Inhibition of transcription, expression, and secretion of the vascular epithelial growth factor in human epithelial endometriotic cells by romidepsin

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Objective: To investigate whether the histone deacetylase (HDAC) inhibitor romidepsin down-regulates *VEGF* (vascular endothelial growth factor) gene expression and abrogates VEGF protein secretion in human epithelial endometriotic cells.
Design: In vitro study with human immortalized epithelial endometriotic cells.
Setting: University hospital.
Patient(s): Not applicable.
Intervention(s): None.
Main Outcome Measure(s): Real-time reverse-transcriptase polymerase chain reaction to evaluate *VEGF* gene expression, immunoblot analysis to evaluate protein expression, and enzyme-linked immunosorbent assay to evaluate VEGF protein secretion into the culture medium.
Result(s): Treatment of 11z human endometriotic cells with romidepsin statistically significantly inhibited *VEGF* gene transcription and down-regulated VEGF protein expression. Moreover, romidepsin abrogated the secretion of VEGF protein into the culture medium. Romidepsin also reduced the expression of hypoxia-inducible factor-1α (HIF-1α), which is implicated in the transcription of the *VEGF* gene, in cobalt chloride-pretreated (to mimic hypoxic conditions) 11z cultures.
Conclusion(s): Romidepsin targets VEGF at the transcriptional level, which subsequently leads to the reduction of secreted VEGF (the "active" form of VEGF). Therefore, romidepsin may be a potential therapeutic candidate against angiogenesis in endometriosis. (Fertil Steril® 2011;95:1579–83. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, HIF-1α, human epithelial endometriotic cells, romidepsin, VEGF expression and secretion

Endometriosis is a common disease affecting 5% to 15% of reproductive-aged women and is defined as the ectopic growth of endometrial tissue. Endometriosis is often associated with dysmenorrhoea, dyspareunia, chronic pelvic pain, and infertility in affected women (1–3). The detailed pathomechanisms are unclear, but retrograde menstruation seems to be a basic requirement (4), and epigenetic alterations may have a role in endometriosis (5).

Like any autotransplanted tissue, the menstrual endometrial tissue shed into the intraabdominal cavity needs to establish an adequate blood supply by formatting new blood vessels (angiogenesis). Angiogenesis is therefore critical for the survival of the endometrial tissue outside the uterine cavity.

Vascular endothelial growth factor (VEGF), one of the most important proangiogenic factors, activates endothelial cells and increases vascular permeability (6, 7). Vascular endothelial growth factor is strongly expressed in endometriotic lesions (8, 9) and is predominantly localized in the epithelium of the endometriotic implants (10). Indeed, compared with controls without endometriosis or eutopic endometrium, significantly high levels of VEGF molecules have been found in the peritoneal fluid of women with endometriosis and in endometriotic lesions themselves (8, 11).

Because of the proangiogenic effect of VEGF, together with the fact that VEGF levels and capillary density are increased in endometriosis, we hypothesize that agents inhibiting angiogenesis and VEGF may be effective in treating endometriosis (12). The currently available inhibitors of angiogenesis either block proangiogenic cytokines or their interaction with their receptors or have a direct inhibitory effect on endothelial cells (13). Some of these antiangiogenic drugs may have a teratogenic potential and have to be handled with care, as endometriosis is a disease affecting women of reproductive age. Another drawback common to all currently available antiangiogenic drugs is that they act against already existing and secreted VEGF molecules.

Our study employs a different approach against VEGF by directly blocking the expression of the *VEGF* gene on the transcriptional level rather than inhibiting its biological function on the protein level (when VEGF is already present). To this aim, we determined the effects of romidepsin on the messenger RNA (mRNA) transcription, the protein expression, and the secretion of VEGF in human epithelial endometriotic cells.

Romidepsin (also referred to as FK-228, Depsipeptide) is a natural bicyclic tetrapeptide-type member of the family of histone

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deacetylase (HDAC) inhibitors, originally isolated from *Chromobacterium violaceum*. The HDAC inhibitors epigenetically modulate the expression of a variety of genes by altering the chromatin structure (14, 15). Romidepsin has recently been shown to inhibit proliferation and activate apoptosis in human epithelial endometriotic cells (16). Romidepsin is also being studied across a range of hematologic malignancies (17, 18) and against solid tumors (19), and its antiangiogenic effect in tumors is acknowledged (20, 21).

MATERIALS AND METHODS

Cell Culture and Drugs

The immortalized human epithelial endometriotic cell line 11z used was provided by Dr. Anna Starzinski-Powitz (Institute of Anthropology and Human Genetics, Johann-Wolfgang-Goethe University, Frankfurt, Germany). The 11z cell line has been generated from primary peritoneal human epithelial endometriotic cells through immortalization by in situ electroporation with SV-40 T-antigen (22). The 11z cells have been previously characterized (23) and were cultured as previously described elsewhere (16). Romidepsin was provided by Gloucester Pharmaceuticals (Cambridge, MA) and a stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at –20°C. Our previous study (16) showed that romidepsin was effective in the lower nanomolar concentration range by producing the accumulation of acetylated histones, up-regulation of p21, and proliferation inhibition in 11z cells. Cobalt chloride was purchased (Sigma Chemical, Buchs, Switzerland), and a stock solution in water was stored at –20°C.

Real-Time RT-PCR

Romidepsin-induced changes of *VEGF* expression on the mRNA-level were determined by real-time reverse-transcriptase polymerase chain reaction (RT PCR) analysis. Total RNA was isolated from 11z cultures (50,000 cells in 200 μL seeded in 96-well plates) left untreated (control) or treated for 24 hours with romidepsin (5 nM, 10 nM, and 50 nM) using the TurboCapture 96 mRNA Kit (Qiagen AG, Hombrechtikon, Switzerland) following the manufacturer's protocol. Real-time RT-PCR was carried out in an iCycler (Bio-Rad, Reinach, Switzerland) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's protocol. The PCR program consisted of denaturating at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. The samples were amplified for 36 cycles. The expression level of *VEGF* was normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the same reaction. The following forward (sense) and reverse (antisense) primers were used. *VEGF* (Biomol Research Laboratories, Plymouth Meeting, PA): 5′-ATC ACG AAG TGG TGA AGT TC-3′ and 5′-TGC TGT AGG AAG CTC ATC TC-3′; *GAPDH* (Microsynth, Anawa Trading SA, Zurich, Switzerland): 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′ and 5′-TCC TTG GAG GCC ATG TGG GCC AT-3′. The experiment was performed in duplicate.

Immunoblot Analysis

Immunoblot analysis was performed from cell lysates prepared from untreated (control) 11z cultures or 11z cultures treated with various concentrations of romidepsin for 24 hours or 48 hours as previously described elsewhere (16). Some cultures were pretreated with 150 μM cobalt chloride ($CoCl_2$) for 7 hours before the addition of romidepsin. The presence of $CoCl_2$ mimics the hypoxic condition, as it prevents oxygen binding to the HIF (hypoxia-inducible factor) prolyl hydroxylases, thus preventing HIF-1α hydroxylation and its subsequent degradation by the proteasome (24). The $CoCl_2$-mediated stabilization and accumulation of HIF-1α induces the transcription of HIF-targeted genes in spite of the presence of oxygen. The protein concentration of cell lysates was determined by the BCA Protein Assay Kit (23227; Pierce, Perbio Science, Lausanne, Switzerland).

We separated the 20-μg cell lysate protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by blotting onto a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Otelfingen, Switzerland) according to standard laboratory protocols. Proteins were detected by the specific primary antibodies and the respective secondary, horseradish peroxidase-conjugated anti-mouse (M15345; Transduction Laboratories, Lexington, KY) or horseradish peroxidase-conjugated anti-rabbit (7074; Cell Signaling, BioConcept, Allschwil, Switzerland) antibodies. The primary antibodies used were: VEGF (ab46154; Lucerna-Chem, Luzern, Switzerland), HIF-1α (ab51608; Lucerna-Chem), acetyl-H3 (9671; Cell Signaling). Mouse anti-α-actin (A5441, Sigma Chemical) was used as the sample loading control. Complexes were visualized by enhanced chemiluninescence (Amersham Biosciences) and autoradiography.

ELISA

The amount of VEGF secreted in the culture medium (supernatant) was determined by enzyme-linked immunosorbent assay (ELISA). Conditioned medium (serum-free) supernatants of untreated and romidepsin-treated (5 nM, 10 nM, 50 nM) 11z cells cultures (20,000 cells in 200 μL, 96-well plate) were collected 24 hours and 48 hours after the addition of romidepsin and stored at –20°C until further use. Equal volumes (50 μL) of the supernatants were subjected to ELISA (Human VEGF ELISA Kit, KHG0111, Invitrogen, Basel, Switzerland), which was performed according to the manufacturer's protocol (including standard curve and positive and negative assay controls). Optical density (OD, absorbance at 450 nm) was measured spectrophotometrically (SpectraFluor Plus Reader; Tecan AG, Männedorf, Switzerland). Background OD (empty well) was subtracted from all OD values. Data are presented as secreted VEGF relative to untreated control (percentage of control) calculated from the respective OD values.

Statistical Analysis

The romidepsin-induced reductions in VEGF mRNA transcription and in VEGF protein secretion were compared with the respective untreated controls. Mean ± standard deviation (SD) values were calculated (where appropriate). Statistical analysis was performed using the two-tailed Student's *t*-test. $P<.05$ was considered statistically significant.

RESULTS

Romidepsin Down-regulates *VEGF* Gene Expression

In tumor cells, HDAC inhibitor-induced *VEGF* gene down-regulation has been shown (24). We therefore determined the effects of romidepsin on *VEGF* gene transcription in 11z cells. A representative real-time RT-PCR data set is shown (Fig. 1). The results demonstrated that a 24-hour treatment of 11z cells with romidepsin (5 nM and 10 nM) produced a statistically significant reduction of the number of VEGF mRNA molecules and hence of *VEGF* gene transcription in a concentration-dependent manner. As compared with the untreated control, the values (mean ± SD) of the romidepsin-mediated reduction were 60 ± 5% (5 nM) and 87 ± 18% (10 nM).

Romidepsin Reduces VEGF Protein Expression and VEGF Secretion

Based on the observed romidepsin-induced down-regulation of *VEGF* gene transcription, we determined the effects of romidepsin on VEGF protein expression and secretion in 11z cells. The representative data set are shown. Immunoblot analysis demonstrated that romidepsin reduced VEGF protein expression in 11z cells after 24 hours. Moreover, an almost complete loss of VEGF protein was evident after 48 hours of treatment (Fig. 2A). Romidepsin treatment also resulted in the statistically significant reduction of the amount of VEGF secreted into the culture medium (see Fig. 2B) in a concentration- and time-dependent manner, as demonstrated by ELISA. As compared with the untreated control, the respective values (mean ± SD of triplicates) for the reduction were 25 ± 3% (5 nM, $P<.01$), 30 ± 7% (10 nM, $P<.01$), and 45 ± 4% (50 nM, $P<.01$) after 24 hours