Gynecologic Oncology 133 (2014) 575–583

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Hypoxia promotes vasculogenic mimicry formation by inducing epithelial–mesenchymal transition in ovarian carcinoma

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HIGHLIGHTS

- Hypoxia is critical to cell plasticity and functionally important for the formation of vasculogenic mimicry.
- Cellular plasticity of ovarian cancer cells was primarily reflected in morphological changes and their pluripotential ability.
- Exploring the role of hypoxia in vasculogenic mimicry may identify new therapeutic approaches.

ARTICLE INFO

Article history:
Received 4 November 2013
Accepted 23 February 2014
Available online 28 February 2014

Keywords:
Ovarian cancer
Vasculogenic mimicry
HIF-1α
Hypoxia
Epithelial–mesenchymal transition

ABSTRACT

Objectives. The functions of hypoxia and subsequent hypoxia-inducible factor-1α (HIF-1α) activation in vasculogenic mimicry (VM) are currently unclear. This study aimed to investigate the effects of hypoxia on VM formation in ovarian cancer, and explore the possible mechanism involved.

Methods. The expression levels of HIF-1α, E-cadherin, vimentin, Twist1, Slug, and VE-cadherin proteins were analyzed by immunohistochemistry in 71 specimens of epithelial ovarian cancer. The results were correlated with VM and survival analysis. We used a well-established in vitro model of a three-dimensional culture to compare VM formation under hypoxia and normoxia in ovarian cancer cell lines SKOV3 and OVCAR3. To explore the potential mechanism, we examined the effects of hypoxia on the mRNA and protein expression levels of both E-cadherin and vimentin.

Results. HIF-1α expression was correlated with loss of E-cadherin expression and up-regulated vimentin expression in 11 of the 18 VM-positive patients. Ovarian cancer with evidence of VM was significantly more likely to have high Twist1, Slug, and VE-cadherin expression levels. VM was observed in vitro under hypoxia. The ovarian cancer cells presented morphological epithelial–mesenchymal transition (EMT)-like changes (more fibroblastoid morphology and loss of cellular cohesiveness) under hypoxic conditions. The mRNA and protein levels demonstrated the induction of EMT after hypoxia. Clinicopathological analysis revealed that both VM and HIF-1α expression levels presented shorter survival durations.

Conclusions. Hypoxia contributed to VM formation by inducing EMT. These results may offer new insights for consideration in ovarian cancer treatment strategies.

Introduction

Ovarian cancer has the highest mortality rate among all gynecological malignancies, and most patients are diagnosed with advanced cancer [1]. Metastatic capacity and growth of malignant tumors are highly dependent on the ability of the tumor to develop new blood vessels. Tumor cell vasculogenic mimicry (VM) describes the ability of aggressive cancer cells to form de novo vasculogenic-like networks in vitro in the absence of endothelial cells or fibroblasts, concomitant with their expression of vascular cell-associated molecules. VM has been shown to occur in various aggressive cancers, including breast [2], prostate [3], melanoma [4], and ovarian cancers. Sood et al. [5] demonstrated that approximately 30% of human ovarian cancers exhibit some degree of tumor cell-lined channels, which is associated with advanced stage, high tumor grade, development of distant metastasis, and poor overall survival. Parvin [6] suggested that the tumor cell-lined channels must also be considered when designing novel antiangiogenic strategies.

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http://dx.doi.org/10.1016/j.ygyno.2014.02.034
0090-8258/

VM formation involving tumor cells mimics endothelial cells consisting of a type of mesenchymal cell, similar to EMT [7]. EMT, a process by which epithelial cells lose their polarity and are converted into a mesenchymal phenotype, is a critical event in inducing morphogenetic changes during embryonic development, organ fibrosis, and tumor metastasis [8–11]. Our recent studies implied that Twist1 and EMT affect VM formation in human hepatocellular carcinoma cells (HCCs) in vivo and in vitro [12].

The hypoxic microenvironment of tumors affects the metabolism, angiogenesis, and survival of cells orchestrated by hypoxia-inducible factor-1α (HIF-1α), depending on tissue specificity. Hypoxia contributes to the progression of various cancers by activating adaptive transcriptional programs that promote cell survival, motility, and tumor angiogenesis [13]. Hypoxia provides tumor cells with greater resistance to anticancer therapies, such as radiation. HIF-1 directly or indirectly regulates the expression of EMT regulators, such as Snail, Zeb1, and SIP1 [14,15]. Given that HIF-1α is a crucial inducer that contributes to tumor EMT, the function of HIF-1α in VM formation in ovarian cancers should be examined. Although the importance of both hypoxia and subsequent HIF-1α activation in tumor angiogenesis is known, their functions in regulating VM are unclear.

In this study, we addressed the functional significance of hypoxia with regard to both increased migration and increased invasion, leading to VM formation in vitro by inducing EMT. We also evaluated the clinical relevance of hypoxia in human ovarian carcinomas with VM. The data may offer alternative targets for therapeutic intervention.

Materials and methods

Patient samples

71 consecutive cases of epithelial ovarian cancer were retrieved from the Tumor Tissue Bank of Tianjin Cancer Hospital. The diagnoses of these ovarian cancer samples were verified by pathologists. All patients underwent laparotomy at Tianjin Cancer Hospital from 1991 to 1999. None had received preoperative chemotherapy or radiotherapy. The clinicopathologic features of the 71 patients are summarized in Supplementary Table 1. We collected paraffin-embedded tumor tissue samples from patients who had not undergone therapy prior to tumor surgical operation. All patients were staged according to the International Federation of Gynecology and Obstetrics surgical staging system. The use of these tissue samples for this study was approved by the institutional research committee.

Immunohistochemistry

Sections of 4 μm in thickness were mounted on slides coated with poly-L-lysine. The slides were deparaffinized in xylene. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 50% methanol for 10 min at room temperature. The sections were rehydrated in alcohol, washed with PBS, and pretreated with citrate buffer (0.01 M citric acid, pH 6.0) for 20 min at 95 °C in a microwave oven. After blocking nonspecific binding sites via exposure to 10% normal goat serum in PBS for 20 min at 37 °C, the sections were incubated overnight at 4 °C with a series of antibodies (Supplementary Table 2). The staining systems used in this study were PicTure PV6000 (Zhongshan Chemical Co., Beijing, China) and Elivision Plus (Zhongshan Chemical Co., Beijing, China). PBS was used instead of the primary antibodies for the negative control. The results were semiquantified according to the method described by Sood et al. [16]. In brief, the percentage of positive cells was rated as: 0 points = 0% to 5%; 2 points = 6% to 50%; and 3 points >50%. Staining intensity was rated in the following manner: 1 point = weak intensity; 2 points = moderate intensity; and 3 points = strong intensity. Points for the expression and percentage of positive cells were added, and an overall score (OS, 0 to 3) was assigned. Tumors were categorized into the following four groups: negative (OS, 0) <5% of the cells stained, regardless of intensity; weak expression (OS, 1) = 1 to 2 points; moderate expression (OS, 2) = 3 to 4 points; and strong expression (OS, 3) = 5 to 6 points.

CD31/PAS double staining

CD31 immunohistochemical staining was applied on the sections before PAS staining. The slides were rinsed with distilled water after diaminobenzidine was used as the chromogen, then treated with 0.5% periodic acid solution for 10 min, and rinsed with distilled water for 2–3-min. In a dark chamber, the slides were treated with Schiff solution for 15–30 min. After distilled water rinsing, sections were counterstained with hematoxylin. Normal gastric mucosa was chosen as a positive control.

Cell culture

The ovarian cell lines used in this study were SKOV3 and OVCAR3 (American Type Culture Collection, Rockville, MD, USA). The derivation and sources of these cell lines were previously reported [2]. These cells were maintained and propagated in vitro by serial passage in RPMI 1640, and supplemented with 10% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Invitrogen). All experiments were performed with 70% to 80% confluent cultures. The day after plating cells, the medium was changed to RPMI-1640 without serum for 24 h. The SKOV3 and OVCAR3 cells were incubated for 48 h in either normoxic or hypoxic conditions in the absence/presence of filtered cobalt chloride (Sigma, final concentration = 150 μM). $CoCl_2$ is a hypoxia-mimicking agent that is widely used in research on cell proliferation, cell differentiation, and various hypoxic responses [17].

Migration and invasion assay

Cell migration assay was performed using Transwell cell culture inserts with 8 μm porosity polyethylene terephthalate filters (Corning). In brief, confluent tumor cells were trypsinized, plated onto the upper Matrigel-coated insert, and allowed to attach to the membrane for 1 h. Filtered $CoCl_2$ was then added to the upper insert, and free-$CoCl_2$ was used as controls. The cells were allowed to migrate for 24 h, after which the upper surface of the membrane was wiped to remove non-migratory cells. The migratory cells were fixed for 15 min with methanol, stained with DAPI (Sigma) for 30 min, photographed using a fluorescent microscope (Nikon, Japan), and counted to compare the differences in cell migration ability between normoxia and hypoxia.

For invasion assay, Matrigel was added to the insert for measuring the ability of cells to invade through the extracellular matrix (ECM). Cells were allowed to invade for 24 h. The cells that invaded through Matrigel and adhered to the bottom of the membrane were fixed, stained with crystal violet solution, cut out, mounted, photographed, and counted. Each experiment was performed in triplicate wells and repeated thrice. The mean values are presented as mean ± SE.

Three-dimensional (3D) culture and in vitro network formation

Matrigel (50 μL, Collaborative Biomedical) was dropped onto glass coverslips and allowed to polymerize for 1 h at 37 °C. The tumor cells were then seeded on top of the gels at high density, and allowed to incubate. The cultures were maintained in RPMI-1640 supplemented with 10% FBS and 0.1% gentamicin sulfate. For in-gel methods, the tumor cells were mixture-seeded with Matrigel to allow them to polymerize. Filtered $CoCl_2$ was added to the medium in the hypoxic group 2 h after polymerization.

The tumor cells were used to evaluate the in-gel and on-gel methods for the formation of cell pipelines. Conditioned medium soluble with 10% FBS was added by pretreatment and continuous treatment regimes. The observation time of the in-gel method was 13 d, whereas that of the