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Hyperbaric Oxygen Inhibits Benign and Malignant Human Mammary Epithelial Cell Proliferation

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Abstract. *Background: Hyperbaric oxygenation (HBO) therapy is the administration of 100%-inhaled oxygen to patients at increased atmospheric pressure. Materials and Methods: We used an in vitro model to examine the effects of HBO on mammary cell proliferation. Normal mammary epithelia, primary tumor and metastatic tumor cells derived from the same patient and immortalized by transfection with the human papilloma virus E6 oncogene, as well as the MCF7 human mammary adenocarcinoma cell line, were studied. Results: HBO (97.9% O_2, 2.1% CO_2, 2.4 atmospheres absolute) inhibited the proliferation of all 4 cell types as measured by light microscopy, [^3H]thymidine uptake, a tetrazolium-based colorimetric assay and a clonogenicity assay. The anti-proliferative effect of HBO was time-dependent ($p<0.01$ for all 4 cell types). Hyperoxia alone (95% O_2, 5% CO_2, 1 atmosphere absolute) and increased atmospheric pressure alone (8.75% O_2, 2.1% CO_2, 2.4 atmospheres absolute) also inhibited proliferation, but their effects were not as profound as HBO ($p<0.01$ when either hyperoxia or increased pressure was compared to HBO for all 4 cell types). HBO enhanced the anti-proliferative effects of melphalan ($p<0.05$), gemcitabine ($p<0.001$) and paclitaxel ($p<0.001$). The clonogenicity assay demonstrated that the effects of HBO were still evident 2 weeks after the exposure ($p<0.01$ for all 4 cell types). Experiments using Hoechst-propidium iodide or annexin V-propidium iodide staining showed no HBO-induced increases in necrosis or apoptosis. Conclusion: HBO inhibits benign and malignant mammary epithelial cell proliferation, but does not enhance cell death.*

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Breast cancer is one of the leading causes of death in American women. These women die because their disease is resistant to surgery, radiation, hormonal therapy and chemotherapy. Some of the women who succumb to breast cancer experience local recurrence of their tumor. Chest wall recurrences are associated with pain, bleeding and ulceration in over 60% of patients (1), and are often accompanied by odor and drainage. As a consequence of their symptoms and the distress caused by watching their tumor grow, these women often suffer miserably, sometimes for years (2). Recurrent tumors are frequently difficult to resect and are often resistant to chemotherapy. If a woman has received a previous course of breast irradiation, toxicity often precludes additional therapy. Given the limited efficacy of state of the art therapy for recurrent breast cancer in the chest wall, it is imperative that novel approaches to treating these tumors be developed.

Hyperbaric oxygen (HBO) therapy is the administration of 100%-inhaled oxygen to patients at increased atmospheric pressure. This relatively non-toxic therapy is used to treat a number of diseases including carbon monoxide poisoning (3) and non-healing wounds (4). In phase III trials performed over two decades ago, HBO was shown to act synergistically with radiation to improve loco-regional control of head and neck cancer (5). The mechanism whereby HBO enhances the effect of ionizing radiation was never elucidated. Numerous animal studies have shown that HBO also enhances the tumoricidal effect of chemotherapy (6-15). The use of HBO in the treatment of solid tumors may have been prematurely abandoned.

In this study, we investigated the effects of HBO on proliferation of normal mammary epithelia, primary tumor and metastatic tumor cells derived from the same patient and immortalized by transfection with the human papilloma virus E6 oncogene (16). The E6 gene product binds to the p53 tumor suppressor and induces its ubiquitin-mediated degradation (17). We used these cell types for 3 reasons.

First, they enabled us to explore whether the effects of HBO differ depending upon the state of cellular transformation. Second, having been passaged only a limited number of times, the transfectants have fewer mutations than cell lines. Third, the transfectants are similar to the many sporadic breast cancers which do not have p53. We also studied the MCF7 human mammary adenocarcinoma cell line which has functional p53. Experiments were controlled for the effects of hyperoxia or increased atmospheric pressure alone. We found that HBO inhibited spontaneous proliferation and enhanced the anti-proliferative effect of chemotherapeutic agents. The effects of HBO could not be attributed to enhanced cell necrosis or apoptosis.

Table I. *HBO suppresses mammary cell proliferation as determined by cell counting*.*

Cell type	Control (cells x 10^5)	HBO (cells x 10^5)
Normal	5.07	3.30
Primary tumor	2.66	2.17
Metastatic tumor	4.90	3.70
MCF7	2.85	1.82

*5x10^4 cells/well were cultured at 37˚C in either 21% O_2, 5% CO_2 at 1 atm abs (control) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (HBO). After a 20-h incubation, the number of adherent cells in each well was counted in triplicate using a hemocytometer counting chamber.

Materials and Methods

Cell strains and cell lines. Normal (H16N2) mammary epithelial and primary tumor (21PT) cells were derived from mastectomy samples from a patient with infiltrating ductal and intraductal carcinoma (18). Metastatic tumor (21MT) cells from the same patient were isolated from a pleural effusion. All 3 lines were immortalized by transfection with the human papilloma virus oncogene E6, as previously described (19, 20). H16N2 cells were cultured in DFCI-1 medium; 21PT and 21MT cells were cultured in α medium (18). The human MCF7 mammary adenocarcinoma cell line (American Type Culture Collection, Rockville, MD, USA) was cultured in Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine modified to contain 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate (Gibco BRL, Gaithersburg, MD, USA) supplemented with 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (Gibco BRL).

Cell proliferation experiments. The methodology for the cell proliferation experiments was a modification of the protocol used by Keane *et al.* (21). Cells were suspended in medium at a density of 2.5x10^5 cells/ml. Two hundred microliters of cell suspension (5x10^4 cells) were added to separate wells of a 96-well plate (Corning Inc., Corning, NY, USA). After incubating for 2 h at 37˚C, 5% CO_2, non-adherent cells were carefully removed from each well by aspiration. Two hundred microliters of either medium alone, medium containing melphalan (Sigma), medium containing gemcitabine (Eli Lilly and Company, Indianapolis, IN, USA), or medium containing paclitaxel (Mylan Pharmaceuticals Inc., Morgantown, WV, USA) were then gently added to each well. The cells were cultured up to an additional 20 h at 37˚C in a hyperbaric chamber, oxygen chamber, or incubator. All experiments using the transformed cell lines were performed in triplicate during the exponential growth phase.

Hyperbaric oxygenation. Experiments were performed in a 37˚C room using a B11-22 Small Animal Hyperbaric Chamber (Reimers Engineering, Inc., Alexandria, VA, USA). For the HBO experiments, the gas in the chamber was flushed for 4 min with 97.9% O_2, 2.1% CO_2 and then pressurized to 2.4 atmospheres absolute (atm abs) over 5 min. We used 2.1% CO_2 at 2.4 atm abs to achieve a CO_2 concentration equivalent to the 5% CO_2 at sea level (1 atm abs) used in conventional incubators. To ascertain the effect of pressure alone, cells were incubated at 37˚C in 8.75% O_2, 2.1% CO_2, 89.15% nitrogen at 2.4 atm abs. We used 8.75% O_2 so that the oxygen concentration would be the equivalent of 21% O_2 at 1 atm abs. To determine the effect of hyperoxia alone, cells were incubated at 37˚C in a chamber containing 95% O_2, 5% CO_2 at 1 atm abs. "Control" cultures for each experiment were placed in an incubator at 37˚C, 21% O_2, 5% CO_2, 74% nitrogen at 1 atm abs. All chambers as well as the incubator were humidified.

[^{3}H]thymidine assay. After the plates had been incubated for 3, 7, 16, or 20 h, 10 μl of 100 μCi/ml [^{3}H]thymidine (New England Nuclear, Boston, MA, USA) were added to each well. After an additional 18-h incubation in 95% air, 5% CO_2, 1 atm abs, 37˚C, the plates were frozen at –70˚C. All cultures were harvested on the same day by aspiration in water with the cellular debris trapped in glass-fiber paper. The filter paper was cut and β-radioactivity was counted in 2 ml of scintillation cocktail (ScintiSafe, Fisher Scientific, Houston TX, USA).

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The MTS assay measures the bioreduction of this tetrazolium salt to an intensely-colored formazan. While this assay actually measures mitochondrial respiration, it is commonly used to determine the relative number of viable cells in a sample (22). MTS (Promega, Madison, WI, USA) was reconstituted at 1.71 mg/ml in Dulbecco's phosphate-buffered saline (PBS). Phenazine methosulfate (PMS, Sigma) was made up at a concentration of 1.53 mg/ml in Dulbecco's PBS. Both solutions were stored in the dark at 4˚C. Just prior to use, 975 μl of MTS were gently mixed with 25 μl of PMS. Immediately after removing the plates from their chambers, 50 μl of the MTS-PMS solution were added to wells containing either media or cell suspension. The plates were incubated in the dark at 37˚C for 45 min before being shaken gently for 15 sec. The optical density (OD) of each well was read at 490 nm using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA, USA). To correct for background absorbance attributable to the media, the mean OD of wells containing cell-free media was subtracted from the OD of wells containing cells. To determine the relative number of viable cells in an experimental well, the OD of the experimental well was divided by the mean OD of wells of unstimulated cells incubated at 37˚C, 21% O_2, 5% CO_2, 1 atm abs. The resulting fraction was multiplied by 100 to yield a relative percentage.

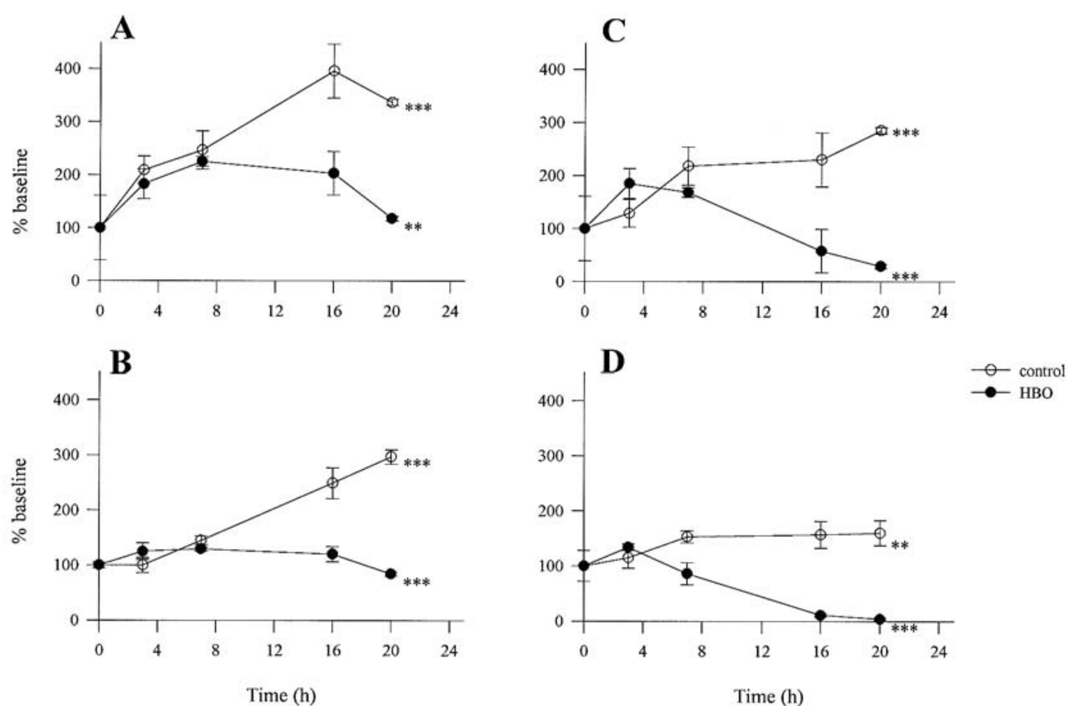

Figure 1. *HBO suppresses mammary cell proliferation as determined by [^{3}H]thymidine assay. (A) Normal mammary epithelia, (B) primary tumor, (C) metastatic tumor, or (D) MCF7 cells (all at 5x10^4 cells/well) were cultured at 37˚C in either 21% O_2, 5% CO_2 at 1 atm abs (control) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (HBO). At specified time-points, [^{3}H]thymidine was added to the wells of 1 control and 1 HBO plate. Both the control and HBO-exposed plates were then incubated for an additional 18 h at 21% O_2, 5% CO_2 at 1 atm abs. For each cell line, the β-radioactivity at each time-point is expressed as a percentage of the radioactivity at time zero. Results for each time-point are shown as the mean±SD of samples from 3 wells. ***$p<0.001$ and **$p<0.01$ using factorial ANOVA. The control and HBO curves were not directly compared in this experiment. For all 4 cell lines, 1 representative of 2 experiments is shown.*

Clonogenicity assay. The clonogenicity assay was performed as previously described (23) with minor modifications. Briefly, all 4 cell types were cultured in 24-well plates (Corning) until they were almost confluent. Some plates were exposed to HBO for 12 h at 37˚C; control plates were incubated concurrently in 95% air, 5% CO_2, at 1 atm abs, 37˚C. Following this incubation, the medium was aspirated and each well was washed once with solution A (a balanced salts solution) (24). Adherent cells were removed from the surface of the wells using 0.025% trypsin plus 0.01% ethylenediaminetetraacetic acid (Sigma). For each cell type, the cells in the original medium, wash and trypsin were recombined and then centrifuged at 4˚C at 1200 xg for 10 min. The cells were resuspended in 1 ml of medium using a pipette to gently agitate them to ensure single-cell suspensions. The cells were counted and 100 cells per condition were transferred to separate wells in a 6-well plate. The plates were incubated in 95% air, 5% CO_2, 1 atm abs at 37˚C for up to 2 weeks to allow colonies to become visible. During this period, the medium was changed every 4 days. Finally, colonies were stained with 2% vol/vol methylene blue in 50% vol/vol ethanol/PBS and counted. The number of colonies in each well was considered to represent the percentage of HBO-exposed cells capable of proliferating.

Hoechst-propidium iodide assay. All 4 cell types were cultured on separate chamber slides until they were almost confluent. Some slides were then incubated for 20 h at 37˚C in the HBO chamber; control slides were incubated concurrently in 95% air, 5% CO_2, 1 atm abs, 37˚C. Following this incubation, the nuclei were stained by adding Hoechst 33342 (Molecular Probes, Eugene, OR, USA) to each well at a final concentration of 4 mg/ml. After incubating for 15 min at 37˚C, the slides were allowed to cool to room temperature and then propidium iodide (Sigma) was added to each well at a final concentration of 5 mg/ml. Five minutes after adding propidium iodide, the dye-containing media was aspirated and cover slides were applied. For each cell type, 300 cells were examined for nuclear morphology and color. Cells having blue nuclei with normal morphology were classified as "live"; cells with blue nuclei with apoptotic morphology were classified as "early apoptotic"; cells with red nuclei with apoptotic morphology were classified as "late apoptotic"; and cells with red nuclei without apoptotic morphology were classified as "necrotic".

Statistical analysis. The data shown in the graphs are expressed as the mean±standard deviation (SD). Factorial analysis of variance and Student's unpaired *t*-test were performed using SPSS, version 11.0, statistical analysis software (SPSS Inc., Chicago, IL, USA).

Results

HBO inhibits mammary epithelial cell proliferation. Normal (N) mammary epithelia, primary tumor (PT) and metastatic tumor (MT) cells derived from the same patient and

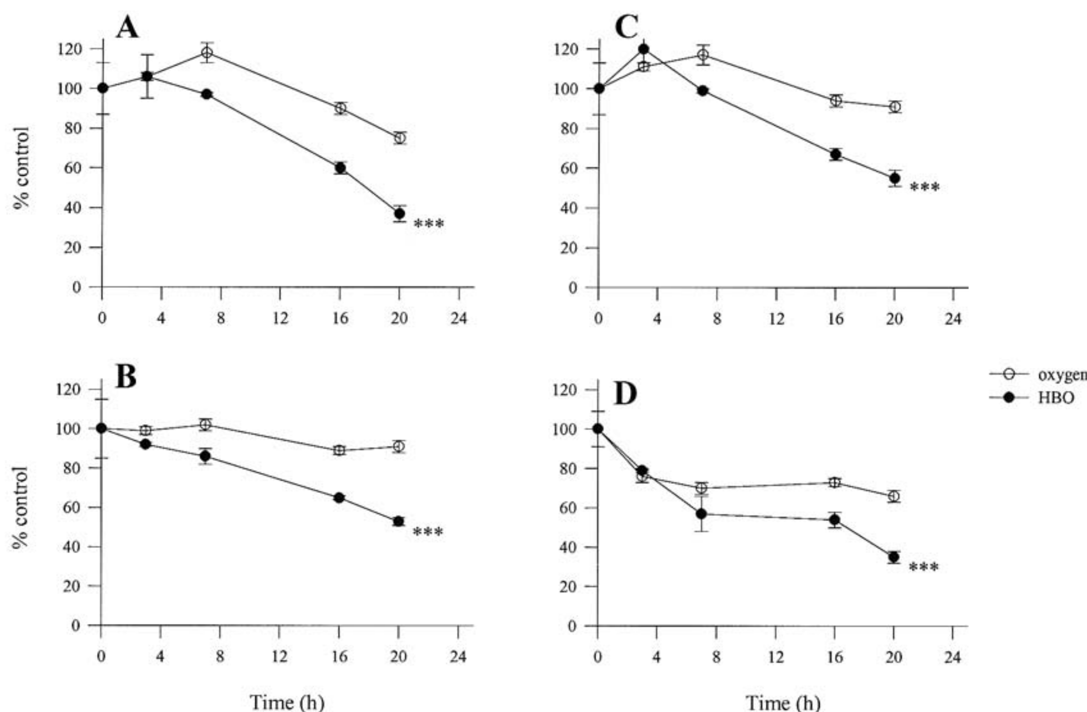

Figure 2. *HBO is a more potent inhibitor of mammary cell proliferation than hyperoxia alone as determined by the MTS assay. (A) Normal mammary epithelia, (B) primary tumor, (C) metastatic tumor, or (D) MCF7 cells (all at 5x10^4 cells/well) were cultured at 37˚C in 21% O_2, 5% CO_2 at 1 atm abs (control); 95% O_2, 5% CO_2 at 1 atm abs (oxygen); or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (HBO). At specified time-points, MTS was added to the wells of 1 control, 1 oxygen-exposed and 1 HBO-exposed plate and the OD at 490 nm measured. For each cell line, the OD for each oxygen and HBO time-point is expressed as a percentage of the OD of the control at that same time. Results for each time-point are shown as the mean±SD of samples from 3 wells. ***$p<0.001$ when comparing HBO-exposed cells to oxygen-exposed cells using factorial ANOVA. For all 4 cell lines, 1 representative of 5 experiments is shown.*

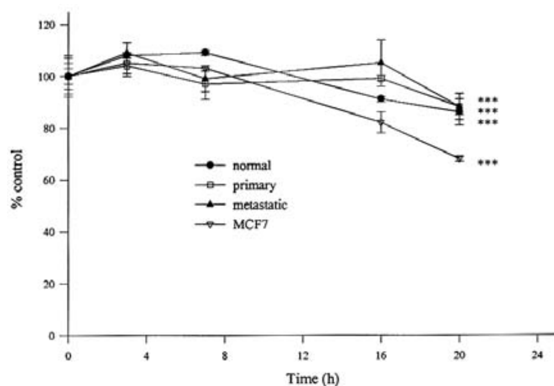

Figure 3. *Increased atmospheric pressure alone inhibits mammary cell proliferation. Normal mammary epithelia, primary tumor, metastatic tumor, or MCF7 cells (all at 5x10^4 cells/well) were cultured at 37˚C in either 21% O_2, 5% CO_2 at 1 atm abs (control) or 8.75% O_2, 2.1% CO_2 at 2.4 atm abs (pressure). At specified time-points, MTS was added to the wells of 1 control and 1 pressure-exposed plate and the OD at 490 nm measured. For each cell line, the OD for each time-point is expressed as a percentage of the OD of the control at that same time. Results for each time-point are shown as the mean±SD of samples from 3 wells. ***$p<0.001$ when comparing pressure-exposed cells to control cells using factorial ANOVA. For all 4 cell lines, 1 representative of 2 experiments is shown.*

immortalized by transfection, as well as the MCF7 mammary adenocarcinoma cell line, were used to study the effects of HBO on mammary epithelial cell proliferation. In the first experiment, the cells were cultured for 20 h in either 21% O_2, 5% CO_2 at 1 atm abs (control) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (HBO) and then cell counts were performed. As shown in Table I, HBO suppressed N, PT, MT and MCF7 cell proliferation by 35%, 18%, 25% and 36%, respectively. Next, we used a [^{3}H]thymidine assay to demonstrate that this effect is time-dependent. As expected, cells cultured under standard conditions proliferated for at least 16 h ($p<0.01$ for all 4 cell types, Figure 1). In contrast, after 7 h of HBO the cell numbers began to decline ($p<0.01$ for all 4 cell types).

HBO inhibits mammary cell proliferation more than hyperoxia alone. To control for the effects of hyperoxia, mammary cells were concurrently exposed to HBO or simple hyperoxia (95% O_2, 5% CO_2 at 1 atm abs). Because the MTS assay is an easy, accurate and non-radioactive surrogate measurement of cell proliferation (22), we used it instead of cell counting or [^{3}H]thymidine for this and subsequent experiments. In contrast to Figure 1 in which

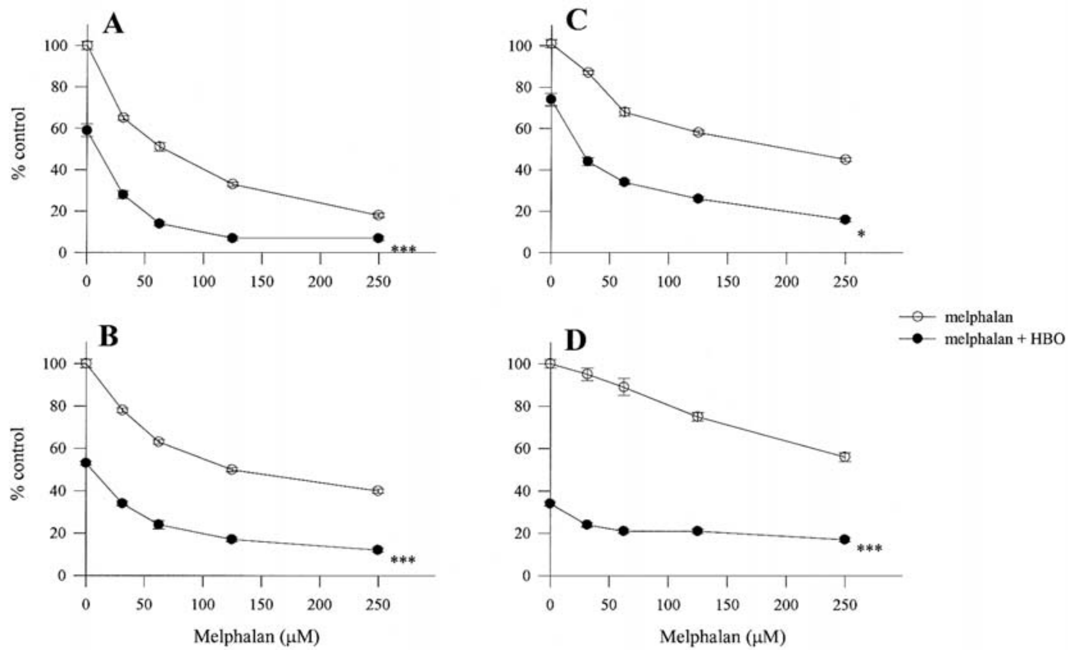

Figure 4. *HBO enhances the anti-proliferative effect of melphalan. (A) Normal mammary epithelia, (B) primary tumor, (C) metastatic tumor, or (D) MCF7 cells (all at 5x10^4 cells/well) were cultured with different concentrations of melphalan in 21% O_2, 5% CO_2 at 1 atm abs (melphalan) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (melphalan + HBO). Controls were incubated without melphalan at 21% O_2, 5% CO_2 at 1 atm abs. After a 20-h incubation, MTS was added to the wells of 1 melphalan, 1 melphalan + HBO and 1 control plate and OD at 490 nm measured. For each cell line, the OD for each melphalan and melphalan + HBO data-point are expressed as a percentage of the control OD at that same time. Results for each concentration are shown as the mean±SD of samples from 3 wells. ***p<0.001 and *p<0.01 when comparing melphalan + HBO-exposed cells to melphalan-exposed cells using factorial ANOVA.*

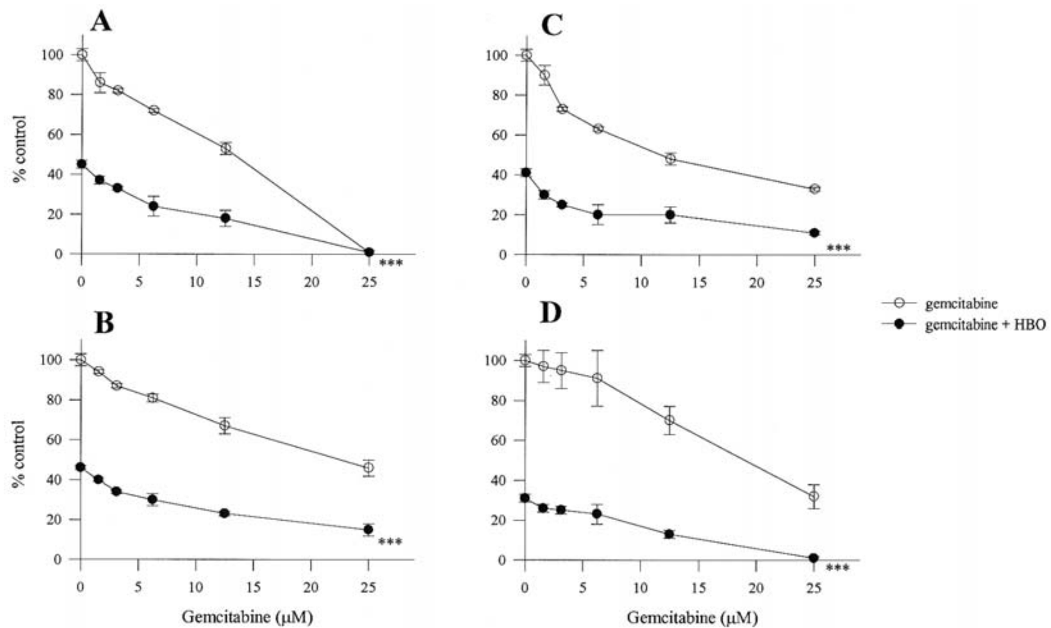

Figure 5. *HBO enhances the anti-proliferative effect of gemcitabine. (A) Normal mammary epithelia, (B) primary tumor, (C) metastatic tumor, or (D) MCF7 cells (all at 5x10^4 cells/well) were cultured with different concentrations of gemcitabine in 21% O_2, 5% CO_2 at 1 atm abs (gemcitabine) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (gemcitabine + HBO). Controls were incubated without gemcitabine at 21% O_2, 5% CO_2 at 1 atm abs. After a 20-h incubation, MTS was added to the wells of 1 gemcitabine, 1 gemcitabine + HBO and 1 control plate and OD at 490 nm measured. For each cell line, the OD for each gemcitabine and gemcitabine + HBO data-point is expressed as a percentage of the control OD at that same time. Results for each concentration are shown as the mean±SD of samples from 3 wells. ***p<0.001 and *p<0.01 when comparing gemcitabine + HBO-exposed cells to gemcitabine-exposed cells using factorial ANOVA. For all 4 cell lines, 1 representative of 2 experiments is shown.*

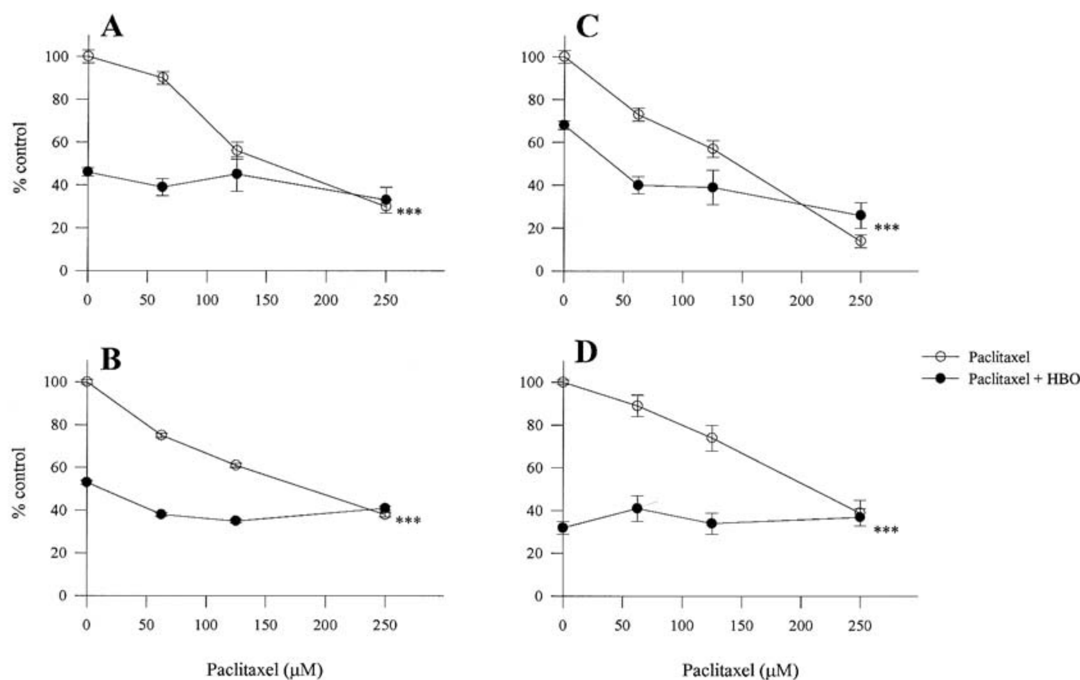

Figure 6. *HBO enhances the anti-proliferative effect of paclitaxel. (A) Normal mammary epithelia, (B) primary tumor, (C) metastatic tumor, or (D) MCF7 cells (all at 5x10^4 cells/well) were cultured with different concentrations of paclitaxel in 21% O_2, 5% CO_2 at 1 atm abs (paclitaxel) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (paclitaxel + HBO). Controls were incubated without paclitaxel at 21% O_2, 5% CO_2 at 1 atm abs. After a 20-h incubation, MTS was added to the wells of 1 paclitaxel, 1 paclitaxel + HBO and 1 control plate and OD at 490 nm measured. For each cell line, the OD for each paclitaxel and paclitaxel + HBO data-point is expressed as a percentage of the control OD at that same time. Results for each concentration are shown as the mean±SD of samples from 3 wells. ***p<0.001 when comparing paclitaxel + HBO-exposed cells to paclitaxel-exposed cells using factorial ANOVA.*

each time-point is standardized to values at time zero, each time-point presented in Figures 2-6 is standardized to control cells cultured in 21% O_2 at 1 atm abs at the same time. As illustrated in Figure 2, hyperoxia alone suppressed proliferation as compared to the control in N by 25%, PT by 9%, MT by 9% and MCF7 cells by 34%. HBO had a more profound anti-proliferative effect than hyperoxia ($p<0.001$ for all 4 cell types); cell numbers declined by 73% in N, 47% in PT, 46% in MT and 65% in MCF7 cells.

Increased atmospheric pressure alone moderately inhibits mammary cell proliferation. In another experiment, cells were incubated at standard atmospheric pressure (21% O_2, 5% CO_2 at 1 atm abs) or exposed to increased ambient pressure alone (8.75% O_2, 2.1% CO_2 at 2.4 atm abs). Figure 3 shows that pressure alone suppressed proliferation in N by 14%, PT by 12%, MT by 12% and MCF7 cells by 32% ($p<0.001$ for all 4 cell types). Because the HBO and pressure exposures both required the use of the hyperbaric chamber, the cells could not be exposed to HBO and pressure alone in the same experiment. However, comparing the data in Figures 2 and 3 shows that the proliferation of all 4 cell types was inhibited more by HBO than by increased pressure alone.

HBO enhances the anti-proliferative effects of chemotherapeutics. Experiments were performed to ascertain whether HBO could enhance the effect of chemotherapeutic drugs. N, PT, MT and MCF7 cells were cultured in the presence of increasing concentrations of melphalan, gemcitabine, or paclitaxel before being exposed to HBO for 20 h. Gemcitabine and paclitaxel were chosen because they are active agents in the treatment of breast cancer. Since cyclophosphamide, the alkylating agent commonly used to treat breast cancer, requires hepatic metabolism for activation, we used another alkylating agent, melphalan, for these experiments. We did not use doxorubicin because its red color interfered with the colorimetric MTS assay. When compared to cells cultured in normoxia at sea level (1 atm abs), HBO enhanced the anti-proliferative effects of melphalan, gemcitabine and paclitaxel ($p<0.05$ for all 3 chemotherapeutics in all 4 cell types; Figures 4-6).

HBO suppresses mammary cell colony formation. To ascertain whether the effects of HBO on mammary cell proliferation are transient or permanent, a clonogenicity assay was performed. Cells were exposed to HBO or normoxia at sea level for 12 h and then 100 cells per condition were transferred to separate wells of a new plate

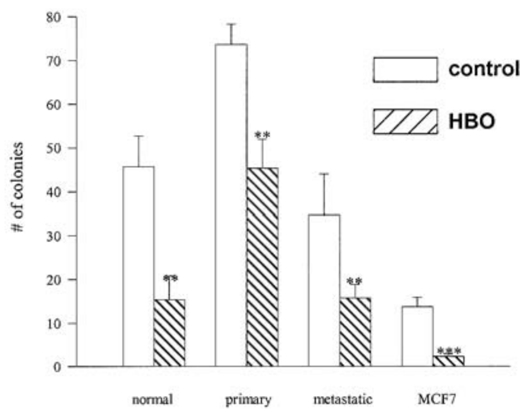

Figure 7. *HBO suppresses mammary cell colony formation. Normal mammary epithelia, primary tumor, metastatic tumor, or MCF7 cells were cultured at 37˚C in either 21% O_2, 5% CO_2 at 1 atm abs (control) or 97.9% O_2, 2.1% CO_2 at 2.4 atm abs (HBO). After a 12-h incubation, 100 adherent cells per condition were transferred to separate wells of a new plate and incubated at 37˚C in 21% O_2, 5% CO_2 at 1 atm abs for up to 2 weeks. When colonies were easily visible, the number of colonies in each well was counted as described (23). Results for each condition are shown as the mean±SD of samples from 3 wells. ***$p<0.001$ and **$p<0.01$ when comparing HBO-exposed to control cells using a Student's unpaired t-test. For all 4 cell lines, 1 representative of 2 experiments is shown.*

and incubated for up to 2 weeks under standard conditions. As shown in Figure 7, HBO-exposed N, PT, MT and MCF7 cells formed 67%, 39%, 55% and 85% fewer colonies than controls, respectively ($p<0.01$ for all 4 cell types). These findings strongly suggest that HBO's anti-proliferative effect is prolonged rather than transient.

HBO does not induce mammary cell necrosis or apoptosis. The previous experiments clearly demonstrate that exposing mammary cells to HBO resulted in decreased numbers of cells compared to the control. HBO could be exerting its effect by suppressing the cells' mitotic rate or by enhancing cell death. To ascertain whether HBO promotes cell death, we performed Hoechst-propidium iodide and annexin V-propidium iodide staining on HBO-exposed cells. Six experiments using Hoechst-propidium iodide and 6 using annexin V-propidium iodide found no increase in apoptosis or necrosis when comparing HBO-exposed to unexposed cells (data not shown). These data suggest that HBO exerts its anti-proliferative effect by inhibiting cell division rather than enhancing cell death.

Discussion

Oxygen is a potential anticancer therapeutic. Mammalian cells require oxygen to proliferate and, under certain conditions, cells also need oxygen in order to undergo programmed cell death (25, 26). Due to their rapid growth rate and limited angiogenesis, solid tumors have hypoxic areas where oxygen concentrations are very low (27, 28). While prolonged hypoxia probably slows tumor growth, it also causes these tumors to be resistant to the tumoricidal effects of radiation (29). By administering l00% oxygen at increased atmospheric pressures, HBO can increase oxygen delivery to cells *in vitro* and solid tumors *in vivo* (30). Although HBO does not deliver as much oxygen to solid tumors as it does to cells in culture, clinical trials have demonstrated that HBO significantly enhances radiation-induced loco-regional control of human head and neck (31, 32) and cervical cancer (33). Smaller trials suggested that HBO was useful in the treatment of bronchogenic cancer (34) and malignant glioma (35). Recently, we demonstrated that HBO acts directly at the cellular level by enhancing radiation-induced apoptosis (36). Due to the cumbersome nature of administering radiation to patients in primitive HBO chambers, the use of HBO in the treatment of solid tumors may have been prematurely abandoned.

In this study, we demonstrated that HBO inhibited mammary epithelial cell proliferation in immortalized cells and a well-established cell line. While using immortalized cells offers the theoretical advantages outlined earlier, these cells are not necessarily more representative of human breast cancer than the MCF7 or other mammary cell lines. Notably, in MCF7 and primary tumor cells the anti-proliferative effect of HBO alone was similar in magnitude to the effects of high concentrations of melphalan, gemcitabine, or paclitaxel alone. These findings suggest that HBO could be effective therapy for breast cancer. Previous investigators have shown that HBO suppresses the proliferation of lymphoma (37), hematopoietic (36, 38), fibroblast (39), sarcoma (15) and prostate cell lines (40), as well as primary keratinocytes (41). In contrast, HBO has been shown to enhance the proliferation of fibroblast cell lines (39), primary fibroblasts (42, 43) and primary endothelial cells (42). These divergent observations may be attributable to differences in cell types, cell density (44), or HBO protocols (39). Whether HBO inhibits or enhances the growth of any given tumor is clinically important. Fortunately, a recent review of the available clinical data concluded that HBO does not enhance malignant growth or metastasis formation (45).

The anti-proliferative effect of HBO was similar in normal breast epithelial cells, primary tumor cells and metastatic tumor cells derived from the same patient. This finding suggests that, under the conditions of this study, HBO's effect was not especially dependent upon the state of cellular transformation and that HBO affects a conserved cellular pathway rather than one which arises during carcinogenesis. Because the p53 tumor suppressor is rapidly degraded in these cells, our data also demonstrated that

HBO does not require functional p53 to exert its anti-proliferative effect. This finding suggests that HBO could be an effective therapy for both p53-wild-type and p53-deficient breast cancers.

When compared to HBO, hyperoxia alone had a significant, but less profound, anti-proliferative effect on mammary epithelial cells. Depending upon the cell type studied, HBO had a 2- to 5-fold greater anti-proliferative effect than hyperoxia. Other investigators have shown that fibroblast (46), endothelial cell (47, 48) and smooth muscle cell (49) proliferation is also blocked by prolonged hyperoxia. Hyperoxia could be exerting its effect by affecting the cell cycle (46) or by enhancing cell death *via* necrosis (50) or apoptosis (51).

While it is possible that HBO's anti-proliferative effect is simply due to enhanced oxygenation, our data suggest that atmospheric pressure has an independent effect on cell number. In this study, we found that normoxia at increased atmospheric pressure modestly suppressed mammary cell proliferation. However, it should be recognized that the changes seen with increased pressure alone could be due to the study design. Cells cultured in 8.75% O_2 were briefly hypoxic while the atmospheric pressure increased from 1 to 2.4 atm abs. This short period of hypoxia could have retarded cellular proliferation when the ambient oxygen returned to normal. Further support for an independent effect of increased ambient pressure comes from previous experiments showing that modest increases in atmospheric pressure cause a small increase in radiation-induced hematopoietic cell apoptosis (36) and a decrease in *in vitro* (52) and *ex vivo* cytokine synthesis (53). Other investigators, using very high atmospheric pressures, have also demonstrated that pressure inhibits proliferation (54, 55) and induces apoptosis (56).

As previously discussed, there are *in vitro* (36) and *in vivo* (31-34) studies showing that HBO enhances the anti-proliferative effects of radiation. While coordinating radiation and HBO treatments could be cumbersome for cancer patients and providers, combining chemotherapy and HBO is more technically feasible. Melphalan (21), gemcitabine (57) and paclitaxel (21, 58) kill breast cancer cells by triggering apoptotic pathways in cells unable to do so themselves. In this study, we found that HBO augmented the anti-proliferative effects of these 3 agents. Our findings are consistent with previous observations that HBO enhanced the anti-proliferative effect of doxorubicin in Burkitt's lymphoma cells (37) and DU-145 prostate cells as well as paclitaxel in PC-3 prostate cells (40). In animals, HBO has been shown to enhance the tumoricidal effect of chemotherapy in spontaneous mammary tumors (6, 10) and other cancers (7-9, 11-15). A small, non-randomized trial of HBO and chemotherapy showed improved survival in patients with hepatic cancer (59). The pronounced anti-proliferative effect seen with the combination of HBO and chemotherapy may be, at least in part, attributable to reactive oxygen species. Administering HBO might enable patients to be treated with lower doses of chemotherapy and thereby suffer fewer side-effects.

The *in vitro* experiments presented in this study confirm a direct effect of HBO on mammary epithelial cell proliferation. Our clonogenicity assay demonstrated that this effect was not due to toxicity and was persistent rather than transient. The persistence of HBO's effect suggests that it may be transmissible to daughter cells. Based upon previous work showing that HBO enhanced apoptosis in hematopoietic cells, we used Hoechst-propidium iodide and annexin V-propidium iodide staining to look for evidence of increased apoptosis or necrosis. Multiple experiments showed no evidence that HBO enhanced cell death. While these experiments did not identify a mechanism whereby HBO inhibited the increase in mammary cell numbers, we believe it is important to know the inhibition is not mediated by inducing cell death. Although we demonstrated that HBO's anti-proliferative activity does not require functional p53, the mechanism whereby HBO suppresses proliferation requires further investigation. Slowing of the cell cycle is one possible mechanism. If HBO induces cell senescence, it might make breast cancer cells less likely to metastasize. The additive effect of HBO and chemotherapeutic agents also merits further study to see if similar results can be achieved with other chemotherapeutic agents and in humans. It would be useful to measure tissue oxygenation in breast cancers *in vivo* both before and during hyperbaric oxygenation. We hope that, ultimately, this research will lead to clinical trials of HBO in women with refractory breast cancer.

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