OBSTETRICS

Anthrax toxin receptor 2 promotes human uterine smooth muscle cell viability, migration and contractility

Joy Yumiko Vink, MD; Pelisa Cheryll Charles-Horvath, MS; Jan Krzysztof Kitajewski, PhD; Claire Vech Reeves, PhD

OBJECTIVE: Previously we demonstrated anthrax toxin receptor 2 knockout (*Antxr2*$^{-/-}$) mice are fertile but fail to deliver their pups at term. This parturition defect is associated with overaccumulation of extracellular matrix proteins and decreased myometrial cell content in the uterus. Myometrial cell loss in *Antxr2*$^{-/-}$ uterine tissue prompted us to evaluate if ANTXR2 is essential for human uterine smooth muscle cell viability and function.

STUDY DESIGN: We subjected human uterine smooth muscle cell to lentiviral-mediated knock down or retroviral-mediated overexpression of ANTXR2. Flow cytometry confirmed lentiviral-mediated knock down or retroviral-mediated overexpression in cell lines vs control. Cell behavior and function in control, lentiviral-mediated knock down and retroviral-mediated overexpression cells were evaluated for apoptosis via TUNEL assay, migration via Boyden chamber assay and with oxytocin-mediated collagen contraction assays. Matrix metalloproteinase activity was evaluated using gelatin zymography. Cell lines and samples were run in duplicate. Student *t* test was used for statistical analysis.

RESULTS: ANTXR2 is expressed by human uterine smooth muscle cell. Human uterine smooth muscle cell-lentiviral-mediated knock down cells exhibited increased apoptosis ($P < .05$) and decreased migration ($P < .05$), although human uterine smooth muscle cell-retroviral-mediated overexpression cells exhibited no change in apoptosis ($P = .91$) and increased migration ($P = .05$) vs control. Human uterine smooth muscle cell-lentiviral-mediated knock down cells contracted significantly less than control, although human uterine smooth muscle cell-retroviral-mediated overexpression cells showed no difference in contractility vs control. Matrix metalloproteinase activity 2 activity appeared slightly decreased in human uterine smooth muscle cell-lentiviral-mediated knock down cells and increased in human uterine smooth muscle cell-retroviral-mediated overexpression cells vs control.

CONCLUSION: ANTXR2 is expressed by human uterine smooth muscle cell and appears important for normal human uterine smooth muscle cell viability, migration and contractility. Further studies are needed to delineate if ANTXR2 is important for normal and abnormal labor patterns.

Key words: anthrax toxin receptor 2, apoptosis, contractility, extracellular matrix, migration, uterine smooth muscle cells

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The anthrax roxin receptor (ANTXR) proteins, ANTXR1 and ANTXR2 (also known as tumor endothelial marker 8 [TEM8] and capillary morphogenesis gene 2 [CMG2], respectively), are most commonly recognized for their ability to bind anthrax toxin. These transmembrane receptors are also known to be critical for angiogenic processes such as endothelial cell proliferation, migration and network formation in part by binding to and interacting with extracellular matrix (ECM) proteins.[1-6]

Recently, we demonstrated that *ANTXR2* is essential for parturition in mice. *Antxr2* knockout (*Antxr2*$^{-/-}$) mice are fertile and carry pregnancies to term, but then fail to deliver their fetuses that results in maternal death or fetal reabsorption.[2,3] Gross analysis of the reproductive tracts revealed enlarged and fibrotic uterine and cervical tissue. Histological analysis of uterine and cervical tissue from the *Antxr2*$^{-/-}$ mice demonstrated excessive accumulation of ECM proteins, particularly, collagen. It also showed striking disruption of smooth muscle cell layers in the myometrium. It was unclear if the disruption of the smooth muscle cell layer was due to the direct effect of absent ANTXR2 expression on the myometrial cells or whether it was secondary to the overaccumulation of collagen/ECM proteins. The loss of smooth muscle cells was so severe in aged mice that the myometrial layers could not be delineated. We hypothesized that the parturition defect in *Antxr2*$^{-/-}$ mice was due in part to decreased smooth muscle cell content and therefore possible dystocia because of inadequate contractile force.

To investigate the mechanism responsible for the aberrant ECM remodeling

From the Division of Maternal-Fetal Medicine (Dr Vink), Department of Obstetrics and Gynecology (Drs Kitajewski and Reeves); Department of Pharmacology (Ms Charles-Horvath); and Department of Pathology and Herbert Irving Comprehensive Cancer Center (Dr Kitajewski), Columbia University Medical Center, New York, NY.

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in *Antxr2*$^{-/-}$ mouse uterine tissue, we evaluated the activity of matrix metalloproteinases (MMPs), enzymes involved in ECM remodeling. MMP2 activity in uterine lysates and conditioned medium from *Antxr2*$^{-/-}$ mouse embryonic fibroblasts was reduced in the *Antxr2*$^{-/-}$ samples. ANTXR2 was found to form a complex with and enhance the activity of MT1-MMP (a membrane bound MMP responsible for MMP2 activation).[3] These findings suggested that disruption of the *Antxr2* gene in mice resulted in decreased MT1-MMP activity and thus decreased MMP2 activation. The lack of MMP2 activity in *Antxr2*$^{-/-}$ mouse likely led to decreased ECM remodeling, eventually caused fibrosis in the reproductive tracts of *Antxr2*$^{-/-}$ mice. Despite these findings, the role of ANTXR2 in normal murine uterine smooth muscle cell function and viability remained unclear. In particular, it was unclear if the uterine smooth muscle cells in the myometrium of the *Antxr2*$^{-/-}$ mouse were being lost because of increased rates of apoptosis and if loss of ANTXR2 disrupted the intrinsic function of smooth muscle cells that includes migration, contraction, and ECM remodeling.

To date, no studies have evaluated ANTXR2 expression on human uterine smooth muscle cells (HUMSC) nor have studies addressed the role of ANTXR2 during normal HUSMC function. Studies have documented that individuals harboring a mutation in the *ANTXR2* gene have a rare, autosomal recessive disease called systemic hyalinosis, which is further characterized as 2 syndromes, infantile systemic hyalinosis (ISH) and juvenile hyaline fibromatosis (JHF).[7-9] Patients with ISH and JHF have abnormal collagen and glycosaminoglycan deposition in various tissues resulting in gingival hypertrophy, progressive joint contractures, osteolysis, osteoporosis, recurrent subcutaneous fibromas, and hyaline depositions.[10] However, studies are lacking on the role of ANTXR2 in human reproductive tissues and during parturition. We sought to evaluate ANTXR2 expression in HUSMC and delineate the role of ANTXR2 in HUSMC function and ECM homeostasis.

Materials and Methods

DNA constructs

For *ANTXR2* knockdown, lentiviral pLKO.1 vectors encoding scrambled control shRNA and shRNA targeting *ANTXR2* transcripts were purchased from Sigma Aldrich (St. Louis, MO). The pLKO.1 vector carries puromycin resistance allowing for selection of shRNA expressing cells. The *ANTXR2* siRNA target sense sequence was 5′-CCTGCAC CTATCCTGAATAAA-3′. For ANTXR2 overexpression, we used a retroviral vector engineered to express recombinant receptor-EGFP fusion protein as previously described.[2,11] HUSMCs (Promocell, Heidelberg, Germany) were cultured in smooth muscle cell growth medium 2 (Promocell) according to manufacturer's instructions. Cells were cultured under standard conditions in a humidified incubator at 37°C, 5% CO_2.

ANTXR2/CMG2 gene silencing and overexpression

Lentivirus was produced in transfected 293T cells and retrovirus was produced in GP2-293 cells and used to transduce HUSMCs as previously described.[2,12] Briefly, lentiviral-containing supernatants and retroviral-containing supernatants were collected 24 hours after transfection, filtered, diluted 1:1 with HUSMC growth medium 2 and added to low passage HUSMC. This process was repeated at 24 hours, 36 hours, and 48 hours. At 48 hours after the initial infection, lentiviral cell lines were selected for 5 days with puromycin (Invitrogen, Carlsbad, CA) at 1 μg/mL and then maintained at 0.5 μg/mL. Retroviral cell lines were selected for 5 days with 300 μg/mL hygromycinB (Invitrogen). *ANTXR2* gene silencing and overexpression were confirmed via flow cytometry as described previously described.[2]

Evaluation of cell viability—apoptosis assay

HUSMC cell lines were seeded at equivalent numbers on type I collagen-coated coverslips. Samples were run in duplicate. After allowing the cells to adhere to the plates, the medium was changed to basal medium. After 48 hours, TUNEL (TdT mediated dUTP Nick End Labeling) staining of cells was carried out using the ApopTag Red In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. DAPI (Vector Laboratories, Burlingame, CA) was used to counterstain nuclei. Cells were visualized on a Nikon ECLIPSE E 800 microscope (Nikon Inc, Melville, NY). Five pictures were taken at 20× magnification (top, middle, bottom, left, and right) for each coverslip and the number of TUNEL-positive cells was counted per high power field. An average of the number of TUNEL positive cells in each of the 5 pictures per coverslip was obtained for each sample and used for analysis. Apoptotic cells were also detected using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Quantification of apoptotic cells was performed using flow cytometry. Samples were run in duplicate.

Evaluation of HUSMC migration—Boyden chamber assay

We performed cell migration assays using a specialized Boyden migration chamber that included a 24-well tissue culture plate with cell culture inserts (BD Falcon, San Jose, CA). The inserts contained an 8-μm pore size polycarbonate membrane that was precoated with matrigel. Briefly, we seeded equal numbers of the HUSMC cell lines on the membrane of the culture inserts. Basal HUSMC medium supplemented with 10 ng/mL of epidermal growth factor (EGF) was added on top of the cells in each insert. As a control in this migration test, the same medium (basal HUSMC medium supplemented with 10 ng/mL of EGF) was also added to the lower compartment of each of the cell lines. To test the migration of HUMSC-R2KD and HUSMC-R2OE cell lines, basal HUSMC medium supplemented with 10 ng/mL of EGF and 20 ng/mL of the chemoattractant, platelet-derived growth factor subunit B (PDGF-B), was added to the lower compartments in another set of each of the cell lines. After 48 hours of incubation at 37°C, the migrated cells were fixed in 4% PFA. The HUSMC from the upper surface of

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