Differential expression of G-protein-coupled estrogen receptor-30 in human myometrial and uterine leiomyoma smooth muscle

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Objective: To determine differential expression of G-protein-coupled receptor 30 (GPR30) in uterine leiomyoma and its matched myometrium.
Design: GPR30 expression examined in both tissues and cultured cells.
Setting: Research laboratories.
Patient(s): Women 35 to 50 years old with uterine leiomyomas.
Intervention(s): Hysterectomy.
Main Outcome Measure(s): GPR30 expression profile.
Result(s): Using Western blot and real-time quantitative polymerase chain reaction analyses, we found that GPR30 was highly expressed in uterine leiomyomas compared with their matched myometrium. In only three out of nine patients examined was GPR30 protein detectable by Western blot analysis in myometrial tissues, but at statistically significantly lower levels than in their leiomyomas. Confocal microscopy revealed the nuclear localization of GPR30 in leiomyoma tissues and cultured leiomyoma smooth muscle cells (SMCs). Treatment with 0.1 μM 17β-estradiol increased mRNA expression of GPR30 in leiomyoma SMCs but decreased expression in myometrial SMCs. Treatment with G-1, a GPR30 agonist, stimulated phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) in both SMC types. PD98059, the MEK inhibitor, completely inhibited G-1-induced phosphorylation of p44/42 in myometrium SMCs, but not in SMCs from leiomyoma.
Conclusion(s): GPR30 is abundantly expressed in uterine leiomyomas, likely resulting from estrogen stimulation. (Fertil Steril® 2013;99:256–63. ©2013 by American Society for Reproductive Medicine.)
Key Words: GPR30, smooth muscle cells, uterine leiomyoma

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Uterine leiomyoma, characterized by proliferation of smooth muscle cells (SMCs), is the most common benign tumor in women. Because leiomyomas occur in women at reproductive age and regress after menopause, estrogen is believed to play a critical role in the pathogenesis of the tumor. Estrogen receptor-α (ER-α) is abundantly expressed in uterine

leiomyomas compared with myometrium (1), and ER-β is expressed in both myometrium and leiomyoma tissues (2). However, the expression of the novel ER G-protein-coupled estrogen receptor 1 (GPER1) or G-protein-coupled receptor 30 (GPR30) (3) has not been investigated in human uterine leiomyomas and their matched myometrial tissues.

Activation of GPR30 promotes endometrial cell proliferation (4). In endometrial cancer cells, GPR30 mediates the proliferative effects induced by 17β-estradiol (E_2) (5). GPR30 also mediates E_2-induced proliferation of several other cells, such as the mouse spermatogonial GC-1 cell line (6). The proliferative effect of GPR30 involves the mitogen-activated protein kinase (MAPK) pathway (7). Additionally, GPR30 signaling induces proliferation and migration of breast cancer cells through connective tissue growth factor (8).

In the reproductive tissues, expression of GPR30 was detected in uterus epithelial cells and mediated a cell proliferative response (9). GPR30 mRNA was also detected in mouse uterine tissue (10). More recently, the presence of GPR30 mRNA and protein was reported in human myometrium obtained at term cesarean deliveries before or after the onset of labor (11). The present study was designed to determine the expression pattern of GPR30 in human myometrium and uterine leiomyoma smooth muscle tissues.

MATERIALS AND METHODS

Tissue samples were obtained from patients as detailed in the next section. G-1 was purchased from Cayman Chemical. Antibody for ER-α was purchased from Santa Cruz Biotechnology. GPR30 antibody and peptide were obtained from Novus Biologicals. Antibodies for p44/42 MAPK and phospho-p44/42 MAPK were purchased from Cell Signaling Technology. Alexa Fluor 568-conjugated secondary antibody was obtained from Invitrogen. The α-tubulin antibody and all other reagents were obtained from Sigma-Aldrich.

Tissue Sample

Uterine leiomyoma and adjacent normal myometrial tissues were obtained from patients (35 to 50 years old) undergoing hysterectomies at the Tianjin Central Hospital for Obstetrics and Gynecology, Tianjin, People's Republic of China, with approval from the hospital ethics board and patients' consent, as previously described elsewhere (12, 13). The endometrium of those patients was in the proliferative phase, which was diagnosed through the dilation and curettage (D&C) procedure and by the pathologists in the Pathology Department of Tianjin Central Hospital for Obstetrics and Gynecology. The patients did not receive any hormone therapy or other medications for at least 3 months before surgery. After surgery, tissues were collected and stored in liquid nitrogen for further extraction of protein and RNA for Western blot and real-time quantitative polymerase chain reaction (RT-qPCR) analyses, respectively. Another fraction of fresh tissues were used for primary tissue culture with enzymatic digestion, as previously described elsewhere (13).

Western Blot Analysis

Cells or homogenized tissues were lysed with ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche Pharmaceuticals). Protein concentration was determined using the bicinchoninic acid assay (BCA) with protein assay reagent (Pierce) according to the manufacturer's instructions. Equal amounts of protein were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to nitrocellulose membrane (Millipore), followed by blocking with 5% nonfat milk, and incubation with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies. The blot signals were visualized using West Pico Chemiluminescent Substrate Kit (Pierce), imaged by Molecular Image Chemidoc XRS System (Bio-Rad Laboratories) and analyzed using Quantity One software (Bio-Rad Laboratories).

In the experiment using a GPR30 blocking peptide, primary antibodies for GPR30 were incubated with a fivefold excess of the blocking peptide in a small volume of Tris buffer saline Tween-20 (TBST) for up to 2 hours at room temperature. After blocking with 5% nonfat milk, two polyvinylidene difluoride membranes with transferred proteins were incubated with untreated GPR30 antibodies and blocking peptide-incubated antibodies separately.

RT-qPCR

Total RNA from tissues or cultured cells was extracted using Trizol Reagent (Takara Biotechnology). Reverse-transcription was performed using 1 μg of total RNA in a 25 μL reaction volume at 42°C for 60 minutes using a polymerase chain reaction machine (Hangzhou Jingle Scientific Instruments). All real-time PCR experiments were performed on a real-time PCR machine (Bio-Rad Laboratories) with QuantiTect SYBR Green PCR kit and specific primers purchased from Takara Biotechnology and Invitrogen. Quantification of gene expression was assessed with the comparative cycle threshold (Ct) method. The relative amounts of mRNA for the target genes were determined by subtracting the Ct values for these genes from the Ct value for the housekeeping gene β-actin (ΔCt). Data are depicted as $2^{-\Delta Ct}$. qPCR was performed as previously described elsewhere (12).

Primary Cell Culture

Human leiomyoma (HL) and matched myometrium (HM) SMCs were isolated from uterine leiomyoma and adjacent normal myometrial tissues, respectively. Leiomyoma and myometrial tissues were cut into small pieces ($\sim$1 mm^3), which were then immersed in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS), 0.2% collagenase, and 50 mg/mL of trypsin inhibitor for 2 to 6 hours at 37°C with continuous agitation until cell suspension became evident. Cells were cultured in phenol red-free DMEM with 10% heat-inactivated FBS. Cells from passages three to five