EFFECTS OF HYPERBARIC OXYGEN EXPOSURE ON EXPERIMENTAL HEAD AND NECK TUMOR GROWTH, OXYGENATION, AND VASCULATURE

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Abstract: *Background.* Hyperbaric oxygen (HBO_2) is used to promote healing in irradiated tissues, but concern persists about the possibility that it may promote residual tumor growth.

Methods. The tumor growth of SQ20B and Detroit 562 head and neck squamous cell carcinoma xenografts were studied after single-dose irradiation and 5×/week HBO_2 treatment at 2.4 atm absolute for 90 minutes. The effect of HBO_2 treatment on tumor hypoxia and vasculature was also examined by immunohistochemical analysis.

Results. HBO_2 treatment increased tumor oxygenation during the treatment interval but did not promote the growth of either irradiated or unirradiated tumors. No increase in tumor vascular endothelial growth factor expression or vascularization was detected.

Conclusions. This study found no evidence for persistent changes in tumor microenvironment or tumor growth promotion caused by hyperbaric oxygen exposure. © 2005 Wiley Periodicals, Inc. *Head Neck* **27:** 362–369, 2005

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The predominant use of hyperbaric oxygen (HBO_2) in the context of cancer therapy has been to improve healing of normal tissues after radiation injury or to promote surgical wound healing in a previously irradiated site (recently reviewed by Feldmeier and Hampson[1]). In particular, HBO_2 treatment is often used in patients with head and neck cancers who have received high doses of radiation and for whom further surgery or dental extraction is required. A proposed mechanism for the effect of HBO_2 on tissue healing is that it creates an oxygen gradient that promotes increased angiogenesis within the hypoxic irradiated tissue.[2,3] However, this raises a concern regarding the use of HBO_2 in the setting of cancer therapy, because such an effect would have the potential for promoting the growth of residual tumor. Several clinical case reports have described rapid progression of tumors occurring after HBO_2

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treatment both within the previously irradiated area and outside of the irradiated area.[4–7] However, on the basis of presently available clinical data, defining or ruling out a causal relationship between HBO_2 treatment and the rapid tumor progression reported in these cases is not possible. Many studies have been conducted in animal models to determine whether HBO_2 has a growth-promoting effect on tumors (reviewed in Feldmeier et al[8]). McMillan et al[9] reported that HBO_2 inhibits the initiation of tumor growth but enhances the growth of pre-existing tumors. However, there is no direct evidence that HBO_2 promotes angiogenesis in tumors, which could promote enhanced growth of established tumors.

When used concurrently with radiation, HBO_2 treatment has been shown to enhance tumor killing in experimental models.[10] In addition, more than a dozen clinical trials have been conducted using HBO_2 treatment concurrent with irradiation in an effort to radiosensitize tumors by increasing their oxygenation at the time of treatment. Although the results of these trials were mixed, a meta-analysis carried out by Overgaard and Horsman[11] of 17 trials including a total of 2026 patients found a 6% increase in locoregional control in the patients receiving HBO_2 treatment. This result was statistically significant.

The effects of HBO_2 treatment on tumor growth when administered subsequent to the course of irradiation have not been as thoroughly studied. Animal studies to date have not examined the effect of HBO_2 exposure on irradiated tumors and, therefore, do not model the typical clinical situation in which HBO_2 is used to reduce radiation-induced normal tissue damage. In this study, we examined HBO_2 treatment–induced changes in tumor oxygenation, changes in tumor vascular density, and expression of the angiogenic growth factor vascular endothelial growth factor (VEGF). Because HBO_2 therapy is commonly used in patients treated for head and neck cancers, we also compared the growth rates of irradiated Detroit 562 and SQ20B human head and neck cancer xenografts tumors that were treated with HBO_2 at a clinically relevant pressure (2.4 atm absolute) and schedule (90 min/day, 5 days per week) with tumors receiving sham treatment.

MATERIALS AND METHODS

Cell Culture. SQ20B and Detroit 562 cell lines were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cultures were maintained at 37°C in incubators with 5% CO_2 and 100% relative humidity. Cells were tested for mycoplasma and for murine pathogens (Research Animal Diagnostic and Investigative Laboratory Impact I profile, University of Missouri). Cells for inoculation were harvested by mild trypsinization, counted, and resuspended in matrigel (B-D Collaborative Research, Franklin Lakes, NJ) immediately before injection.

Reagents. To monitor tumor hypoxia, EF5 [2-(2-nitro-1H-imidazole-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)acetamide], 10 m*M* in 5% dextrose, was administered intravenously at a dose of 0.01 mL/g immediately before the last HBO_2 treatment. EF5 binding was detected using a fluorochrome (Cy3)-conjugated monoclonal antibody specific for EF5 (ELK3-51). Measurement of hypoxia using this system has been detailed previously.[12–14] All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and were of reagent or tissue culture grade.

Tumor Generation and Irradiation. Pathogen-free male Ncr-nu/nu mice were obtained from Taconic Labs (Germantown, NY). At 5 weeks of age, mice were inoculated by subcutaneous injection into the right thigh with 3×10^6 Detroit 562 cells or 6×10^6 SQ20B cells in 100 μL of matrigel suspension. Tumors were measured twice a week with calipers in three mutually perpendicular diameters (a, b, and c), and the volume was calculated as $V = (\pi/6) \times a \times b \times c$. Treatment of newly inoculated mice was initiated 1 day after tumor injection. In studies of established tumors and tumor regrowth after irradiation, treatment (irradiation and HBO_2) was initiated after tumors were established and reached a defined volume (approximately 30 mm^3 Detroit 562 or 90 mm^3 SQ20B). Tumor irradiation was performed with a 250-kV orthovoltage irradiator (Philips RT 250) through a 0.2-mm copper filter with shielding of nontumor sites. The source-to-tumor target distance was 30 cm, with a monitored dose rate of 2.6 Gy/min for a total single fraction dose of 6 Gy.

All studies were carried out under University of Pennsylvania Animal Care and Use Committee–approved protocols.

HBO_2 Treatment. Mice in fully ventilated stainless steel cages were exposed to HBO_2 or pressurized air in a 41-L steel hyperbaric chamber

(manufactured by Thermatech Ltd., Kaukauna, WI). The chamber was flushed with 100% oxygen for 3 minutes at normal pressure, followed by gradual compression to 2.4 atm absolute pressure at 100% oxygen over 3 minutes. HBO_2 exposure was maintained at 2.4 atm for 90 minutes with continuous gas exchange (>5 L/min flow rate), followed by gradual decompression over 3 minutes. Control animals underwent compression and decompression as described previously under air. Pressurization for control groups was maintained for 10 minutes. Treatments were given daily, five times per week until termination of the studies dictated by tumor burden. The range of treatment times was from 13 days (Figure 2, SQ20B unirradiated controls) to 28 days (Figure 1, Detroit 562).

For tumor hypoxia studies, animals received a single intravenous EF5 injection immediately before hyperbaric treatment. Animals were killed 90 minutes after EF5 injection at the conclusion of HBO_2 exposure, and tumors were snap frozen in embedding medium for sectioning.

Determination of Hypoxia and Vascular Density in Tumors. Frozen tumor specimens were sectioned (10 μ*M*) by cryostat and fixed in 4% paraformaldehyde for 60 minutes at 0°C. Sections were then stained with the EF5-specific Cy3-conjugated ELK3-51 mouse monoclonal antibody.[15] For vessel studies, sections were stained with rat anti-mouse vascular epithelial cell antigen (CD31) monoclonal antibody (Pharmingen, San Diego, CA), which stains for the platelet/endothelial cell adhesion molecule 1 (PECAM-1), followed by affinity-purified mouse anti-rat immunoglobulin (IgG) (Jackson ImmunoResearch, West Grove, PA). CD31-stained sections were re-fixed in 4% paraformaldehyde (20 minutes at 0°C) and stained with Cy3-conjugated ELK3-51 for photomicroscopy and vessel analysis. Images were acquired under epifluorescent illumination as described previously.[16] Quantitation was accomplished by acquiring gray-scale TIFF images with an intensity scale from 0 to 255. Pixel intensities of each image were used to derive the final absolute fluorescence intensity using the following equation:

$$I_{\text{corrected}} = \frac{I_{\text{image}}}{I_{\text{standard}}} \times 1000 \times \frac{T_{\text{standard}}}{T_{\text{image}}}$$

where I is the measured intensity and T is exposure time. Vascular densities were analyzed using OpenLab software and are expressed as the ratio between the sum of CD31-positive (vascular) area and the total area of the tumor tissue in each image.

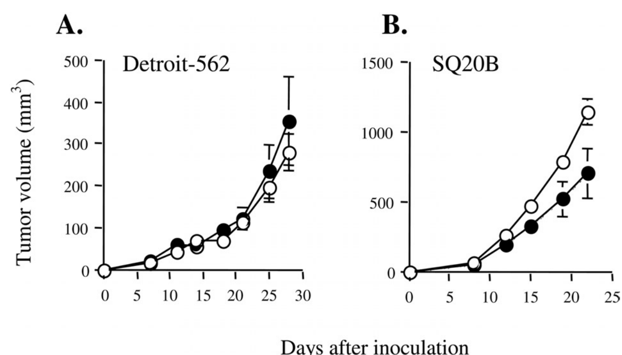

FIGURE 1. HBO_2 treatment does not promote growth in newly inoculated tumors. HBO_2 treatment was started 1 day after subcutaneous inoculation of nude mice with tumor cells (3×10^6 Detroit 562 cells or 6×10^6 SQ20B cells suspended in matrigel) and continued daily (5 days/week) until the end of the experiments (28 days for Detroit 562 and 23 days for SQ20B). Open symbols represent control tumors. Solid symbols represent HBO_2-treated tumors. Tumor measurements were made in three perpendicular directions. The volumes were derived by multiplying these three measurements and correcting to a spheroid using a factor of π/6. Error bars denote standard errors. (**A**) Detroit 562 tumors. Each treatment group contained seven mice. (**B**) SQ20B tumors. There were six mice per treatment group.

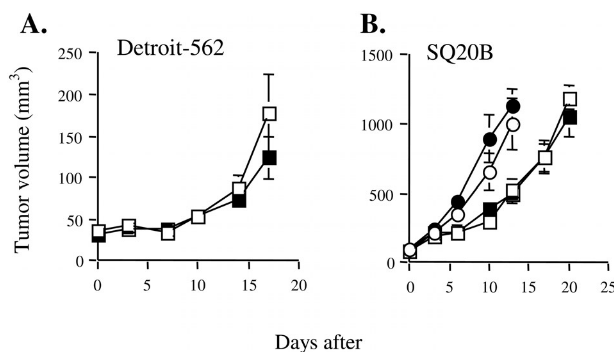

FIGURE 2. HBO_2 treatment does not promote the growth of established or irradiated tumors. Established tumors were irradiated with 6 Gy (square symbols, A and B) or sham-irradiated (circles, B only). HBO_2 treatment was started 1 day after irradiation of tumors and continued until the end of the experiments. Solid symbols represent HBO_2-treated tumors. Open symbols represent tumors that did not receive HBO_2. Error bars denote standard errors. (**A**) Detroit 562 tumors. Each treatment group contained 14 mice. (**B**) SQ20B tumors. There were six mice per treatment group, with the exception of the irradiated, HBO_2-treated group, which had seven mice.

Protein Extraction and Western Blot Analysis. Protein isolation and quantitation and Western blotting were performed as described previously.[17] VEGF was detected using a mouse mono-

clonal antibody (AB-4, LabVision Corp, Freemont, CA). Protein loading was verified by reprobing membranes with a mouse anti-β actin antibody (Sigma). Primary antibody binding was detected with peroxidase-labeled sheep anti-mouse antibody. Blots were developed by use of enhanced chemiluminescence (ECL; Amersham Corp., England).

RESULTS

HBO_2 Does Not Promote Growth of Nonirradiated or Preirradiated Tumors. The primary question that we set out to answer was whether HBO_2 exposure delivered at a clinically relevant pressure and schedule would promote the growth of human head and neck carcinoma xenograft tumors. The experimental HBO_2 treatment was based on a common clinical protocol in which patients are treated daily for 90 minutes 5 days a week with 2.4 atm absolute. We tested the effect of this treatment on tumor growth by examining both established and newly inoculated head and neck squamous cell carcinoma xenograft tumors grown in nude mice. These two situations mimic residual established tumor, as well as newly seeded tumor cells that have yet to establish tumor vasculature. The growth rates of the newly inoculated tumors treated daily with 90 minutes HBO_2 or receiving sham treatment of 10 minutes compressed air did not differ significantly (Figure 1). If anything, there was a slight delay in the growth of HBO_2-treated SQ20B tumors relative to controls. To test for HBO_2 effects on established tumors and tumors that were irradiated, the experiment was modified so that tumors were allowed to grow to a palpable size (approximately 50 mm^3), at which time they were irradiated with 6 Gy. A single fraction of 6 Gy was chosen, because this dose is noncurative but yields a measurable growth delay in these tumor xenografts. HBO_2 treatment was initiated the day after irradiation and continued daily to the end of the experiment. In this protocol, HBO_2 treatment had no significant effect on tumor growth for either Detroit 562 (Figure 2A) or SQ20B (Figure 2B) tumors after irradiation. The effect of HBO_2 treatment on established SQ20B tumors was also examined in the absence of irradiation. No significant difference in growth was seen between HBO_2-treated and air-treated control groups in these unirradiated tumors (Figure 2B).

HBO_2 Exposure Transiently Reduces Hypoxia in Head and Neck Tumor Xenografts. The preceding experiments demonstrated that HBO_2 therapy did not increase tumor growth. To document whether tumor oxygenation was altered in this model by HBO_2 treatment, we examined tumor hypoxia using the nitroimidazole EF5, which forms covalent protein adducts in viable hypoxic cells in a manner that is inversely proportional to oxygen concentration in the physiologic range. This reagent has been used extensively to assess tumor oxygenation in a number of studies.[12–14] To detect HBO_2-mediated changes in tumor oxygenation, EF5 was injected immediately before HBO_2 treatment. Images of tumors were acquired after immunohistochemical staining for EF5 (Figure 3), and the data are presented as the staining intensities of the brightest 50% of cells and the brightest 5% of cells on an absolute fluorescence scale (Table 1). The levels of staining in sham-treated xenografts were within the expected range. Control Detroit 562 tumors showed 50th and 95th percentile staining intensities of 42 and 83, respectively. These values were both reduced during HBO_2 treatment (to 21 and 33, respectively). Comparison of HBO_2 or control treatment SQ20B tumors also showed a reduction in hypoxia at the 50th percentile in one experiment in which tumors had only moderate levels of hypoxia. A second experiment with

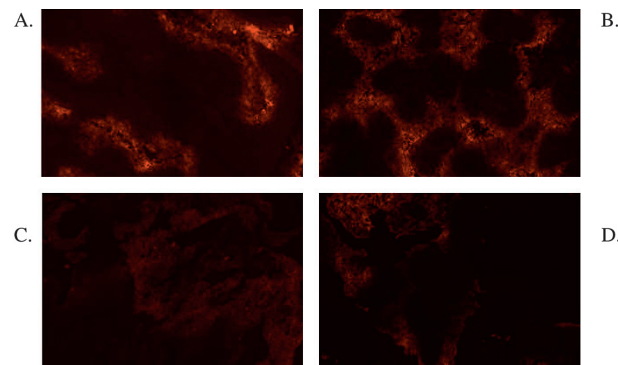

FIGURE 3. HBO_2 exposure reduces hypoxia in head and neck squamous cell carcinoma xenograft tumors. Top panels (**A** and **B**): Control animals were injected with EF5 in parallel to HBO_2–treated mice but were not exposed to hyperbaric oxygen. Bottom panels (**C** and **D**): The hypoxic cell marker EF5 was injected intravenously just before HBO_2 treatment. Three hours after EF5 injection (90 minutes after completion of HBO_2 treatment) tumors were harvested for analysis. Frozen tissue sections were stained for EF5 binding with the EF5-specific monoclonal ELK3-51 (red). Left (**A**. **C**.): SQ20B tumors. Right (**B**. and **D**.): Detroit 562 tumors. Images intensities have been corrected for differences in exposure times.

Table 1. Quantitation of tumor hypoxia during HBO_2 treatment.

	Fluorescence measurements	
	50th percentile intensity	95th percentile intensity
Detroit 562		
Control	42 ± 12	83 ± 10
HBO_2 treated	21 ± 3	33 ± 4
SQ20B		
Experiment 1		
Control	34 ± 1	54 ± 2
HBO_2 treated	25 ± 4	52 ± 5
Experiment 2		
Control	52 ± 11	79 ± 19
HBO_2 treated	37 ±/−6	65 ±/−12

Note. Fluorescence intensity measurements were made from EF5/ELK3-51 stained tumors. The numbers presented are the mean fluorescence intensities for cells in the 50th and 95th percentile of EF5 binding within the tumor. Detroit tumors: control, n = 2; HBO, n = 3. SQ20B tumors: exp. 1, control, n = 8; HBO, n = 6; exp. 2, control, n = 4, HBO, n = 3. Details of image acquisition and analysis have been published previously.[30]

SQ20B tumors, in which tumors were more hypoxic at the time of treatment, showed an effect on both 50th and 95th percentile staining, although intertumor variability was present (Table 1). The results of these studies show that tumor reoxygenation did occur during HBO_2 exposure in these two tumor xenograft models.

HBO_2 Exposure Does Not Alter Vascular Endothelial Growth Factor Expression or Vascularization of Tumor Xenografts. One hypothesis for the mechanism of HBO_2 activity is that it promotes angiogenesis by increasing VEGF expression.[18] To test whether this was seen in head and neck squamous cell carcinoma xenografts, we examined whether tumor vascularization or VEGF expression was altered by HBO_2 treatment. Protein samples were collected from tumors treated with HBO_2 for 4 weeks (20 treatments; Detroit 562) or 3 1/2 weeks (17 treatments; SQ20B) and analyzed by Western blotting. VEGF protein levels did not differ between HBO_2-treated and air-treated tumors, although there was variation in VEGF levels between individual tumors within the different groups (Figure 4). These data show that there was no consistent alteration in VEGF expression after HBO_2 exposure in these two tumor models. We then examined whether HBO_2 promotes tumor angiogenesis in these tumors. Endothelial cells in frozen tumor sections were stained using an antibody to the platelet endothelial cell adhesion molecule-1 (PECAM-1 CD31 antigen) (Figure 5A, top). The area of CD31 staining was determined after thresholding of the signal (Figure. 5A, bottom), and this was used to quantify vascular density. Similar vascular density was observed between HBO_2-treated and control tumors both in Detroit 562 (Figure 5B) and SQ20B (Figure 5C).

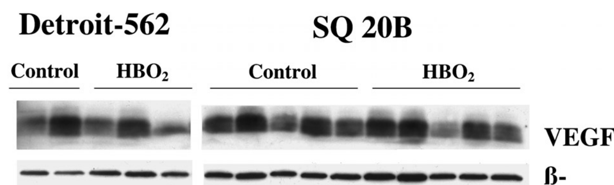

FIGURE 4. Vascular endothelial growth factor (VEGF) expression is not altered by HBO_2 treatment. Proteins were extracted from frozen tumors and analyzed by Western blotting for VEGF. Equal sample loading was monitored by probing for β-actin.

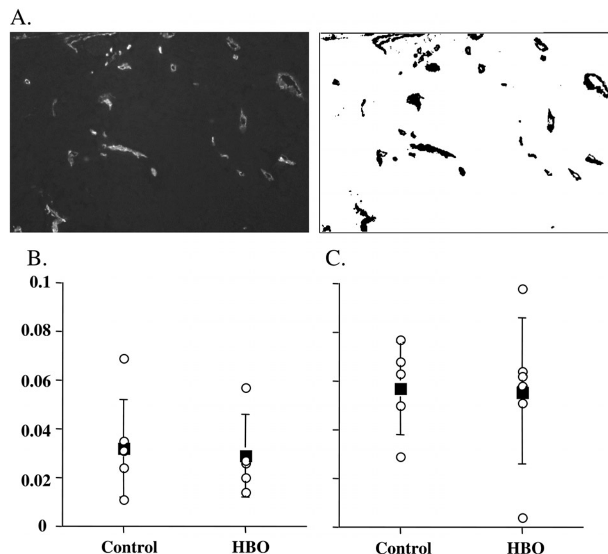

FIGURE 5. Tumor vascularization after HBO_2 therapy. (**A**) Frozen tissue sections were stained for vascular endothelium using monoclonal to CD31 (Pharmingen). The left panel shows the original image acquired from fluorescence microscopy of an SQ20B control tumor. The right panel shows the vascular area in this tumor section defined by thresholding of the original image. Tumors treated for 28 days (Detroit 562, **B**) or 23 days (SQ20B, **C**) with HBO_2 were harvested 90 minutes after the last treatment. Vascular density is reported as the ratio of vascular area to total tissue area. Vascular density was analyzed using OpenLab software (Improvision). Open circles show individual tumors. Solid squares represent mean values of each treatment group. Error bars indicate 1 standard deviation from the mean.

DISCUSSION

Numerous well-accepted indications for HBO_2 exist, including carbon monoxide poisoning, compromised skin grafts, acute traumatic wounds,

chronic nonhealing diabetic ulcers, crush injuries, burns, gas gangrene, and compartment syndrome.[19–23] HBO_2 has also been used in patients with radiation-induced late effects such as osteoradionecrosis and soft-tissue necrosis in an effort to promote tissue healing (reviewed in Feldmeier and Hampson[1]). However, when given in patients who have been treated for cancer, there has been a concern that increasing tissue oxygenation may promote the growth of occult tumor cells, leading to tumor recurrence. In a series of patients with cervical carcinoma treated with radiotherapy and HBO_2 for radiosensitization, Johnson and Lauchlan[7] reported a higher than expected incidence and unusual pattern of metastases. In response to their data, several groups investigated the effects of HBO_2 in animal models. Two studies have shown some evidence of increase in the growth of the primary tumor or in distant metastases.[9,24] In the report by Shewell and Thompson,[24] there was an increase in pulmonary metastases in animals with spontaneous tumors treated with HBO_2 compared with air but no increase in metastases in animals with transplanted tumors. In the study by McMillan et al,[9] HBO_2 was associated with a decrease in the *number* of tumors but an increase in their *size* in an anthracene-induced hamster cheek pouch model. The authors hypothesized that the tumor-enhancing effect was due to increased oxygenation in tissues and increased angiogenesis in tumors. However, more than a dozen studies have shown either no enhancement by HBO_2 treatment on the growth of tumors or a slight inhibition in animal models (reviewed in Feldmeier et al[25]).

Despite these animal studies, there are still reservations about referring patients with a history of malignancy for HBO_2 therapy. This skepticism is largely due to anecdotal reports in the literature that followed the initial report by Johnson and Lauchlan in 1966.[7] Bradfield et al[5] reported on four patients whose head and neck cancers progressed rapidly after HBO_2 exposure. Another report cited a single patient with cervical carcinoma in which the possibility was raised that HBO_2 treatment contributed to rapid recurrence.[6] Overall, these reports cannot be considered conclusive.

Additional questions about a possible effect of HBO_2 treatment on tumor growth arise, because HBO_2 exposure does seem to influence the production of growth factors in normal cells. In an in vitro study of fibroblasts by Kang et al,[26] basic fibroblast growth factor (bFGF) and VEGF expression were increased on the first day of HBO_2 exposure but returned to control levels by day 3 and remained there through the 7-day time course of the experiment. Enhanced fibroblast growth was not generally observed in cells treated at 1 to 3 atm HBO_2, with the exception of the 2 atm treatment group, which showed increased cell numbers on days 5 and 7 of the assay. Increases in VEGF production have been reported in wounding models.[18] In a porcine partial-thickness skin graft model, Kalns et al[27] found the converse to be true. VEGF expression and vascularization were reduced over the short term by HBO_2 treatment. Our results in tumors in which VEGF expression is abundant in the absence of HBO_2 exposure demonstrate no change caused by HBO_2 treatment. The difference between the results in normal wound and cell models and those obtained in tumors may relate to the genetic deregulation of angiogenesis in tumors. Although a wound does promote expression of VEGF and other growth and angiogenic factors, the upregulation is still controlled and seems to respond to changes in oxygenation to a greater extent than that observed in the tumors examined here.

Because there is some lingering doubt as to whether HBO_2 therapy is safe for patients with possible residual cancer, and because the effect of HBO_2 therapy on tumor regrowth after irradiation has not been reported, we decided to rigorously investigate these questions in an animal model. We used two different head and neck squamous cell carcinoma lines, SQ20B and Detroit 562, and subjected mice to HBO_2 treatment after subcutaneous implantation of the tumor cells. HBO_2 therapy was initiated either 1 day after inoculation to mimic microscopic occult disease or at a time when the tumors had grown to a macroscopic size to mimic a nest of established residual disease. In some cases, tumors were irradiated before the onset of HBO_2 therapy. These experimental designs thus simulated the case of a patient previously treated with radiation therapy, who had a recurrence of the tumor and might be treated with HBO_2 therapy before an attempted salvage operation. In none of our experiments did we find that HBO_2 therapy increased the rate of tumor growth.

Because HBO_2 therapy has been shown to increase tissue oxygenation,[28,29] we next determined whether HBO_2 therapy increased tumor oxygenation in our model. We used the drug EF5 to directly assess oxygenation. As expected, HBO_2 treatment led to increased tumor oxygenation. Other studies have obtained results using Eppen-

dorf oxygen electrodes in patients during and immediately after HBO_2 exposure that are similar to ours.[30] Thus, although tumors in our experiments were better oxygenated during HBO_2 therapy, this did not promote their growth. In support of this, we did not find that repeated HBO_2 therapy increased tumor VEGF expression or caused changes in tumor vascularization, consistent with the idea that the transient increase in tumor oxygenation does not lead to long-term effects in tumors.

Our studies are in agreement with a number of other animal studies that have shown no increase in tumor growth after HBO_2 therapy. We have extended these observations by using HBO_2 therapy in mice whose tumors have been irradiated, thus more faithfully recapitulating the clinical situation in which HBO_2 is used to attenuate radiation-induced normal tissue damage. In contrast to the earlier studies, we have carefully examined tumor vascularization, VEGF expression, and tumor oxygenation to show that HBO_2 therapy does not result in long-term alterations in these parameters known to be important in tumor growth. In conclusion, our laboratory studies agree with most clinical studies that have failed to show an enhancing effect of HBO_2 therapy on tumor growth. Furthermore, our data provide experimental evidence that the growth of residual tumor is not enhanced by HBO_2 treatment, whether the tumor has been previously irradiated or not.

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