Cancer Biology & Therapy 10:4, 386-390; August 15, 2010; © 2010 Landes Bioscience

Oxygenation inhibits ovarian tumor growth by downregulating STAT3 and cyclin-D1 expressions

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Key words: oxygenation, ovarian cancer, hypoxia, STAT3, cyclin-D1

Hypoxia, which is commonly observed in many solid tumors, is a major impediment to chemo- or radiation therapy. Hypoxia is also known to overexpress/activate signal transducer and activator of transcription 3 (STAT3) leading to tumor progression as well as drug resistance. We hypothesized that increased oxygenation of the hypoxic tumor may have an inhibitory effect on STAT3 activation and hence tumor-growth inhibition. Mice containing human ovarian cancer xenograft tumor were exposed to hyperbaric oxygen (HBO; 100% oxygen; 2 atm; 90-min duration) daily, for up to 21 days. Mice exposed to HBO showed a significant reduction in tumor volume, with no effect on body weight. STAT3 (Tyr 705) activation and cyclin-D1 protein/mRNA levels were significantly decreased up on HBO exposure. Interestingly, HBO exposure, in combination with weekly administration of cisplatin, also significantly reduced the tumor volume; however, this group of mice had drastically reduced body weight when compared to other groups. While conventional wisdom might suggest that increased oxygenation of tumors would promote tumor growth, the results of the present study indicated otherwise. Hyperoxia appears to inhibit STAT3 activation, which is a key step in the ovarian tumor progression. The study may have important implications for the treatment of ovarian cancer in the clinic.

Introduction

Hypoxia plays a significant role in cancer therapy. It reduces the sensitivity of tumors to radio- or chemotherapeutic agents.[1-3] Hence, there have been several approaches to oxygenate the hypoxic tumor before radiation or chemotherapy. One approach is to improve systemic oxygen delivery by increasing the dissolved oxygen concentration in plasma using special hemoglobin preparations, perfluorochemicals or hyperbaric oxygenation (HBO).[4,5] HBO treatment involves the administration of 100% oxygen under higher-than-atmospheric pressure. The additional pressure, coupled with inspiration of pure oxygen, substantially increases the amount of oxygen dissolved in the plasma. The relatively high levels of oxygen deliverable under HBO exposure make this approach attractive. Several studies in experimental animal tumors have shown that HBO reduces the radioresistance of certain types of tumors[6-9] and enhances the anticancer activity of doxorubicin and other alkylating agents.[10,11] Clinical trials have been initiated to study the effect of HBO inhalation on the radiotherapy for many types of malignant tumors.[12] HBO exposure has been shown to increase tumor radiosensitivity in both pre-clinical and clinical studies.[13,14]

Although, HBO exposure has been shown to enhance the radio- or chemosensitivity of tumor, the effect of HBO alone on the progression of malignancy is uncertain. HBO has been previously contraindicated in the treatment of malignancy due to the concern that hyperoxia may stimulate tumor growth via re-oxygenation of hypoxic tumor cells and increased neovascularization.[15,16] Repeated HBO exposures have been shown to induce angiogenesis leading to an increase in vascular density in ischemic, necrotic and gangrenous tissues.[17] Some recent studies have reported an increase or early onset of metastases with HBO therapy. Johnson et al. reported a tumor stimulatory effect with HBO, with increased metastases in patients with cervical cancer[18]. This has been supported by other experimental studies and clinical trials.[15] There are also other studies that showed a tumor inhibitory or negligible effect with HBO treatment.[19,20] On the other hand, a few studies have shown a suppressive effect of HBO on tumor growth.[13,21,22]

Recently, we have reported that solid tumor xenografts of ovarian cancer were severely hypoxic, with the partial pressure of oxygen (pO_2) about 2 mmHg.[23] We observed that hypoxic exposure of a human ovarian cancer cell line (A2780) in vitro resulted in a substantial increase in the STAT3 phosphorylation (Tyr705) which could be reversed within 12 hours after re-exposure of the cells to normoxia. Because activation of STAT3 directly regulates both cell-proliferation and survival genes that provide growth advantages to tumor cells, we hypothesized that oxygenation of the ovarian tumor in vivo may have an attenuating effect on STAT3 activation and hence inhibit tumor growth.

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Submitted: 02/24/10; Revised: 05/20/10; Accepted: 05/21/10
Previously published online: www.landesbioscience.com/journals/cbt/article/12448
DOI: 10.4161/cbt.10.4.12448

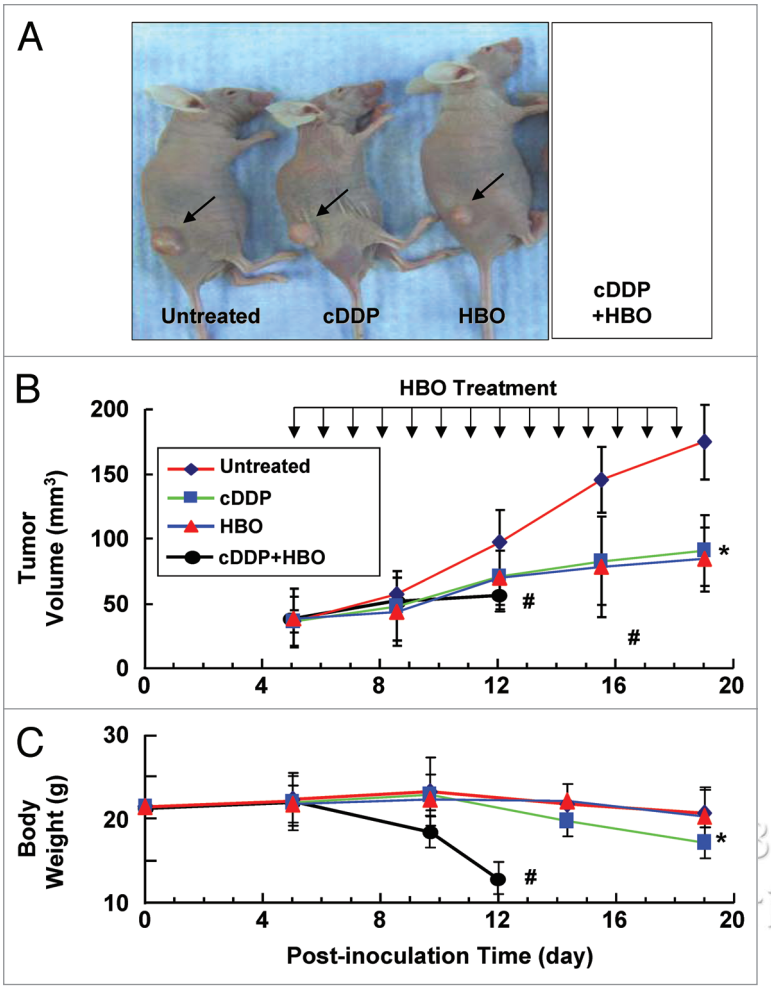

Figure 1. Effect of HBO on the growth of A2780 ovarian xenograft tumor in mice. (A) Picture of tumor-bearing mice shown at the end of the observation period. Arrows indicate tumors. (B) Tumor volume. Both HBO and cisplatin-treated (cDDP) animals had significantly (*$p < 0.05$; N = 6 mice/group) reduced tumor volume when compared to untreated controls. (C) Body weight of the animals during the treatment period. Cisplatin-treated animals had significantly (*$p < 0.05$; N = 6 mice/group) reduced body weight when compared to untreated controls and HBO-treated animals. # indicates a fourth group of animals, treated with both cisplatin and HBO that were euthanized on day 12 of the experimental period due to extreme loss of body weight.

In the present study we evaluated the effect of HBO treatment on the tumor growth and STAT3 activation in a murine xenograft model of human ovarian cancer. The results demonstrated that HBO treatment resulted in the attenuation of STAT3 activation leading to inhibition of tumor growth.

Results

Effect of oxygenation on tumor growth and body weight. Representative animals from each of the experimental groups that survived to completion of the study are shown in **Figure 1A**. The HBO or cisplatin group of animals had significantly reduced tumor volume when compared to untreated group (**Fig. 1B**). There was no significant difference in the tumor volumes between the HBO- and cisplatin-treated groups. Nonparametric statistical analysis showed that there was a significant loss of body weight in the cisplatin treatment group when compared to the untreated group at days 12, 15 and 18 ($p = 0.0079$ for each of these time points). Significant differences in body weight were also observed between the cisplatin + HBO treatment group and the control group on days 5 and 10 ($p = 0.0079$ for these time points). No significant difference in body weight was found between the HBO-treated group and the control group ($p > 0.3070$ for all time points). The cisplatin + HBO group of mice were sacrificed prior to the planned conclusion of the study due to an extreme loss of body weight (**Fig. 1C**). These results suggested that HBO treatment alone was capable of inhibiting tumor growth, without any adverse effect on body weight.

Effect of oxygenation on STAT3 and cyclin D1 expression. Tumor tissues excised from HBO-treated animals showed significantly reduced pSTAT3 (Tyr705) expression when compared to untreated controls (**Fig. 2A**). The treatments had no significant effect on the pSTAT3 (Ser727) or total STAT3 levels. We further observed that the expression of the cell-proliferation gene cyclin D1 was inhibited in HBO-treated animals (**Fig. 2B**). In addition, we confirmed through immunohistochemical analysis that pSTAT3 levels were reduced in the HBO group (**Fig. 2C**). These results suggested that inhibition of ovarian tumor growth by HBO may be due to inhibition of pSTAT3 and cyclin D1 expressions.

Discussion

The present study has demonstrated the effect of hyperbaric oxygenation on the growth of a human ovarian cancer xenograft tumor in mice. HBO exposure, administered daily for 21 days, significantly attenuated tumor growth, which correlated with the inhibition of STAT3 activation and cyclin D1 expression. Recently, we reported that hypoxia (1% O_2) stimulated STAT3 activation in human ovarian cancer cells (A2780) grown in vitro.[23] We observed that activated STAT3 was more highly expressed than the traditional markers of hypoxia, HIF-1α or VEGF, in hypoxia-cultured ovarian cancer cells. This is noteworthy, as the activation of STAT3 is a key factor in the development of cellular resistance to chemotherapy.[24,25] The results of the present study show that STAT3 activation is modulated by tumor tissue oxygenation.

In addition to STAT3 activation, it is likely that other signaling mechanisms could likewise be affected by a systemic increase in oxygen levels. The cell-proliferation gene cyclin D1 was downregulated in HBO-treated animals. We did not observe any changes in HIF-1α or VEGF expression levels (data not shown). These results are consistent with previous reports from similar studies conducted on lung metastases, in which HBO had an inhibitory effect on tumor growth.[26]

We also used HBO as an adjuvant treatment in combination with the well-known ovarian cancer chemotherapy agent, cisplatin. This combination therapy significantly reduced the tumor volume within 2 weeks when compared with untreated animals and animals treated with HBO alone. However, this group of mice had significantly reduced body weight when compared to all other groups. At present, we do not know the precise etiology for this drastic weight loss observed in the combination treatment animals, and whether or not HBO treatment would enhance the systemic toxicity of cisplatin and/or cause additional organ damage and failure. It has been postulated that HBO administration promotes the formation of reactive oxygen species, thereby inducing oxidative stress within the tissues.[27] In some studies, HBO has been shown to induce oxidative injury,[28,29] while others claim a preventive effect.[30-32] However, whether HBO acts as an oxidant promoter or as an antioxidant agent remains a matter of debate.

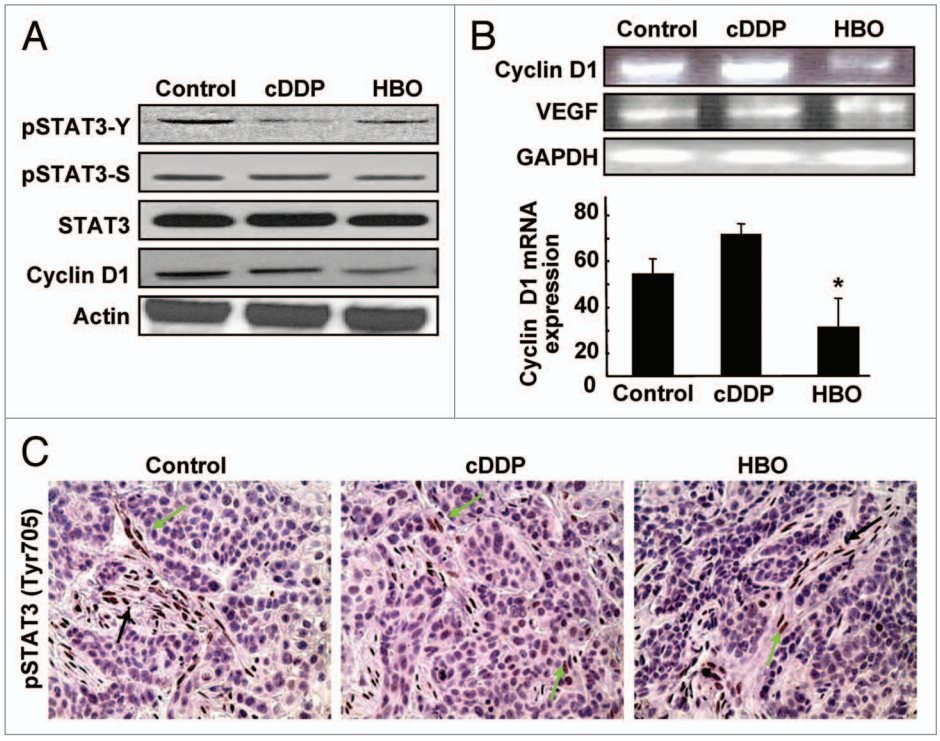

Figure 2. Effect of oxygenation on pSTAT3 and Cyclin D1 expression. Western blot analyses were performed on tissues from excised A2780 tumor xenografts in untreated mice, and mice treated with cisplatin (cDDP) or HBO. (A) Both treatments reduced the pSTAT3 (Tyr705; Y) and cyclin D1 levels, but not pSTAT3 (Ser727, S) or total STAT3 levels. (B) Cyclin D1 and VEGF mRNA levels were analyzed by RT-PCR. *$p < 0.05$ (N = 3). (C) Immunohistochemical staining showing pSTAT3. Arrows represent positively-stained areas.

HBO therapy has been investigated as a means by which to prevent cisplatin-mediated nephrotoxicity,[33,34] and otoxicity.[35] Aydinoz et al.[34] reported significant body-weight loss in cisplatin as well as cisplatin + HBO-treated rats. However, the most dramatic weight loss was observed in the cisplatin only-treated group. Atasoyu et al.[33] have observed a high mortality rate in rats receiving cisplatin and cisplatin + HBO. Further, Yassuda et al.[35] noted that 2 of 5 guinea pigs treated with combination therapy died prior to the completion of the study. Thus far, the evidence suggests that even under differing treatment conditions, combination therapy utilizing cisplatin and HBO should be avoided. The effect of HBO administration on tumor growth has also been contradictory, with some studies reporting that HBO produces a growth-stimulatory effect, while others report a growth-suppressive effect. Feldmeier et al.[15] extensively reviewed experimental and clinical data and concluded that HBO exposure had no primary or metastatic tumor stimulatory effect.

Our investigation was intended as a simple pilot study on the effect of HBO on ovarian tumor growth, primarily from a molecular signaling standpoint. By re-oxygenating ovarian tumor tissue, it is possible to decrease the levels of activated STAT3, thereby reducing the oncogenic effects affiliated with constitutive activation of this oncogene. While combination therapy using cisplatin may not be appealing, in light of our experimental results and those reported by others, we see an opportunity to develop new chemotherapeutic agents that could be used in combination with HBO as an additional means of treatment for ovarian cancer patients. Future studies may also include additional investigation into other signaling mechanisms that may be responsible for HBO attenuation of ovarian tumor growth.

Materials and Methods

Materials. Antibodies directed against pSTAT3 (Tyr705 and Ser727) were purchased from Cell Signaling Technology (Beverly, MA). VEGF, cyclin D1, HIF-1α and STAT3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cisplatin (cis-dichlorodiamine platinum, cDDP) was obtained from Sigma.

Ovarian cancer xenograft tumors in mice and experimental groups. A2780 human ovarian cancer cells (5×10^6 cells in 60 µl of PBS) were subcutaneously injected in the back of 6-week-old BALB/c nude mice (National Cancer Institute). All treatments were initiated on day 5, when the tumor size reached 2–4 mm. The mice were divided into four groups, containing 8 mice per group: (i) Control (untreated); (ii) HBO (only); (iii) Cisplatin (only); (iv) HBO + Cisplatin (combination). HBO was administered daily for 3 weeks. Cisplatin (100 mg/kg; i.p.) was administered once per week for three weeks.

HBO protocol. Animals were placed in cages in a custom-built small-animal hyperbaric chamber (PolyFab, Boston Plastics Manufacturing; Wilmington, MA). HBO (100% O_2) was

administered at a pressure of 2 atm for 90 min. A minimum of 15-min pressurization and depressurization was allowed for the animals to adjust to the changes in pressure. HBO was administered daily, for up to 21 days. The tumor size was measured twice per week using a digital vernier caliper and the tumor volume was calculated from the orthogonal dimensions (d_1, d_2, d_3) using the formula (d_1 x d_2 x d_3) x $\pi/6$. The animals were euthanized at the end of the experimental period. Tumor tissues were collected and subjected to western blotting and immunohistochemical analyses.

Immunoblot analysis. The xenografted tumor tissue samples were homogenized on ice and the tissue lysates were prepared in nondenaturing lysis buffer containing 10-mM Tris-HCl (pH 7.4), 150-mM NaCl, 1% Triton X-100, 1-mM EDTA, 1-mM EGTA, 0.3-mM phenylmethylsulfonyl fluoride, 0.2-mM sodium orthovanadate, 0.5% NP40, 1-μg/ml aprotonin and 1-μg/ml leupetin. The tissues lysates were centrifuged at 10,000 xg for 20 min at 4°C, and then supernatant was separated. The protein concentration in the lysates was determined using a Pierce detergent-compatible protein assay kit. For western blotting, 25 to 50 μg of protein lysate per sample was denatured in 2x SDS-PAGE sample buffer and subjected to SDS-PAGE on a 10% or 12.5% tris-glycine gel. The separated proteins were transferred to a PVDF membrane and the membrane was blocked with 5% nonfat milk powder (w/v) in TBST containing 10-mM Tris, 100-mM NaCl and 0.1% Tween 20 for 1 h at room temperature, or overnight at 4°C. The membranes were then incubated with the primary antibodies mentioned previously. The bound antibodies were detected with horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG or HRP-labeled donkey anti-rabbit IgG (Amersham Pharmacia Biotech) using an enhanced chemiluminescence detection system (ECL Advanced Kit). Protein expressions were quantified using Image Gauge (version 3.45) software.

Immunohistochemistry. Tumor tissues were fixed in formalin and embedded in paraffin. Six-micron-thick tissue sections were obtained and used for hematoxylin and eosin staining. For immunofluorescence staining, 8-μm-thick tissue sections were serially rehydrated in 100%, 95% and 80% ethanol after deparaffinization with xylene. Slides were kept in steam for 30 min and then washed in PBS (pH 7.4) three times for 5 min each. The tissue sections were first incubated with 2% goat serum and 5% bovine serum albumin in PBS (to reduce nonspecific binding), and then by incubation for 4 h with an anti-mouse anti-pSTAT3, followed by incubation with secondary antibodies (1:1,000 dilutions) conjugated to horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG or HRP-labeled donkey anti-rabbit IgG (Amersham Pharmacia Biotech). The tissue slides were visualized using a Nikon fluorescence microscope.

Reverse-transcription PCR (RT-PCR). Total RNA isolated from ovarian tumor tissue was prepared with TRIzol (Life Technologies, Grand Island, New York) according to the manufacturer's instructions. RNA quantification was done using spectrophotometry. Reverse transcription RT-PCR analysis for the mRNA expressions in cyclin D1, VEGF and the internal control GAPDH was carried out using a GeneAmp PCR System Veriti thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s), and extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

Statistical analysis. Due to the small sample size, non-parametric Wilcoxon rank sum tests were performed for paired comparisons. Bonferroni corrections were used to adjust the significance level for multiple comparisons. A p-value of less than 0.0083 (=0.05/6) was considered significant with Bonferroni corrections for the non-parametric tests. Otherwise, a p value of less than 0.05 was considered significant.

Acknowledgements

We acknowledge the financial support from the Kaleidoscope of Hope Foundation for Ovarian Cancer Research.

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