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Hyperbaric oxygen alone or combined with 5-FU attenuates growth of DMBA-induced rat mammary tumors

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Abstract

We tested the hypothesis that hyperbaric oxygen (HBO) alone and with chemotherapy (5-FU) attenuates tumor growth of DMBA-induced tumors in rats. Six series were performed: (1) Controls (air and vehicle 0.9% NaCl i.p.), (2) 5-FU (0.2 mg/kg i.p.), (3) HBO (2 bar for 90 min and vehicle), (4) HBO and 5-FU, (5) HBO (11 days) and air (next 12 days), (6) HBO (23 days). All treatments were applied on days 1, 4, 7, 10 (Series 1–4), as well as on days 14, 17 and 23 (Series 5–6). Tumor diameter increased by 76.7 and 41.2% in untreated controls and in the 5-FU group, respectively, after 10 days. Tumor size fell by 17–24.2% in the HBO groups and by 35.5% when combined with 5-FU ($P < 0.05$ compared to HBO). HBO treatment reduced the total number of blood vessels in the tumors. After completion of HBO treatment tumor size increased, but statistically insignificant, during the next 12 days.

Keywords: Hyperbaric oxygen; 5-FU; Tumor-reduction; Angiogenesis; Immunohistochemistry; Rats

1. Introduction

Failure of radiotherapy as well as chemotherapy of solid tumors has been ascribed to the presence of insufficiently perfused areas of the tumor [1–3]. Hyperbaric oxygen (HBO) has been shown to increase tumor radiosensitivity both in basic and clinical studies [4–7]. This potentiation is believed to reflect an increase in the proportion of well-oxygenated tumor cells, by an increase in the amount of dissolved oxygen in the plasma [7]. It has also been proposed to reflect an induced angiogenesis, since repeated hyperbaric oxygen exposures have been demonstrated to increase vascular density in ischemic, necrotic and gangrenous tissue [8–10]. HBO can therefore probably also be used in conjunction with chemotherapy to overcome resistance by increasing both uptake of the anticancer drug by the tumor and the susceptibility of tumor cells to the drug. Tissue pO_2 values after HBO breathing have been previously shown to increase from an average of 33 up to 221 mm Hg after 60 min at 2 bar in humans. Most importantly, the pO_2 was maintained at 10% or more above pre-compressed values for more than 3 h, which is very different from arterial blood where pO_2 is rapidly normalized [11]. Elevated pO_2 is expected also in malignant tumors

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and an HBO effect should therefore continue for some time after treatment. HBO can be toxic and several studies have investigated if HBO in conjunction with radiotherapy could have a cancer promoting effect [12,13]. However, published information failed to support a growth-enhancing effect by HBO [14]. Nevertheless, a few studies have shown a suppressive effect of HBO on tumor growth [15–17]. Against this background, we asked the following questions studying DMBA-induced mammary tumors in rats: Does HBO by itself have a effect on tumor growth and angiogenesis? If so, how many exposures are required to obtain maximal effect and what happens after the HBO exposures are completed? Would HBO enhance the effect of chemotherapy (5-FU)?

2. Material and methods

2.1. Animal and tumors

Female Sprague–Dawley (Møllegård, Denmark) rats ($n = 44$) were given 16 mg dimethyl-α-benzanthracene (DMBA) dissolved in olive oil by gavage at the age of 7 weeks [18]. The experiments were performed when the rats were 13–15-weeks-old, having reached a body weight of 250–300 g and developed multiple tumors. Only one tumor from each animal was used. The experiments were approved by the Norwegian Committee for Animal Research.

2.2. Treatment

Series	n	Gas	pO_2 (bar)	Drug	Duration days
1	8	Air	0.2	NaCl	11
2	14	Air	0.2	5-FU	11
3	8	HBO	2.0	NaCl	11
4	8	HBO	2.0	5-FU	11
5	8	HBO	2.0	NaCl	11
		Air	0.2	NaCl	12
6	8	HBO	2.0	NaCl	23

The HBO and drugs were given on day 1, 4, 7, 10 (Series 1–4), in addition to 14, 17 and 23 (Series 5–6). In Series 4 the HBO treatment was administered immediately after 5-FU injection.

2.3. Measurement of tumor growth

Tumor-size was measured externally by calipers, during a short isofluran (Rhone-Poulenc Chemicals) and N_2O anesthesia in an anesthetic chamber (Ohmeda: BOC Health Care, Weast-Yorkshire, England). The volume of the tumor was calculated as: $\pi/6 \cdot (a)^2(b)$, where a is the smallest and b is the longest length of the tumor.

2.4. Hyperbaric chamber and exposure procedure

The rats were placed in the hyperbaric chamber in litter-free cages ($590 \times 385 \times 200$ mm^3) and showered lightly with water prior to entering the pressure chamber, to avoid the danger of fire in a pure oxygen atmosphere. The oxygen concentration in the chamber was monitored continuously by an oxygen cell (C3, Middelsborough, England). When 98% oxygen was reached the chamber was pressurized with oxygen over approximately 5 min to 2 bar, and this pressure was maintained for 90 min. The chamber atmosphere was flushed for 5 min at 30 and 60 min with pure oxygen. The temperature and humidity were held at approximately 22°C and 100%, respectively.

2.5. Morphology

After the rats were killed by pentobarbital, sections from the primary tumors were fixed in 4% phosphate buffered formalin, embedded in paraffin and stained with haematoxylin and eosin. The sections (between epithelial cells, in the capsule and stroma) were examined for inflammatory reactions (density of lymphocytes and granulocytes). Inflammation was graded semi-quantitatively as: 0; no inflammation, 1; slight, 2; moderate and 3 severe inflammation.

2.6. Immunohistochemical vessel staining and microvessel density

Frozen sections (5 μm) were fixed in acetone for 10 min and incubated for 60 min with the mouse anti-rat CD-31 (550300, Pharmingen) diluted 1:200, at room temperature. The reaction was detected by

applying rabbit anti-mouse (Z0259, DAKO) 1:37.5 for 30 min and mouse APAAP (D0651, DAKO) 1:25 for 30 min. The color was developed with New Fuchsin solution and counterstained with hematoxylin. Intra-tumor microvessels were counted in 1 hot spot area (area having the highest vessel density), at 400 × magnification, in five consecutive fields (each 0.16 mm^2) and given as the mean of five fields as vessels/mm^3, according to the commonly used method described by Weidner [19]. The total amount of blood vessels/mm^2 tumor area was calculated as: blood vessels/mm^2 X (tumor volume on day 11/tumor volume on day 0).

2.7. Statistics

One-way analysis of variance (Anova) followed by Bonferroni and Student's *t*-test was used for statistical analysis of tumor growth between groups, angiogenesis and inflammatory differences. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. HBO effect as a stand-alone treatment

The suppressive effect on tumor volume found after only 4 HBO exposures at 2 bar, was dramatic and rather unexpected (Fig. 1). A few studies support a similar suppressive effect of HBO on tumor growth, although these studies were performed either at higher oxygen partial pressure or with considerably more HBO exposures. Tumor colonies in the rat lung showed suppression after 16–21 HBO exposures at 3 bar [15], and this was suggested as a local oxygen effect in the lung. Also, growth of DMBA-induced tumors in hamsters after 20 HBO exposures at 2.4 bar was delayed [17].

The effect of HBO recorded in this study is probably an effect of increased pO_2 rather than the pressure per se, since Dettmer et al. [16] showed no statistical difference in primary tumor volume between 1 bar air ($pO_2 = 0.2$ bar) and 3 bar air ($pO_2 = 0.6$ bar). Additionally, Metrovic et al. [15] showed that an oxygen-nitrogen mixture to 3 bar had no effect on tumor growth while 3 bar HBO reduced tumor volume.

Tumor survival and growth, in their hypoxic environments, are the results of a number of adaptive mechanisms mediated at least in part by the transcription factor hypoxia-inducible factor-1 (HIF-1) [20]. HIF-1 is a heterodimer composed of two subunits, where HIF-1α is the subunit that determines its biological function. Increased HIF expression is probably mediated via the adaptor protein Shc in endothelial cells [21]. Thus, preventing or terminating HIF-1α transactivation has the potential to interfere with tumor growth. HIF-1α activity is tightly regulated by oxygen-dependent control, and in the well oxygenated state HIF-1α is rapidly degraded [22]. Thus, it would seem that hyperoxia might be a switch for turning off the HIF-1α and thereby tumor growth.

HIF has also another important effect. It mediates the vascular endothelial growth factor (VEGF) gene expression, which is important in angiogenesis and thereby blood and energy availability. The VEGF level has been shown to be pO_2-dependent both in vitro and in vivo [23]. Hypoxia enhances HIF and thereby also VEGF gene expression in nonmalignant tissues [23]. In tumors VEGF mRNA levels are often elevated. Since, the VEGF level as HIF has been shown to be pO_2-dependent, it is reasonable to assume that an elevated pO_2 as in the present study would reduce HIF and VEGF levels and thereby reduce angiogenesis in the tumors. The present results

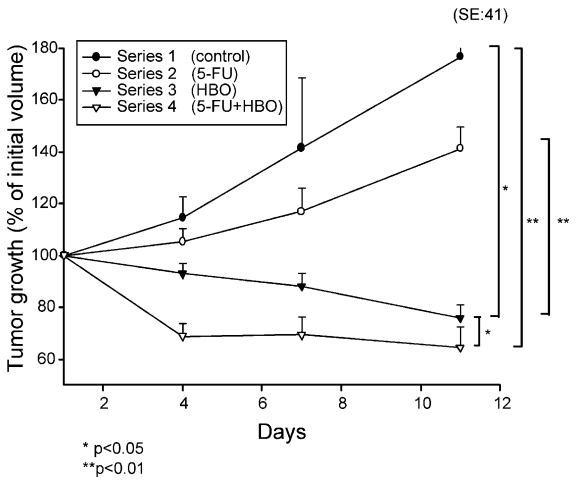

Fig. 1. The effect of 5-FU and HBO on tumor growth—alone or in combination. Treatments were given day 1, 4, 7 and 10. Values represent means ± SE.

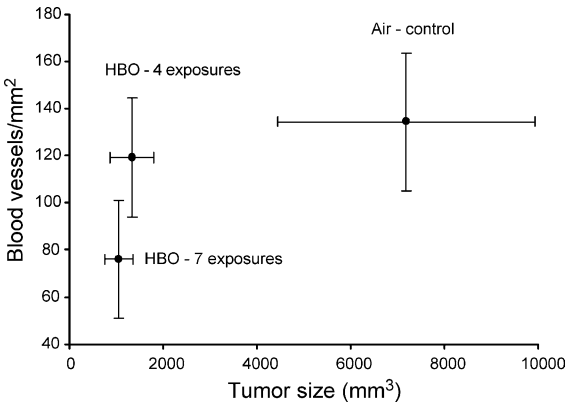

Fig. 2. The relationship between tumor size and blood vessels per mm^2 before and after HBO exposures. Values represent means ± SE. The tumor size was significantly different between the groups ($P < 0.05$, One-way Anova), while the blood vessels/mm^2 was not significant different between the groups.

support such an anti-angiogenic effect of elevated pO_2. By estimating the total quantity of blood vessels/mm^2 tumor area we found a significant reduction from 264 to 75 and further to 33 in air (control), HBO (four exposures) and HBO (seven exposures), respectively. The difference between the groups was highly significant ($P < 0.002–0.03$). The correlation between tumor size and blood vessels/mm^2 is visualized in Fig. 2.

The histological examination of the present tumors also revealed enhanced inflammation ($P < 0.05$) when estimating the combined inflammatory response in: capsule, epithelium and stroma together after HBO exposures (Series 3) when compared to control (Series 1). The most pronounced changes ($P < 0.03$) were found after long time exposure (Series 6). The total ($n = 5$ in each Series) semi-quantitative inflammation grade in Series 1, 3 and 6 were 16, 21 and 24, respectively. The enhanced inflammation, could be a response to the anti-angiogenic effect or by itself an additional reason for the suppression of the tumor growth.

Reactive molecular species of oxygen have been proposed to promote tumor growth [24,25]. A possible explanation of the present HBO anti-tumor effect in the rats could be the difference in the activity of an oxygen radical scavenger, superoxide dismutase (SOD), between normal and tumor cells. A low mitochondrial SOD (Mn SOD) activity lower cytosol (Cu–Zn SOD) activity in tumor cells [26]. Since the exposure to HBO is accompanied by increased production of oxygen radicals, the diminished SOD activity could raise the susceptibility of tumor cells to HBO. On the other hand, HBO might increase MnSOD, which has previously been shown to inhibit cell growth in vitro in malignant cells [26]. Any combination of the factors above could have produced the suppressive effect observed on tumor growth following HBO exposure.

In the present study, the HBO effect was maximal after 4 HBO exposures (Fig. 3). However, continued HBO exposures kept tumor size significantly below day 1 levels. This seems to imply that HBO exposure reduces the tumor to a certain size and thereafter prevents its further growth. After completing 4 HBO exposures, the slight increase in tumor size was found not statistically significant different from day 1 (Fig. 3). Tumor growth therefore seems to be suppressed for at least 12 days after HBO completion

3.2. Combined HBO and 5-FU effect

Despite dramatic advances in the effectiveness of chemotherapy, the current treatments are still far from fully effective. HBO has been used extensively and successfully in radiotherapy to increase tumor radiation sensitivity and tumor oxygenation [2–3,7,17]. 5-FU is often used as a primary agent for chemotherapy

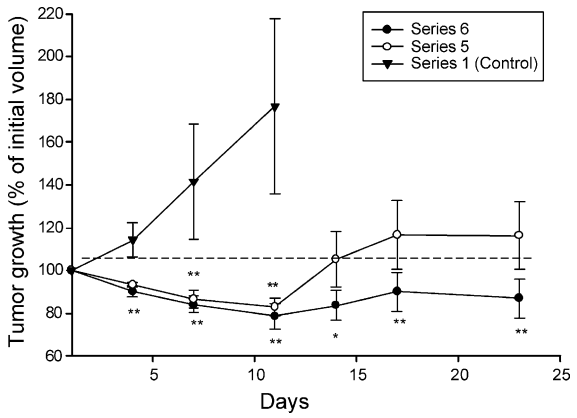

Fig. 3. The after-effect of four HBO exposures (Series 5) and the effect during multiple HBO exposures (Series 6) on tumor growth. Dashed line indicates the level for initial tumor-size. Treatments were given day 1, 4, 7 and 10. Values represent means ± SE. Statistical significance is given as: $*P < 0.05$ vs. day 1, $**P < 0.01$ vs. day 1.

of solid tumors. The 5-FU by itself induced a slight reduction in tumor growth compared to control (41.2 vs. 75% increase) (Fig. 1). The HBO alone, *reduced* tumor volume by 17–24.5% and the combined treatment reduced tumor size by 35%, and therefore constitutes 50–70% of the total reduction of the combined therapy. The remaining effect must be a result of increased effect of 5-FU either due to enhanced uptake into the tumor [6], and/or an HBO-induced increase in sensitivity of the tumor cell to chemotherapy.

4. Conclusions

(1) HBO alone suppressed DMBA tumor volume by 17–24.2% after 4 HBO exposures as compared to an increase of 76.7% in controls and was accompanied by a 72–87.5% reduction in numbers of blood vessels in the tumor. (2) Maximal HBO effect on tumor growth was obtained after only four exposures to 2 bar pure O_2 for 90 min. (3) HBO in combination with 5-FU, reduced tumors size significantly more than chemotherapy alone. (5) After completion of HBO treatment, tumor size increased insignificantly during the next 12 days.

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