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The adhesion phenotype manifests an altered metabolic profile favoring glycolysis

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Objective: To determine whether metabolic markers are differentially expressed in normal and adhesion fibroblasts with and without hypoxia exposure.
Design: Prospective experimental study.
Setting: University research laboratory.
Patient(s): Fibroblasts established from normal peritoneum and adhesion tissues from the same patients.
Intervention(s): In vitro experiments on normal peritoneal and adhesion fibroblasts under normal and hypoxic (2% O_2) conditions.
Main Outcome Measure(s): Expression of metabolic markers, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose transporter 1 (GLUT1), hypoxia inducible factor (HIF)-1α, hexokinase 2 (HK2), lactose dehydrogenase A (LDHA), and pyruvate dehydrogenase alpha 1 (PDHA1) were measured using real-time reverse transcription polymerase chain reaction; adenosine triphosphate (ATP), HIF-1α, and lactate levels were assessed with ELISAs.
Result(s): Baseline mRNA levels of GAPDH and HIF-1α were increased, while GLUT1 and PDHA1 were decreased in adhesion as compared with in normal peritoneal fibroblasts. There was no change in baseline levels of HK2 or LDHA between the cell lines. Hypoxia increased protein levels of HIF-1α and mRNA levels of GAPDH, GLUT1, and HK2 and decreased levels of PDHA1 in both cell lines. Hypoxia increased LDHA mRNA levels in normal peritoneal fibroblasts. Baseline levels of lactate and ATP were lower in adhesion as compared with in normal peritoneal fibroblasts. In response to hypoxia, there was an increase in lactate in both cell lines and a decrease in ATP in normal fibroblasts.
Conclusion(s): Adhesion fibroblasts manifested an altered metabolic profile, which favors the glycolytic pathway, and is further altered by hypoxia. Targeting these specific metabolic markers during surgery can be an important therapeutic intervention minimizing the development of postoperative adhesions. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Postoperative adhesions, metabolic markers, glycolysis, hypoxia, adhesion phenotype

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Postoperative adhesions represent abnormal fibrous connection between different anatomic peritoneal surfaces and are frequently associated with potentially devastating complications such as infertility, ectopic pregnancy, bowel obstruction, chronic abdominal and pelvic pain, and difficult reoperations (1–6). These complications may be encountered in either the immediate postoperative period or as late as decades postsurgically (7–10). Even with the application of microsurgical and laparoscopic techniques, both of which are associated with reduction in surgical trauma, postoperative pelvic adhesions still form in as many as 95% of patients within the first week of major gynecologic surgery (7). While laparoscopic lysis of adhesions is considered an option for treatment of adhesion-related complications such as chronic pelvic pain, adhesions have been shown to reform at one or more sites in almost all patients who undergo adhesiolysis (11–13). In addition, between 12% and 51% of patients also develop de

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novo adhesions after laparotomy or operative laparoscopy (7, 12, 14).

It has been hypothesized that tissue hypoxia, as a result of vascular injury and loss during surgery, plays an important role in the development of the "adhesion phenotype" (15, 16). The adhesion phenotype is characterized by a significant increase in the basal levels of extracellular matrix proteins (such as type I and III collagens and fibronectin), growth factors (such as transforming growth factor [TGF]-β1 and tumor necrosis factor-α), and angiogenic factors (such as vascular endothelial growth factor [VEGF]) (16). The mechanism by which hypoxia induces the development of the adhesion phenotype is believed to involve endogenous production of $O_2^{\bullet -}$. Indeed, scavenging $O_2^{\bullet -}$ by superoxide dismutase, a powerful antioxidant, restores the adhesion phenotype marker (TGF-β1 and type I collagen) levels in adhesion fibroblasts to levels observed in normal peritoneal fibroblasts (17). Furthermore, scavenging $O_2^{\bullet -}$ during hypoxia exposure protects against the development of the adhesion phenotype in normal peritoneal fibroblasts (17).

In response to hypoxia and the subsequent oxidative stress, however, there occurs a metabolic shift from aerobic to anaerobic metabolism through alteration of the activity of glycolytic enzymes (18, 19). Central to this shift, is hypoxia inducible factor (HIF)-1α-dependent transcriptional regulation of virtually all of the genes in the glycolytic pathway, as well as promotion of cell survival and consequently the development of tissue fibrosis (19, 20). Specifically, these genes include hexokinase 2 (HK2), which phosphorylates glucose to produce glucose 6-phosphate, thereby committing glucose to the glycolytic pathway, the glucose transporters GLUT1 and GLUT3, and genes involved in glycolysis (phosphoglucose isomerase [PGI], phosphofructokinase [PFK] 1, aldolase, triose phosphoisomerase [TPI], glyceraldehyde 3-phosphate dehydrogenase [GAPDH], phosphoglycerate kinase [PGK], phosphoglucomutase [PGM], enolase, pyruvate kinase [PK], and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [PFKFB1–4]), as well as genes involved in decreasing mitochondrial function (pyruvate dehydrogenase kinase [PDK] 1 and myc-associated factor X [MAX] interacting protein [MAX-1]) (19, 21).

Hypoxia-inducible factors are O_2-sensitive transcription factors that induce the expression of pyruvate dehydrogenase kinase (PDK), an enzyme that regulates the entry of pyruvate into the mitochondria (22–25). In the presence of activated PDK, pyruvate dehydrogenase E1-alpha (PDHA1), the enzyme responsible for the conversion of pyruvate into acetyl-CoA, is inhibited, thereby limiting the entry of pyruvate into the mitochondria, where glucose oxidation can occur (25). Pyruvate is alternatively converted to lactate, resulting in an 18-fold reduction in net adenosine triphosphate (ATP) production (23, 25). Hypoxia also stimulates the activity of lactate dehydrogenase A (LDHA), an enzyme that converts pyruvate into lactate. Thus, hypoxia-induced oxidative stress hinders conversion of pyruvate into acetyl-CoA, increases lactate production, and possibly plays a role in the development of the adhesion phenotype (25).

In this study we sought to demonstrate that the adhesion phenotype manifests an altered metabolic profile, which is further enhanced by hypoxia. This may provide an insight into the metabolic mechanisms of adhesion development.

MATERIALS AND METHODS

Source and Culture of Human Fibroblasts

We have previously described the method used for collection and isolation of fibroblasts (26). At entry into the abdominal cavity in women undergoing laparotomy surgery for pelvic pain, we excised adhesion tissue and normal parietal peritoneal tissue from the anterior abdominal wall lateral to the midline incision (27, 28). Normal peritoneum was at a minimum 7.6 cm from any adhesions. Subjects did not have an active pelvic or abdominal infection and were not pregnant. All patients gave informed written consent to tissue collection, which was conducted under a protocol approved by the Wayne State University Institutional Review Board.

Harvested tissue samples were immediately placed in standard medium (Dulbecco's modified Eagle medium [DMEM] containing 10% fetal bovine serum [FBS] and 2% penicillin and streptomycin). Tissues were cut into small pieces in a sterile culture dish and transferred into a fresh T-25 flask with 3 mL of dispase solution (2.4 U/mL; GIBCO BRL, Life Technologies). The flasks were incubated overnight at 37°C in an environ-shaker (LAB-LINE Instruments). Samples were then centrifuged for 5 minutes at 1,400 *g*, transferred into a fresh T-25 flask with prewarmed DMEM medium, and placed in 37°C incubator (95% air and 5% CO_2); outgrowth of fibroblasts generally took 2 weeks. Once confluence was reached, the cells were transferred to 100-mm^2 tissue culture dishes and cultured in standard media with 10% FBS. Thereafter, the confluent dishes were subcultured by trypsinization (1:3 split ratios). Studies were conducted using passage 3–5 cells to maintain comparability and to ensure the normal phenotype of cells was not compromised.

Hypoxic Treatment of Fibroblasts

All hypoxic experiments were performed in an airtight modular incubator chamber (Billups-Rothenberg), which was deoxygenated by positive infusion of 2% O_2 in a CO_2-nitrogen gas mixture for 5 minutes. Cultures were then placed in a standard humidified tissue incubator. There were no statistically significant differences in viability by crystal violet or trypan blue exclusion (data not shown). Parallel cultures were placed in normal conditions (20% O_2) for all time points. Cells were harvested after 24 hours. All experiments were performed in triplicate.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA isolation. Total RNA was extracted from human normal peritoneal and adhesion fibroblasts using the RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer.