC, as discussed above, the contractility responses of MMC muscle strips to KCl were significantly reduced. The reason for this pronounced difference between function and structure is not clear and cannot be explained by the classical aspects of bladder physiology models of smooth muscle and nerve interactions. Despite the short gestation in fetal rats, with MMC ultrastructural changes within the bladder wall, for example, in electrical coupling between cells, in receptor populations, and/or the spread of spontaneous and evoked activity from one part of the bladder to another may have already occurred. Another possible explanation may involve abnormalities in the pathways important in modulating contractile protein activity, such as changes in the calcium- and calmodulin-dependent myosin light chain phosphorylation (27). Finally, abnormal expression and release of molecules, i.e., vasoactive intestinal polypeptide, endothelin-1, tachykinins, and angiotensins, that have been shown to be important modulators of detrusor smooth muscle contraction (3, 4, 28) may also be involved in the development of MMC-associated NBD. Whether these hypotheses account for the present results and if so, whether changes in ultrastructure and modulating factors are inherent or represent early consequences of the abnormal innervation in MMC bladders are important questions that need to be addressed.

In summary, the rat model of RA-induced MMC offers a unique tool to evaluate the biomolecular mechanisms of NBD in MMC and to improve our understanding of the pathophysiological processes involved in the development of myelodysplastic bladders. We showed that the peripheral neural supply deteriorates throughout gestation and that despite normal structural development, the functional status of the detrusor smooth muscle has already been altered in this short gestational model. Therefore, further experiments to assess the exact sequence and timing of smooth muscle maldevelopment and the contribution of nonneuronal and nonmuscular modulators of contractility to bladder dysfunction in MMC-associated NBD are warranted. If long gestational studies reveal that the progressive neural injury is associated with abnormal smooth muscle development and deterioration of NBD, early fetal intervention in selected cases may prove beneficial (14, 15, 19, 22, 29, 32, 37, 39, 44, 54).

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