Hypoxia Arrests Ovarian Carcinoma Cell Cycle Progression, but Invasion Is Unaffected

Ana Krtolica and John W. Ludlow

Department of Biochemistry, University of Rochester School of Medicine and Dentistry [A. K., J. W. L.,] and the Division of Developmental Therapeutics [J. W. L.], University of Rochester Cancer Center, Rochester, New York 14642

ABSTRACT

Although hypoxic cells are generally resistant to radiation and chemical therapies designed to halt the spread of neoplastic disease, few investigations have been carried out with regard to the molecular mechanisms responsible for this phenomenon. Here, we report of the development of an in vitro model system with which to study the molecular mechanisms involved in the proliferation and invasion of human ovarian carcinoma cells under hypoxia. Results from [3H]thymidine incorporation experiments indicate that hypoxia triggers cessation of ovarian carcinoma cell DNA synthesis. Flow cytometry analysis of cellular DNA content for hypoxic cultures revealed that cell cycle progression was arrested. This arrest was found to be reversible upon reoxygenation of the cultures. Concomitant with this growth arrest is hypophosphorylation of pRB and a reduction in cyclin A abundance, suggesting that hypoxia induces growth arrest by regulating the activities of these crucial cell cycle-regulatory proteins. In vitro invasion assays revealed that hypoxia has no appreciable effect on the invasive ability of these cells. Immunoblotting established that the detected proteolytic activity was due to the matrix metalloproteinase MMP-2, the Mr 72,000 type IV collagenase that is most closely associated with the metastatic phenotype in vitro and in vivo. These data support the notion that populations of ovarian carcinoma cells are capable of surviving and invading extracellular matrix during hypoxic conditions and, after a more suitable oxygen environment is reached, giving rise to new cell colonies.

INTRODUCTION

Over 90% of all ovarian cancers are believed to result from the malignant transformation of ovarian surface epithelium. The most prevalent mechanism of ovarian cancer spread is by direct extension of tumor cells from the primary tumor into the peritoneum, omentum, and bowel surfaces (1). Although peritoneal invasion seems to be the most relevant process in the development of ovarian carcinoma leading to poor patient outcome of the disease, there have been few reports dealing with ovarian carcinoma invasion and the potential mechanisms involved (2—4). Additionally, although the correlation between proteolysis and invasion has been firmly established for many other tumor types (5), little information exists on the role of proteinases, particularly the matrix metalloproteinases, in the dissemination of ovarian carcinoma (3, 6).

Ovarian cancer cells that have detached from the primary tumor mass, either spontaneously or perhaps as a result of surgical debulking, and are at the initial stages of attachment and penetration into the peritoneal stroma can experience lower oxygen levels than those exposed on the surface of the vascularized tumor (7). It has been well established that cells that are subjected to reduced oxygen availability, also referred to as hypoxia, often demonstrate resistance to both radiation and chemical therapies designed to halt their growth and proliferation (8—10). It has also been observed that patients who undergo surgical cytoreduction to 0.5 cm or less have a greater survival advantage after chemotherapy than do patients with larger masses (11). One can infer from these data that a threshold number of remaining tumor cells maintain proliferative and invasive capability, despite postoperative therapy. Taken together, it is tempting to speculate that resistant hypoxic cells play a role in maintaining a critical threshold of neoplastic cells capable of repopulating the patient.

Published work from our own laboratory (12, 13) is consistent with the hypothesis that arrest of cellular proliferation by hypoxia results from disrupting the synthesis, degradation, and posttranslational modifications of at least two cellular proteins, the product of the retinoblastoma susceptibility gene, pRB, and cyclin A. Both of these cellular proteins are known to be involved in regulation of the cell division cycle. These data suggest that hypoxia may have selective effects on components within cell cycle regulatory pathways, thereby disrupting critical feedback within these pathways that would otherwise couple alterations in regulatory factors to the requirement that cells overtly progress through the cell cycle.

In this report, we present an in vitro model system that we have used to study the effect of hypoxia on ovarian carcinoma cell proliferation and invasion. The data indicate that while the cell division cycle is reversibly arrested, possibly due to the modulation of critical cell cycle regulatory protein activities, hypoxic ovarian carcinoma cells maintain their invasive properties. In addition, we have established that the detected proteolytic activity of these cells was due to the matrix metalloproteinase MMP-2, the Mr 72,000 type IV collagenase that is most closely associated with the metastatic phenotype in vitro and in vivo.

MATERIALS AND METHODS

Cell Growth Conditions. OW-1, SAU, and SKA are continuous lines of ovarian carcinoma cells derived from patient isolates (14). These cells were maintained in DMEM (GIBCO) containing 10% fetal bovine serum at 37°C in a humidified, 5% CO2-containing atmosphere.

Establishing Hypoxic Cultures. Cells were grown to 40—60% confluence on glass dishes as described above. Cells were made hypoxic for 24 h in evacuation chambers by intermittent application of vacuum and sparging with 95% N2-5% CO2 over a 2-h period. Cells were analyzed at this point (as 2-h hypoxic cells) or maintained under hypoxic conditions in the presence of 100 mM diethanolamine (an oxygen scavenger) at 37°C for the indicated periods of time, as described previously (15). Under these conditions, oxygen levels drop to ≤10 mm Hg by 8—12 h of hypoxia. After 24 h of hypoxia, cells were either harvested or reoxygenated for the indicated periods of time by replacing the spent medium with fresh medium and incubating the cultures at 37°C in a humidified, 5% CO2-containing atmosphere.

Colony-forming Assays. Four plates of OW-1, SAU, and SKA cells were plated at approximately 30—40% confluency. Two plates were made hypoxic as described above and cultured at 37°C for 24 h. The remaining two plates served as the aerobic controls. At this time, adherent cells were trypsinized from the plates, and the viable cell number was determined by counting trypsin-extracting cells in a hemocytometer and by replating these cells under aerobic conditions at 500 and 100 cells/60-mm dish. After 10 days, the media was discarded from all five plates of each set, and the cell monolayer was washed with PBS. The cells were then fixed and stained for 10 min in 70%
methanol containing crystal violet. At this time, the stained cells were washed with distilled water and air dried for 24 h before counting the number of colonies, which are defined as containing 50 or more cells.

**Cell Cycle Analysis.** Aerobic and hypoxic cells were harvested by trypsinization and low-speed centrifugation. The resulting cell pellet was then washed three times with PBS before fixation in cold 70% ethanol (−20°C) and stored at this temperature until flow cytometric analysis. Fixed cells (at least 5 × 10⁶ cells) were then centrifuged (55 × g for 10 min), resuspended in 1 ml of PBS containing 1 mg/ml RNase A, and incubated at room temperature for 30 min. Propidium iodide was added to a final concentration of 10 μg/ml, and flow cytometry was performed on a Profile II flow cytometer (Coulter counter; Coulter, Hialeah, FL). DNA histogram analysis was performed using the EPICS cytological DNA software package version 2.0.

**[³H]Thymidine Pulse Labeling and Quantitation.** Aerobic and hypoxic cells (1 × 10⁵) attached to the bottom of 35-mm dishes were labeled with [³H]thymidine (2 μCi/ml). Following incubation at 37°C for 30 min, the labeled cells were lysed on ice with 1 ml of RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS]. Immediately thereafter, this lysate was precipitated on ice for 30 min with 10% trichloroacetic acid. The precipitable counts were recovered by filtration through GF/A glass filters, washed extensively with 5% trichloroacetic acid, rinsed with 95% ethanol, and then allowed to air dry before quantitation by liquid scintillation spectroscopy.

**In Vitro Invasion Assay.** Invasion assay under hypoxic and aerobic conditions was performed as described previously (16). Briefly, polycarbonate filters of Transwell chambers were coated with 10 μl of Matrigel, and 2 × 10⁵ cells were seeded/insert. Medium was added to both lower and upper chambers. After incubating the chambers for 24 h at 37°C, the inserts were stained with hematoxylin (17), and the number of cells on the underside of the membrane was counted under optical microscope or prepared for scanning electron microscopy (18). In the later case, representative fields of the underside of the membrane were photographed for each insert, and cells were counted. Both procedures used to visualize cells for quantitation yielded comparable results.

**Gelatin Zymography.** We assayed for proteolytic activity using gelatin substrate gels. As described previously (19–21), nonheated samples were mixed with SDS sample buffer without reducing agent and applied to 9% acrylamide separating gels copolymerized with 1 mg/ml gelatin. After electrophoresis and removal of SDS from gel, they were incubated at 37°C overnight to allow for proteolytic activity. Gelatinolytic enzymes were detected as transparent bands on the background of Coomassie Blue-stained gelatin. To confirm that detected enzyme bands belong to the matrix metalloproteinase family and thus are Ca²⁺ dependent, after removal of SDS, an identical gel was preincubated for 1 h at 4°C with 0.5 mM EGTA and then incubated overnight in reaction buffer containing 0.5 mM EGTA. An almost complete reduction in proteolytic activity was observed.

**SDS-Gel Electrophoresis and Western Blotting.** All cells were lysed in EBC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, and 0.5% NP40] containing 10 μg/ml of the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonylfluoride for 15 min at 4°C. The lysates were cleared by centrifugation at 14,000 × g for 10 min. Electrophoresis was performed in 7.5% SDS-polyacrylamide gels (22) using 100 μg of total cell lysate protein (23) for each sample lane. After electrophoresis, the proteins were transferred to nitrocellulose paper (24) in buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% v/v methanol, and 0.01% SDS (pH 8.5). Residual protein binding sites on the nitrocellulose were blocked by incubation for 30 min in TBS [25 mM Tris-HCl (pH 8.0) and 150 mM NaCl] containing 4% BSA. Then, the nitrocellulose was incubated in TBS containing 2% BSA and a 1:1000 dilution of the anti-pRB monoclonal antibody PMG3-245 (PharMingen), a 1:100 dilution of anti-cyclin A monoclonal antibody C160 (25, 26), or a 1:1000 dilution of rabbit anti-M, 72,000 type IV collagenase antiserum, a kind gift from Dr. Stetler-Stevenson (NIH, Bethesda, MD). Following three washes of 10 min each with TBS containing 0.5% Tween-20 (TBST), the nitrocellulose was probed with horseradish peroxidase-conjugated anti-IgG (Promega) and developed for chemiluminescence (Amersham) according to the manufacturer’s instructions.

**RESULTS**

Cell survival of hypoxic OW-1, SAU, and SKA cultures was compared to that of aerobically maintained control cultures. Trypan blue exclusion revealed virtually no difference in cell viability between cells exposed up to 24 h of hypoxia and the aerobic controls (data not shown). The proliferative ability of hypoxic cells upon returning to an aerobic environment was found to vary between 20 and 65% of that measured for control cultures (Fig. 1). Taken together, these data were interpreted to mean that although hypoxic conditions do not result in appreciable cell death of these ovarian carcinoma cells, their proliferative ability upon recovery in an aerobic environment is negatively affected. This result was not entirely unexpected, however, since hypoxia has been shown to inhibit cell proliferation in a variety of cell types (12, 27).

To further address this issue of hypoxia negatively affecting cellular proliferation, [³H]thymidine incorporation and flow cytometric analysis were used to establish the influence of hypoxia on DNA synthesis and cell cycle progression in these cell lines. As shown in Fig. 2, hypoxic conditions resulted in 80 to 90% inhibition of DNA synthesis in all three of the cell lines examined, as evidenced by the appreciable reduction in [³H]thymidine incorporation. Flow cytometry of asynchronously growing ovarian cancer cell cultures revealed that, when compared to aerobic controls, SKA and OW-1 seem to reversibly arrest in G1 or at the G1-S border during hypoxia, as evidenced by a reduction in the S and G2-M peaks of the DNA histogram (Fig. 3). Hypoxic SAU cells do not appear to accumulate in any particular phase of the cell cycle before growth arrest, as evidenced by an undetectable change in the hypoxic DNA profile compared to the aerobic control. Upon recovery in aerobic conditions, the previously hypoxic cells resume cell cycle progression, as evidenced by the re-emergence of the S and G2-M peaks in the DNA histograms. Taken together, it appears that hypoxia reversibly blocks cell cycle progression in these ovarian carcinoma cells.

We have shown previously that, concomitant with cell cycle arrest in untransformed cells, hypoxia induces reversible hypophosphorylation of the product of the retinoblastoma susceptibility gene, pRB, and a reduction in cyclin A abundance. Since hypophosphorylated pRB is active with respect to suppressing cell cycle progression (28) and

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**Fig. 1.** Colony-forming ability of ovarian carcinoma cells recovering from hypoxia. Cell suspensions of 24-h hypoxic cultures were diluted and then maintained under normoxic conditions to assess their proliferative ability as defined by their proficiency in forming multicellular colonies. By dividing the number of multicellular colonies observed by the number of cells used to seed the culture dish (either 100 or 500) and multiplying by 100, the percentage of attached cells, representing the percentage of cells capable of proliferating, was obtained. The colony-forming ability of hypoxic cells recovering in aerobic conditions (C) was compared to that of parallel cultures maintained in aerobic conditions (B). Each column represents the mean for five samples for each cell line. Bars, SEM for two independent experiments.

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abundant cyclin A is needed to progress through S phase and into mitosis (29), these data support the notion that hypoxia blocks cell cycle progression by regulating the activity of these key cell cycle regulatory elements. Immunoblot analysis of protein extracts from OW-1, SAU, and SKA cells exposed to hypoxic conditions and then allowed to recover in an aerobic environment demonstrated similar results (Fig. 4). During exposure to hypoxia, pRB accumulates preferentially in its hypophosphorylated, growth-suppressive form, while levels of cyclin A decrease to an undetectable level. These effects are reversed upon recovery in an aerobic environment. Taken together with the data presented above (Fig. 3), hypoxia appears to reversibly block ovarian carcinoma cell proliferation, perhaps by modulating the activities of at least two different cell division cycle-regulatory proteins.

It was also of interest to determine the effect of hypoxia on ovarian carcinoma cell invasiveness, since this step in the metastatic process is of clinical importance to patient treatment efficacy (30). To this end we established an in vitro invasion assay on Matrigel-covered Transwells (16). We performed assays in aerobic conditions to evaluate invasiveness of the cells used. As can be inferred from Fig. 5, OW-1 and SKA were highly invasive under aerobic conditions. In contrast, SAU did not invade significantly. When these cells were exposed to hypoxia, their invasive abilities did not appreciably alter. These differences in invasiveness between the SAU line and the OW-1 and SKA lines are not due to differences in motility, because all three of these cell lines showed high motility under hypoxic and aerobic conditions (data not shown). To our knowledge, this is the first direct assessment of invasiveness for human ovarian tumor-derived cell lines under hypoxic conditions.
Fig. 4. Western blot analysis of aerobic, hypoxic, and hypoxic-recovered ovarian cancer cell lysates for pRB and cyclin A. Whole-cell lysates were prepared from 24-h hypoxic cultures (24hH; left lanes), 24-h hypoxic cultures allowed to recover for 24 h in aerobic conditions (24hR; middle lanes), and 24-h cultures maintained in aerobic conditions (24hA; right lanes). The ovarian cancer cell line is indicated at the top of each panel. Equal quantities (100 μg) were separated by SDS-gel electrophoresis and transferred to nitrocellulose; then the imprinted membrane was incubated with antibody specific for pRB or cyclin A. The blots were then reacted with horseradish peroxidase-conjugated antibody and developed for chemiluminescent detection. The positions of the slower-migrating, phosphorylated form of pRB (top arrow), the faster-migrating, unphosphorylated form of this protein (middle arrow), and cyclin A (bottom arrow) are indicated at the right of the figure.

Fig. 5. Invasive ability of hypoxic ovarian carcinoma cells. Invasion assays were performed as described in “Materials and Methods.” The number of invasive cells from 24-h hypoxic cultures (●) and aerobic control cultures (□) was obtained by counting the number of cells that penetrated the Transwell membrane. Each column represents the mean from five trials for each cell line.

**DISCUSSION**

The purpose of this study was to develop an in vitro model system with which to study the effect of hypoxia on ovarian carcinoma cell proliferation and invasion. In so doing, we strive to gain a better understanding of the cellular response to hypoxia at the molecular level. Data is presented that supports the conclusion that hypoxia can reversibly arrest ovarian carcinoma cell cycle progression. Concomitant with this arrest is hypophosphorylation of pRB and a decrease in

In vivo, lysis of the extracellular matrix, most commonly basement membrane, is often considered to be the hallmark of the invasion (31). This lysis is performed by matrix-degrading enzymes secreted either by tumor-stimulated stromal cells surrounding tumor or by the tumor cells themselves. In the case of the in vitro invasion assay used in our experiments, the only source of the needed matrix-degrading activity could have been tumor cells. Thus, we decided to assay for matrix-degrading proteins that are associated with invasion and metastasis in multiple in vitro and in vivo studies (32–36). Matrix metalloproteinases are readily detectable by zymography, a type of substrate electrophoresis in which the proteolytic activities of proteins, separated according to their molecular weights, are detected. We analyzed by zymography cell lysates and serum-free conditioned medium from these ovarian carcinoma cell lines using gelatin as a substrate. Major gelatinolytic activity was found in the conditioned medium from all three cell lines, with an apparent molecular weight of Mr 72,000 (Fig. 6). Immunoblotting using anti-MMP-2-specific polyclonal antibodies (Fig. 7) identified MMP-2, an enzyme whose activity is closely associated with invasion, as the most probable enzyme responsible for this proteolytic activity. The effect of hypoxia on gelatinolytic activity was measured by gelatin zymography of the conditioned medium from 24-h hypoxic cells and compared to the activity from aerobically maintained cultures (Fig. 8). The samples were normalized to equal cell number since hypoxic cultures are cell cycle arrested (Figs. 2 and 3). As shown, samples from hypoxic cultures demonstrated only partial inhibition of gelatinase activity.

Fig. 6. Gelatin zymography of ovarian carcinoma cell lines maintained in aerobic conditions. Conditioned medium and lysates from ovarian carcinoma cells were analyzed for their ability to degrade gelatin. All samples were normalized to equal cell number. As shown, abundant proteolysis at approximately M, 72,000 (72 kD) can be detected in conditioned medium from 24-h aerobic cultures for all three lines tested and in lysate from only one of these lines, SAU.

Fig. 7. Western blot analysis of conditioned medium from aerobically maintained ovarian carcinoma cells for type IV collagenase. Proteins contained in the medium from 24-h aerobic cultures were separated by SDS-gel electrophoresis and transferred to nitrocellulose; then the imprinted membrane was incubated with antibody specific for MMP-2. The blots were then reacted with horseradish peroxidase-conjugated antibody and developed for chemiluminescent detection. HT1080 is a human fibrosarcoma cell line (ATCC CCL 121) used as a positive control for MMP-2. The position of MMP-2 is indicated by the arrow at the right of the figure. The slower-migrating band is the proform of MMP-2.
cycin A abundance. Upon reoxygenation in aerobic conditions, these cells progress through the cell cycle concomitant with pRB hyperphosphorylation and an increase in cycin A abundance. It has been shown previously that hypophosphorylated pRB negatively regulates cell cycle progression (28), and abundant cycin A is needed for S-phase progression into mitosis (29). Taken together with a previous report from this laboratory (12), these data further support the notion that hypoxia reversibly blocks the progression of these ovarian carcinoma cells through the phases of the cell cycle by modulating the activity and abundance of key cell cycle-regulatory elements.

In vitro invasion assays did not detect any alteration in ovarian carcinoma cell invasiveness under hypoxic as compared to aerobic conditions. This result may be somewhat surprising when considering that hypoxia blocks cell cycle progression, which in turn blocks cell proliferation, as well as down-regulation of overall cellular protein expression (37). With this in mind, one may predict that hypoxic cells would not be invasive due, perhaps, to a lack of proteolytic enzyme production. As shown here, this does not appear to be the case, because there is only partial inhibition of gelatinolytic activity under hypoxic conditions. Taken together with the observation that ovarian carcinoma cell invasiveness is the same for hypoxic and aerobic cultures, it may be suggested that gelatinolytic activity levels remain above the threshold amounts needed for invasion in hypoxia. Such partial inhibition may be due to hypoxia-induced changes in the specific activity of proteolytic enzymes, which may be independent of enzyme abundance. Our observation that the noninvasive SAU cell line also demonstrates proteolytic activity points to the involvement of other factors in the invasive process, a notion more and more prominent in the recent literature (5, 38). Indeed, although type IV collagenases are readily detectable with the gelatin substrate gels, another group of matrix metalloproteinases commonly involved in invasion, the stromelysins, show preference for the substrate casein. In preliminary experiments, we used casein zymography to detect two weak bands around Mr 35,000 and Mr 45,000 in conditioned medium from all three of these ovarian carcinoma cell lines. Although caseinolytic activity seemed to be much weaker than the one directed toward gelatin, it is quite probable that both of them contribute toward the lysis of the matrix during invasion. Although additional studies need to be carried out to elucidate the molecular mechanism behind ovarian carcinoma cell invasion, the fundamental observation that these hypoxic and cell cycle-arrested cells are still capable of invading extracellular matrix in vitro provides a unique opportunity to address this issue.

In closing, the data presented above suggest that at least some populations of ovarian carcinoma cells are capable of surviving and invading extracellular matrix during hypoxic conditions and, after a more suitable oxygen environment is reached, giving rise to new cell colonies. Clinical studies indicate that frequent relapse of ovarian cancer patients results most likely from repopulation by disseminated cancer cells that have detached from the primary tumor mass prior to or during surgical removal (7, 39). If the in vitro results described in this report are taken together with this clinical observation, the following explanation for patient repopulation by neoplastic ovarian cells may be argued. Since we expect locally metastatic ovarian carcinoma cells in vivo to experience low oxygen availability following detachment from the primary tumor mass, it is of substantial interest that hypoxic ovarian carcinoma cells do not experience appreciable diminution of their invasive potential. Such cells are capable of surviving postoperative radiation and chemotherapy as a result of the hypoxia-induced resistance. These cells are also able to invade the peritoneum and penetrate through extracellular matrix, reaching a more suitable oxygen environment. Such cells may then reenter the cell division cycle, resulting in an increasing number of neoplastic cells capable of invading an expanding area of surrounding tissue. Such a situation most surely contributes toward the formation of a secondary tumor mass. Understanding the cellular response to hypoxia at the molecular level is essential for the development of rational interventions designed to enhance the destruction of hypoxic cancer cells. Once such knowledge is gained, improvement in patient treatment efficacy may be further facilitated.

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