Smoothelin-A Is Essential for Functional Intestinal Smooth Muscle Contractility in Mice

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Background & Aims: **In patients with chronic intestinal pseudo-obstruction, intestinal motility is disturbed by either nervous or myogenic aberrations. The cause of the myogenic form is unknown, but it is likely to originate in the contractile apparatus of the smooth muscle cells. Smoothelins are actin-binding proteins that are expressed abundantly in visceral (smoothelin-A) and vascular (smoothelin-B) smooth muscle. Experimental data indicate a role for smoothelins in smooth muscle contraction. A smoothelin-deficient mouse model may help to establish the role of smoothelin-A in intestinal contraction and provide a model for myogenic chronic intestinal pseudo-obstruction.** ***Methods:*** **We used gene targeting to investigate the function of smoothelin-A in intestinal tissues. By deletion of exons 18, 19, and 20 from the smoothelin gene, the expression of both smoothelin isoforms was disrupted. The effects of the deficiency were evaluated by pathologic and physiologic analyses.** ***Results:*** **In smoothelin-A/B knockout mice, the intestine was fragile and less flexible compared with wild-type littermates. The circular and longitudinal muscle layers of the intestine were hypertrophic. Deficiency of smoothelin-A led to irregular slow wave patterns and impaired contraction of intestinal smooth muscle, leading to hampered transport in vivo. This caused obstructions that provoked intestinal diverticulosis and occasionally intestinal rupture.** ***Conclusions:*** **Smoothelin-A is essential for functional contractility of intestinal smooth muscle. Hampered intestinal transit in smoothelin-A/B knockout mice causes obstruction, starvation, and, ultimately, premature death. The pathology of mice lacking smoothelin-A is reminiscent of that seen in patients with chronic intestinal pseudo-obstruction.**

The principal function of smooth muscle cells (SMCs) in the intestinal tract is to enable propulsion and mixing of food,[1] which improves the digestion of complex molecules and the absorption of nutrients. Coordinated contractions of the circular and longitudinal smooth muscle layers are responsible for peristalsis of the gastrointestinal tract. Contractions of SMCs are slower than those of skeletal and cardiac myocytes, but are more sustained. Hence, the composition of the contractile apparatus of SMCs differs from that of the striated muscle cells. In both cell types, actin–myosin interactions are at the basis of contraction. The contraction of striated muscle is well understood and accessory proteins, such as troponins, are known to be part of the organization of the contractile apparatus and determine the mode of contraction.[2,3] The architecture and composition of contractile elements in SMCs, however, still is not understood fully. The contractile apparatus of SMCs is connected with the cytoskeleton via dense bodies. It consists of an actin–myosin axis complemented with structural muscle proteins, including α-actinin and tropomyosin, and more smooth muscle–specific proteins such as calponin, caldesmon, and smoothelin.[4,5]

Based on their expression pattern, smoothelins have been described as proteins specific for fully differentiated smooth muscle. The 2 major isoforms are smoothelin-A in visceral tissues such as the digestive tract, bladder, and prostate, and smoothelin-B in vascular tissues.[6,7] Both are found only in actively contracting smooth muscle tissues. Under pathologic conditions with impaired function of smooth muscle, such as aneurysms and restenosis, expression of smoothelins rapidly decreases.[8,9] In cultured SMCs, smoothelins colocalize with smooth muscle α-actin (α-SMA) stress fibers.[8,10] Recently, we

Abbreviations used in this paper: α-SMA, smooth muscle α-actin; CIP, chronic intestinal pseudo-obstruction; ICC, interstitial cell of Cajal; SMC, smooth muscle cell; *Smtn*, smoothelin gene.

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showed in vitro that smoothelins can bind physically to α-SMA under normal physiologic conditions.[11] These findings point toward a direct role of smoothelin in contraction.

If SMCs are brought into culture, smoothelin expression is down-regulated rapidly,[6,10] concomitant with their modulation toward a synthetic phenotype. This hampers in vitro investigations of the function of smoothelin in smooth muscle contraction. Therefore, assessment of the function of smoothelins in intestinal contractility requires an in vivo approach. Here, we report the interruption of the smoothelin gene in mice, leading to elimination of both smoothelin-A and smoothelin-B. The smoothelin knockout mice (*Smtn-A/B*$^{-/-}$) show dysfunction of intestinal motility and contractility and die prematurely. The observed phenotype displays pathologies reminiscent of intestinal diverticulosis, chronic intestinal pseudo-obstruction (CIP), and hollow visceral myopathy in humans.

Materials and Methods

Generation of *Smtn-A/B*$^{-/-}$ and *Smtn-B*$^{-/-}$ Mice

To generate *Smtn-A/B*$^{-/-}$ mice, we replaced part of exon 18 and exon 19–20 with a neomycin resistance gene under the control of the thymidine kinase promoter in reverse orientation (Figure 1*A*). The targeting vector contained the thymidine kinase gene for negative selection. After electroporation of the *Pvu*II-linearized construct into mouse L129/Sv embryonic stem cells, we selected neomycin-resistant clones with G418 (Invitrogen, Carlsbad, CA) and 1-[2-deoxy]2-fluoro-β-D-arabinofurasonyl (Invitrogen). DNA from resistant clones was screened by Southern blotting after *Pst*I restriction digestion, using the 3′ probe indicated in Figure 1*A*. Embryonic stem cells from 2 independent targeted clones were injected into C57Bl/6 blastocysts and implanted into pseudopregnant C57Bl/6 females. Mating of the resulting chimeric males to C57Bl/6 females led to germline transmission of the targeted allele as detected by Southern blotting (Figure 1*B*). Because the mice had a mixed background (L129/Sv and C57Bl/6), we used littermates as controls. In the food transit experiment we used age- and sex-matched controls. The generation of smoothelin-B knockout mice (*Smtn-B*$^{-/-}$) is described elsewhere (Rensen et al, unpublished data). In these mice, exons 3–6 of the smoothelin gene are removed and smoothelin-B synthesis is absent; however, smoothelin-A synthesis is not affected. All animal studies were performed according to protocols approved by the Committee on Animal Experimentation of the University of Maastricht.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from various tissues with Tri reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands). Reverse transcription was performed using 1 μg of RNA in the RevertAid First-Strand complementary DNA synthesis kit (Fermentas, St. Leon-Rot, Germany). Expression of smoothelin messenger RNA in *Smtn-A/B*$^{-/-}$ mice was investigated by reverse-transcription polymerase chain reaction using smoothelin-B–specific primers 1F 5′-CCAGGGGGCAGTATGAAGAC-3′ and 1R 5′-CGCAGGTGGTTGTAGAGCGA-3′ and common smoothelin primers 2F 5′-GAGGAGCGCAAGCTGATCA-3′ and 2R 5′-CTGCTGGTGCTGAGAAGGGT-3′. Reverse-transcription polymerase chain reaction products were cloned and sequenced.

Western Blot

Intestinal tissue homogenates ($n = 5$) were prepared in buffer (.25 mol/L sucrose, .01 mol/L Tris-HCl pH 7.4, 2 mmol/L ethylenediaminetetraacetic acid) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentration was measured with the BCA Protein Assay Kit (Pierce, Rockford, IL) and 15 μg was loaded onto a 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. Proteins were blotted on a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences, Roosendaal, The Netherlands) and blocked overnight in phosphate-buffered saline containing .2% Tween-20 and 5% Marvel at 4°C. α-SMA was detected using the monoclonal antibody 1A4 (DAKO, Glostrup, Denmark) and a secondary rabbit anti-mouse antibody conjugated with horseradish peroxidase (DAKO). Smooth muscle myosin heavy chain was detected with the rabbit polyclonal immunoglobulin G bt-562 (Campro Scientific, Veenendaal, The Netherlands) followed by donkey-anti-rabbit horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Bands were visualized by enhanced chemiluminescence. Subsequently, signals were digitized and analyzed with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Histology, Immunohistochemistry, and Electron Microscopy

Organs from mice aged from 0 days to 6 months were fixed overnight in 3.7% formaldehyde in phosphate-buffered saline, embedded in paraffin, sectioned, and stained with H&E. Sirius Red staining was performed for the detection of collagen.

Samples of intestine (and several other tissues) were snap-frozen in liquid nitrogen–precooled isopentane and embedded in OCT Tissue Tek compound (Sakura, Chicago, IL). Cryostat sections were stained with biotinylated mouse monoclonal R4A specific for smoothelin.[12] The ABC-peroxidase kit (Vector Laboratories, Inc, Burlingame, CA) was used for detection, followed by diaminobenzidine tetrahydrochloride staining and hematoxylin counterstaining. Interstitial cells of Cajal (ICCs) were identified by antibody against c-kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).[13]