Oxidative stress: a key regulator of leiomyoma cell survival

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Objective: To determine the effects of attenuating oxidative stress with the use of dichloroacetate (DCA) on the expression of key redox enzymes myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) as well as on apoptosis.
Design: Prospective experimental study.
Setting: University medical center.
Patient(s): Cells established from myometrium and uterine fibroid from the same patients.
Intervention(s): Cells were exposed to normal (20% O_2) or hypoxic (2% O_2) conditions for 24 hours with or without DCA (20 μg/mL), a metabolic modulator that shifts anaerobic to aerobic metabolism.
Main Outcome Measure(s): Nitrate/nitrite (iNOS activity indicator), iNOS, Bcl-2/Bax ratio, MPO, and caspase-3 activities and levels were determined by means of Greiss assay, real-time reverse-transcription polymerase chain reaction, and ELISA. Data were analyzed with the use of SPSS by means of one-way analysis of variance with Tukey post hoc analysis and independent *t* tests.
Result(s): MPO, iNOS, and nitrate/nitrite expression were higher in leiomyoma than in myometrial cells, and they were further enhanced by hypoxia in myometrial cells. Treatment with the use of DCA decreased MPO, iNOS, and nitrate/nitrite levels and negated the effect of hypoxia in both types of cells. Leiomyoma cells showed less apoptosis, as indicated by both caspase-3 activity and the Bcl-2/Bax ratio, than myometrial cells. Hypoxia further decreased apoptosis in myometrial cells with no further effect on leiomyoma cells. Treatment with DCA resulted in increased apoptosis in both types of cells, even in the presence of hypoxia.
Conclusion(s): Shifting anaerobic to aerobic metabolism with the use of DCA resulted in an increase in apoptosis in leiomyoma cells and protected myometrial cells from the acquisition of the leiomyoma-like phenotype. (Fertil Steril® 2017;■:■–■. ©2017 by American Society for Reproductive Medicine.)
Key Words: Dichloroacetate, leiomyoma, oxidative stress, apoptosis, myometrium

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Uterine leiomyomas are the most common benign tumor of the reproductive tract occurring in over half of American women of reproductive age (1). Fibroids are more prevalent in African-American women, in whom they generally tend to be larger and more numerous and produce more severe symptoms (1). Although often asymptomatic, leiomyomas have the potential to cause severe pain, menorrhagia, pelvic pressure, and obstructive urinary and bowel symptoms. In addition, fibroids are known to be associated with infertility, miscarriage, and increased rates of cesarean section (2). Myomectomy is standard therapy for fibroids, although some women eventually require uterine artery embolization or even hysterectomy (1). The molecular mechanisms underlying leiomyoma pathogenesis have yet to be established.

Fibroids possess abnormal vasculature that leads to a hypoxic microenvironment, which may contribute to altered levels of redox enzymes (3). Hypoxia and the resultant oxidative stress have been shown to be a major factor in common profibrotic gynecologic disorders such as fibroids, endometriosis, and postoperative adhesions (4–7). Indeed, it has been shown that uterine fibroids are characterized by an impaired antioxidant cellular enzymatic system, further suggesting a role for oxidative stress in the pathogenesis of fibroids (6). In addition, the role of prooxidant enzymes, such as myeloperoxidase (MPO) and inducible nitric oxide

Received November 29, 2016; revised April 10, 2017; accepted April 19, 2017.
N.M.F. has nothing to disclose. M.S.A. has nothing to disclose. I.M. has nothing to disclose. M.G.S. has nothing to disclose. A.A.-H. has nothing to disclose. M.P.D. reports grants and contract support from National Institutes of Health/National Institute of Child Health and Human Development, Abbvie, Bayer, and Patient Centered Outcomes Research Institute/Agency for Healthcare Research and Quality, and is a stockholder and on the Board of Directors of Advanced Reproductive Care. G.M.S. has nothing to disclose.
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Fertility and Sterility® Vol. ■, No. ■, ■ 2017 0015-0282/$36.00
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http://dx.doi.org/10.1016/j.fertnstert.2017.04.015

synthase (iNOS), in the regulation of apoptosis has been established (8–10). MPO, an abundant hemoprotein previously known to be present solely in cells of hematopoietic origin, including neutrophils, neutrophil precursors, and macrophages, plays a role in immune surveillance and the inflammatory response (11, 12). We were the first to report that MPO is expressed in epithelial ovarian cancer cells and tissues, a finding that has since been confirmed by others (12–14). iNOS is another key prooxidant enzyme that is responsible for nitric oxide (NO) synthesis in response to inflammation (12). These observations highlight a potential significant role for both iNOS and MPO in the pathogenesis of fibroids.

Hypoxia has been shown to be a key regulator of MPO and iNOS in several gynecologic disorders (10, 12, 15). Previous studies have shown that shifting anaerobic to aerobic metabolism with the use of dichloroacetate (DCA) ameliorated the effects of hypoxia and the resultant oxidative stress by inducing apoptosis in other gynecologic conditions (9, 10). DCA is a metabolic modulator that causes a subsequent decrease in oxidative stress through the stimulation of oxidative phosphorylation in the mitochondria rather than the use of glycolysis for generating energy (9, 10).

In the present study, we sought to determine the effect of attenuating oxidative stress, by means of DCA shifting anaerobic to aerobic metabolism, on the expression of key redox enzymes, MPO and iNOS, and on apoptosis in leiomyoma compared with normal myometrial cells.

MATERIALS AND METHODS

Cell Culture

Human uterine cells were a kind gift from Dr. Darlene Dixon, which were derived as described previously (6, 7). Briefly, these cells were isolated directly from myometrium or fibroid tissue at the time of hysterectomy. Once in culture, these myometrial (USMC) or fibroid (DD) cells dominate the culture environment; authenticity was confirmed by the expression of the appropriate smooth muscle cell markers, such as α-smooth muscle actin, vimentin, and F-actin, as previously reported (16, 17). Cells were immortalized through the induction of telomerase activity with the use of a retroviral vector containing human telomerase reverse transcriptase, which allows them to bypass their normal programmed senescence (18).

Human leiomyoma and a myometrial cell lines were cultured and maintained with the use of the SmGM-2 Bullet Kit (Lonza), which included smooth muscle cell basal medium containing 5% fetal bovine serum, 0.1% insulin, 0.2% human fibroblast growth factor β, 0.1% gentamycin–amphotericin 1000, and 0.1% human epidermal growth factor.

Treatment of Cells

Cells (2.5×10^6) were cultured under normal (95% air and 5% CO_2) or hypoxic (2% O_2) conditions with or without DCA (20 μg/mL) for 24 hours in a 37°C incubator. The dose of DCA was chosen based on previous studies (9, 10). For treatment with hypoxia, cultures were placed in an airtight modular incubator chamber (Billups-Rothenberg) with a positive infusion of 2% O_2 in a 5% CO_2-nitrogen gas balanced mixture. Hypoxic cultures were then placed in a standard humidified tissue incubator. All experiments were performed in triplicate.

RNA and Protein Extraction

RNA isolation. Total RNA was extracted from human myometrial or leiomyoma cells with the use of the RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer.

Reverse transcription. A 20-μL cDNA reaction volume containing 1.0 μg RNA was prepared with the use of the Superscript VILO Master Mix Kit (Life Technologies) according to the manufacturer's protocol. Measurement of the amount of RNA in each sample was performed with the use of a Nanodrop Spectrophotometer (Thermo Fisher Scientific).

Real-time reverse-transcription polymerase chain reaction primer design and control. Optimal oligonucleotide primer pairs for real-time reverse-transcription polymerase chain reaction (RT-PCR) amplification of reverse-transcribed cDNA were selected with the aid of the software program Beacon Designer (Premier Biosoft). Sequences of the oligonucleotides used for amplification of β-actin, Bax, Bcl-2, MPO, and iNOS mRNA are described in Supplemental Table 1 (available online at www.fertstert.org). Quantitative RT-PCR was performed with the use of the Express Sybr GreenER qPCR Supermix Kit (Life Technologies) and the Cepheid 1.2f detection system. Real-time RT-PCR was performed in a 25-μL total reaction volume including 12.5 μL Express Sybr GreenER qPCR Supermix, 1 μL cDNA template, and 0.2 μmol/L each of target-specific primers designed to amplify a part of each gene. Standards with known concentrations and lengths (base pairs [bp]) were designed specifically for β-actin (79 bp), Bax (85 bp), Bcl-2 (75 bp), MPO (106 bp), and iNOS (103 bp) with the use of the Beacon Designer software, allowing for construction of a standard curve using a tenfold dilution series. A specific standard for each gene allows for absolute quantification of the gene in number of copies, which can then be expressed per microgram of RNA.

Real-time RT-PCR reaction conditions are described in Supplemental Table 1 and as follows: An initial cycle was performed at 95°C for the time specified in Supplemental Table 1, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing per Supplemental Table 1, followed by a final cycle at 72°C for 30 seconds to allow completion of product synthesis. After real-time RT-PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. Samples were all normalized to β-actin. A control sample, containing all of the reaction components except for the template, was included in all experiments. All experiments were performed in triplicate.

Nitrate/Nitrite Colorimetric Assay

Total nitrate and nitrite concentration, as an indication of NO end-products, was determined by means of the Greiss method

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