Effects of danazol on endothelial cell function and angiogenesis

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Objective: To determine the effects of the androgen-like synthetic steroid, danazol, on endothelial cell function and angiogenesis.
Design: In vitro cell culture models were designed to investigate three critical steps involved in angiogenesis: endothelial cell proliferation, invasion, and tube formation.
Setting: Research laboratory at a level one trauma center.
Patient(s): Commercially available human umbilical vein endothelial cells (HUVEC) were purchased from a pooled patient source.
Intervention(s): The HUVEC cells were treated with danazol at concentrations ranging from 1 to 100 μM and evaluated using a series of angiogenesis assays.
Main Outcome Measure(s): Absolute cells numbers were quantified using colorimetric methods and fluorescent dyes for cells exposed to danazol in both proliferation assays and invasion chambers. AngiQuant vl.33 software was used to evaluate the formation of capillary-like structures on extracellular matrix gels in the presence of danazol.
Result(s): Endothelial cells scrutinized by our in vitro models exhibited decreased proliferation (up to 86%) and tube length (up to 115%) in the presence danazol. Cellular invasion through extracellular matrix, however, was not apparently affected by danazol under the conditions used.
Conclusion(s): Danazol interfered with two of the three steps of angiogenesis studied in vitro. This data may help elucidate the mode of action for danazol in vivo. (Fertil Steril® 2007;88(Suppl 2):1065–70. ©2007 by American Society for Reproductive Medicine.)

Key Words: Danazol, endothelial cell, HUVEC, angiogenesis, cell proliferation, tube formation, steroid receptors

It has long been accepted that steroid hormones, especially ovarian sex hormones, can affect endothelial cells and vascular function. Rapid angiogenesis or the formation of new blood vessels in the endometrium corresponds to the estrogen surge of the menstrual cycle (1). Clinical studies have also demonstrated that women are more prone to cardiovascular diseases following menopause and that estrogen restoration reduces these risks (1). Estrogen can promote angiogenesis in human umbilical vein endothelial cells (HUVEC) in vitro by initiating proliferation, migration, and differentiation (2). In animal models, rat vascular endothelial cells exhibit enhanced proliferation in the presence of steroids (3), and estrogen receptor knockout mice demonstrate impaired angiogenesis induced by basic fibroblast growth factor (bFGF) (4).

Danazol (17α-pregna-2,4-dien-20-yno[2,3-D]-isoxazol-17-ol), a synthetic analog of 17α-ethinyl testosterone, has been widely used in the clinical management of endometriosis (5). In vitro, danazol exhibits antiproliferative effects on endometrial cells when cocultured with peripheral blood monocytes, in the presence of estrogen, or when grown on extracellular matrix proteins (6–8). Binding studies using endometrial tissue have demonstrated that danazol binds with varying affinities to several steroid hormone receptors (SHR), including glucocorticoid, progesterone (PR), estrogen (ER), and androgen receptors (AR), and can displace steroids from chaperone proteins (9). Translocation of danazol/steroid receptor complexes can be varied. AR complexes move freely into the nucleus while ER and PR complexes remain in the cytosol (9). Functional ER and AR have been identified in endothelial cells (10), providing potential targets for danazol.

Angiogenesis has been implicated in the pathogenesis of endometriosis; notably, lesions are surrounded with blood vessels derived from the surrounding vascular system (11). We hypothesized that danazol functions at the endothelial cell level by interfering with the progression of angiogenesis. To explore the ability of danazol to affect endothelial cell function, three distinct stages of angiogenesis were investigated using in vitro models. Endothelial cell proliferation was studied, representing the initial build up of cell mass after initiation of angiogenesis. As angiogenesis progresses, endothelial cells must escape the extracellular matrix and migrate toward the angiogenic site following a chemotactic gradient.

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Finally, endothelial cells must differentiate and assemble to form tubelike structures in a three dimensional space. For the first time, we describe the ability of danazol to directly affect endothelial cell function.

MATERIALS AND METHODS

Cell Culture

Primary HUVEC and endothelial cell medium-2 (EGM-2) growth medium were obtained from Cambrex (Walkersville, MD). The cells were cultured in proprietary medium supplemented with 2% fetal calf serum (FCS), hydrocortisone, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), ascorbic acid, epidermal growth factor (EGF), heparin, and GA-1000 in tissue culture flasks at 37°C and 5% CO_2 unless otherwise stated. Subculturing was performed using trypsin when 60% to 80% confluence was obtained, as specified by the supplier. All other reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. It is important to note that the FCS used for these experiments was not charcoal depleted of hormones.

Cell proliferation assay Cryopreserved ampules of passage 2 HUVEC cells were thawed and plated in 96-well tissue culture plates at 5,000 cells/cm^2. We prepared 50 mM stock solutions of danazol in ethanol before each experiment. To help keep danazol in solution, the FCS content of the medium was increased to 5% for these experiments. The cells were treated with complete EGM-2 medium containing final concentrations of danazol ranging from 0.1 to 100 μM in triplicates. We performed 24-, 48-, and 72-hour incubations, and cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). In short, medium was aspirated from each well, and the cells were washed with 200 μL of Hepes buffered saline (HBSS) from Cambrex warmed to 37°C. Then 100-μL diluted CellTiter solution (15 μL stock + 85 μl EGM-2 containing only 0.1% FCS) was added to each well and incubated for an additional 4 hours. Optical density (OD) was determined in a microplate reader set at 530 nm by subtracting the value of blank well containing CellTiter solution alone from all experimental wells. Data presented as OD ± standard deviation. The percentage decrease was determined as follows: ([Mean untreated OD − Mean experimental OD]/Mean untreated OD) × 100. The final concentration of ethanol in the wells was less then 0.2% and had no effect on cell proliferation or viability.

Invasion assay BioCoat Matrigel Invasion Chambers were purchased from BD Biosciences (San Jose, CA). Inserts were rehydrated at 37°C with 500 μL of HBSS for 2 hours before use in a humidified incubator. Trypsinized passage 8 HUVEC cells were washed twice and placed in warm EGM-2 containing only 0.1% FCS, and then 100,000 cells were added to the upper chamber of the invasion insert in a total volume of 250 μL. Danazol and control compounds were added to the upper reservoir to final concentrations of 10 μM and 100 μM. To initiate invasion, 750 μL EGM-2 supplemented with 5% FCS was added to the bottom chamber, and the plates were incubated for 24 hours. Noninvasive cells were removed from the upper chamber with moistened cotton swabs, and then the inserts were washed twice with HBSS. The inserts were then submerged in 10 μM calcein AM prepared in HBSS and incubated for 4 hours. Fluorescence was determined in a microplate reader at 485-nm excitation and 595-nm emission. For this experiment, LY294002 and the structurally similar but inactive compound LY303511 served as positive and negative controls, respectively.

Tube formation assay To investigate the formation of capillary-like structures by HUVEC cells, the Angiogenesis System: Endothelial Cell Tube Formation Assay (BD Biosciences) was used according to the manufacturer's protocol. In brief, 15,000 passage 8 HUVEC cells were seeded onto rehydrated matrigel plugs in 96-well tissue culture plates in the presence of EGM-2 containing 5% FCS and VEGF, EGF, and IGF-1 Cambrex growth-factor supplements. Danazol was added to final concentrations of 25 μM, 50 μM, 75 μM, or 100 μM as well as the control compounds LY294002 and 303511 at 50 μM. After 18 hours, the wells were photographed using a Hamamatsu Photonics Deutschland GmbH (Herrsching, Germany) camera mounted to an Olympus BX51WI (Olympus America Inc., Center Valley, PA). To determine tube formation length, the images captured using Wasabi v1.4 (Hamamatsu Photonics) were enhanced and filtered using MatLab R14 (The MathWorks, Natick, MA) and analyzed by AngiQuant v1.33 (Antti Niemistö, Institute of Signal Processing, Tampere University of Technology, Tampere, Finland). The percentage of inhibition was determined as follows: ([(Mean tube length with growth factors − Tube length untreated control) − (Mean tube length experimental − Tube length untreated control)]/(Mean tube length with growth factors − Tube length untreated control)) × 100.

Statistical Analysis

All data are presented as experiments performed in triplicate. Differences between subsets were analyzed using Student's *t*-test in Microsoft Excel as well as the nonparametric Mann-Whitney test using Analyse-it software (Leeds, England, United Kingdom). $P \leq .05$ was considered statistically significant.

RESULTS

Danazol Attenuates HUVEC Cell Proliferation in Both a Time- and Dose-dependent Manner

Culturing primary HUVEC endothelial cells in the presence of danazol decreased the OD observed after treatment with Promega CellTiter proliferation assay reagent in a time- and dose-dependent fashion (Fig. 1). The CellTiter assay is based on the reduction of the assay solution by dehydrogenase enzymes to a formazan dye that directly correlates to cell number. Wells were initially seeded with 5000 cells per well, which had proven in preliminary experiments to be on the low end of the detection limit for the assay. Danazol