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

Enrico Danzer, Darcie A. Kiddoo, Robert A. Redden, Lauren Robinson, Antoneta Radu, Steve A. Zderic, Edward J. Doolin, N. Scott Adzick, and Alan W. Flake

1The Center for Fetal Research, and 2Division of Urology, The Children’s Hospital of Philadelphia, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

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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 IV 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 KCl and bethanechol stimulation compared with OIL and RA bladders. Bladder 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 advanced 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 pathophysiological 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 anatomic poorly emptying bladder to a noncompliant hyperreflexic bladder with or without detrusor sphincter dyssynergia resulting in urinary incontinence and, in selected circumstances, hydronephrosis, pyelonephritis, 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 (two-hit hypothesis). First, defective neuronal 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 dark-light 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 craniocervical junction 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-mysin (Biomed Technology, Stoughton, MA, 1:100); mouse-anti-α-sarcinmeic 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 [Polymer 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 Permunt (Fisher Scientific).

Immunoreactivity for α-SMA and neurotubulin-β-III was detected using fluorescein isothiocyanate-conjugated secondary antibody (FITC-antimouse, Vector Lab, Burlinghame, 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-
There was massive destruction of the dorsal part of the spinal cord, indicating severe alterations in the cytoarchitecture. Arrowheads mark the skin lesion.

**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 flattening and erosion of the dorsal portion. Magnification: ×10 for A, B, and C; ×50 for D, E, and F.

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Statistical analysis. All numerical data are presented as means ±

RESULTS

General observation. All MMC lesions were confined to the lumbosacral area of the fetus and, similar to observed patho-

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logical 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 trans-
vex 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 re-
duced (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 contrac-
tile 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-, MMC-, and RA-exposed fetal bladders developed a transient contractile force in re-
sponse 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 achieved was significantly less (P = 0.002)
compared with OIL and RA muscle strips. The mean maxi-
mum 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 bladders 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 gen-
erated 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
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-sarcotomeric 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-sarcotomeric actin. Desmin immunoreactivity revealed a dense network of varicos 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
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 intense well-defined pattern of neurotubulin-β-III-positive nerve varicose 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 bladders 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 stimulation. 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 cholinergic and non-specific contractile properties, respectively,

Fig. 4. Double immunofluorescence staining for α-SMA (green-fluorescence) and neurotubulin-β-III (red-fluorescence) at embryonic day 16 (E16), 18, 20, and 22 days of gestation in OIL, MMC, and RA bladders. Nuclei were stained with DAPI. No difference in α-SMA expression pattern was observed throughout gestation between groups. A–C: at E16 smooth muscle differentiation begins near the serosal edge of the bladder, as defined by α-SMA-positive spindle-shaped cells. No neurotubulin-β-III-positive nerve fibers were seen in OIL, MMC, and RA bladders. D–F: at E18, the differentiation of smooth muscle in OIL, MMC, and RA bladders progressed from the periphery toward the bladder lumen. Numerous neurotubulin-β-III-positive nerve fibers and varicosities were found. Staining intensity and expression pattern for neurotubulin-β-III of MMC bladders were similar to OIL and RA bladders. G–I: at E20, in all groups, the amount of α-SMA-positive smooth muscle cells and fascicles increased progressively. While in OIL (G) and RA (I) bladders, staining intensity for neurotubulin-β-III-labeled cells significantly increased, only a few scattered nerve fibers were found in MMC bladders (H, arrowhead). J–O: at term (E22) an intense well-defined pattern of heavily neurotubulin-β-III-stained neuronal ganglons and nerve bundles were seen within the detrusor muscle of OIL (J, M) and RA (L, O). However, in MMC bladders, the intense neuronal network was almost completely absent within the longitudinal and circular muscle layer of the detrusor muscle (K, N) and only a few neurotubulin-β-III-positive thin nerve fibers were found (arrowhead). Magnification: ×10 for A–O.
NEUROGENIC BLADDER DYSFUNCTION IN FETAL RATS WITH MMC

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Fig. 5. Representative Western blots showing α-SMA (A), desmin (B), myosin (C), collagen III (D), and collagen I (E) protein expression within the bladder detrusor of OIL, MMC, and RA fetuses. Fifty micrograms of total protein were loaded per well. No differences in α-SMA, desmin, myosin, and collagen III protein expression between groups were found. Collagen I protein expression was significantly decreased (P = 0.003) in MMC bladders vs. OIL and RA bladders.
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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