The Enhancing Effects of Hyperbaric Oxygen on Mouse Skin Carcinogenesis

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Abstract. The effects of hyperbaric oxygen (HBO) on mouse skin two-stage chemical carcinogenesis were examined. Six-week-old inbred CD-1 female mice were divided into the following five groups: group 1, normoxia and application of 25 nmol 7,12-dimethylbenz[a]anthracene (DMBA) and 8.5 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA) (n=19); group 2, HBO and DMBA/TPA (n=21); group 3, HBO and DMBA/acetone (n=3); group 4, normoxia and acetone (n=3); and group 5, non-treatment group (n=5). HBO was started at the same time as DMBA. Mice were euthanized at 23 weeks after the start of the experiment. Mice in group 2 showed the occurrence of tumors at 8 weeks after the beginning of the experiment, while the occurrence of tumors in mice in group 1 was observed beginning at 9 weeks. There was a difference in occurrence among low-grade papillomas, high-grade papillomas and SCCs in both groups 1 and 2 by the χ²-test at end of the experiment (p<0.05). The Ki-67 labeling indices of tumors revealed that the percentages of positive cells in low-grade papillomas in groups 1 and 2 were 15.27 ± 2.54% and 29.67 ± 2.82%, respectively (p<0.01). The results suggested that the tumors in group 2, which was treated with HBO, were more progressive than those in group 1, which was not treated with HBO. In this study, HBO accelerated tumor cell proliferation and advanced tumor progression in skin carcinogenesis by DMBA/TPA. (DOI: 10.1293/tox.2013-0046; J Toxicol Pathol 2014; 27: 67–72)

Key words: hyperbaric oxygen, skin, neoplasm, two-stage chemical carcinogenesis, mice

Introduction

In general, hypoxia within tumor tissues plays a significant negative role in the treatment of malignant neoplasms, because the angiogenesis, evasion of apoptosis and increased glycolytic rate are all adaptations made by tumors in the hypoxic microenvironment1–2. To improve therapeutic efficacy, recent efforts have been concentrated on the concept of eliminating the hypoxic state of tumors in order to remove the driving force behind these adaptations. Hyperbaric oxygen (HBO) therapy has been considered to control the hypoxia of the tumor microenvironment and possibly improve treatment outcome. HBO therapy refers to breathing pure (100%) oxygen under increased atmospheric pressure3–5. This potential capacity is believed to reflect an increase in tumor cells and cause hypoxic situation by increased amount of dissolved oxygen in the tissue. HBO may elevate blood levels of active oxygen, which would generate free radicals and cause cellular DNA damage in tissues6. However, the effect of utilizing HBO for cancer treatments has not been clarified yet.

HBO has been reported to increase tumor radiosensitivity both in basic and clinical studies7. HBO has been used as combination treatment with chemotherapy and radiation therapy for malignant tumors8. In our University Hospital, HBO therapy has been used for wound healing, recovery of radiation-injured tissues and cancer treatment in neurosurgery and radiation oncology9. However, many clinicians and researchers do not yet recognize HBO therapy as an effective mechanism of cancer treatment. It still remains controversial in cancer treatment9, 10.

Therefore, the role and modifying mode of HBO with regard to tumors need to be analyzed. In this study, we examined the modification effects on tumors developed under an HBO environment in skin two-stage chemical carcinogenesis using 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA)11.

Materials and Methods

Animals, chemicals and HBO

A total of 51 six-week-old inbred CD-1 female mice (Japan SLC, Hamamatsu, Japan) were housed in cages with access freely to pelleted diet (CE-2, CLEA Japan, Inc., Japan) and drinking water and exposed to a 12-hour light-dark cycle during the experimental period. Mice were di-
vided into the following five groups: group 1, normoxia and DMBA/TPA (n=19); group 2, HBO and DMBA/TPA (n=21); group 3, HBO and DMBA/acetone (n=3); group 4, normoxia and acetone (n=3); and group 5, non-treatment group (n=5) (Fig. 1). Animal care and experiments were approved by the University of the Ryukyus Animal Ethics Committee and carried out in accordance with the guidelines for animal experimentation of the University of the Ryukyus. For two-stage chemical carcinogenesis, the dorsal skin of mice was shaved using surgical clippers. After a 1-week quarantine period, 25 nmol DMBA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.2 ml acetone per mouse was topically applied to mice once except in group 5. After 2 weeks, we began twice-weekly applications of 8.5 nmol TPA (EMD Chemicals, San Diego, CA, USA) in 0.2 ml acetone per mouse in groups 1 and 2 (Fig. 1), and this was continued until the end of the experiment. Acetone was applied to mice in groups 3 and 4 instead of TPA.

After DMBA was applied, mice in groups 2 and 3 were placed in a hyperbaric chamber (Barotec Hanyuda Co., Ltd., Tokyo, Japan) to be exposed to HBO. HBO was administered at a pressure of 2.2 ATA (atmospheres absolute) for 90 minutes. A minimum of 15 minutes of pressurization and depressurization was allowed for animals to adjust to the changes in pressure. HBO was administered 5 days a week. Mice were euthanized under deep anesthesia at 23 weeks from the start of the experiment (Fig. 1).

**Measurement of tumor growth**

Skin was examined for the presence of tumors, and the size and location of tumors were recorded. We counted the number and multiplicity of skin tumors in each mouse. Tumor size was measured externally by caliper at sacrifice. The volume of the tumor was calculated as:

\[ V = \frac{4}{3} \pi (a^3)(b) \]

where (a) is the minor and (b) is the major axis (mm) of the tumor.

**Histological analysis**

Histopathologically, the skin tumors in groups 1 and 2 that were larger than 3.5 mm in diameter were examined by hematoxylin and eosin (HE) staining. According to the criteria of Conti et al., papillomas were judged based on two categories: low-grade papilloma, which is a well-differentiated hyperplastic lesion with no atypical cells or with very few atypical cells in the basal layer, and high-grade papilloma, which is a lesion with more than two-thirds of the thickness of the epithelium occupied by atypical cells. For inflammation, the induced inflammation state was divided into persistent and active; persistent: it appears almost lymphocyte infiltration in tumoral stroma with slightly edema; active: it appears predominantly neutrophil infiltration with lymphocytes in tumoral stroma, with increased and dilated vessels.

**Immunohistochemical analysis**

In order to measure cell proliferation in the skin tumor, the Ki-67 labeling index (LI) was determined. Immunohistochemical staining was performed as described in our previous study. The embedded tissues were cut into 4-μm sections and then stained using anti-Ki-67 antibody (Dako, Carpinteria, CA, USA) and an LSAB Kit (Dako). Five hot spots within each tumor were selected, and the number of positive cells (dense brown precipitate restricted to the nuclei) in 500 cells for each tumor was counted to determine the Ki-67 LI, which was defined as the proportion of positive cells. The histopathological diagnosis and Ki-67 LI evaluation were confirmed by multiple pathologists.

**Statistical analysis**

Data obtained in this study are presented as means ± SEM (standard error of the mean). We used InStat (GraphPad Software, La Jolla, CA, USA) for data analysis. Welch’s t test or the χ²-test was used to determine the significance of
Results

All mice survived throughout the experimental period. There were no significant differences in the initial or final body weights between mice in all groups. The appearance of tumors in group 2 occurred at 8 weeks after the beginning of the experiment, whereas they began to appear in group 1 at 9 weeks. At 12 weeks, the incidences of tumors in groups 1 and 2 were 20% and 38%, respectively (Fig. 3). Ten of 19 mice in group 1 and 14 of 21 mice in group 2 had macroscopic tumors on the surface of dorsal skin at the end of the experiment (Fig. 2 and Table 1). Final incidences of tumors in groups 1 and 2 were 53% and 67%, respectively (Table 1). The final multiplicities of tumors in groups 1 and 2 were 3.30 ± 0.87 and 3.35 ± 0.64, respectively (Table 1). There were no significant differences in tumor incidence and multiplicity between groups 1 and 2. Although the average volume (21.75 ± 9.03 mm³) of tumors in group 2 was greater than that in group 1 (13.81 ± 4.63 mm³), there was no significant difference between these groups (Table 1). No effects on the skin were observed in groups 3, 4 and 5. In addition, none of the other organs were affected by HBO in any group.

Histopathologically, the skin tumors larger than 3.5 mm in diameter in group 1 included 11 low-grade papil-
HBO has been applied to clinical practice, however, the effect of HBO on tumors has not been clarified. HBO therapy has been used in clinical medicine in combination with radiotherapy or chemotherapy for cancer treatment, but no obvious answer has been reported concerning the efficacy of HBO alone against tumors. In this study, the experiment was designed to examine the effect on tumor cells actually in an environment similar to a living body in a mouse chemical carcinogenesis model. The results showed that the tumor volume in group 2 was greatly increased compared with that of group 1; that is, HBO hastened the growth of tumors, although there was no statistical difference (Fig. 2 and Table 1). Pande et al. also reported a similar result, i.e., there was accelerated growth and progression of tumors after HBO therapy. Furthermore, McMillan et al. reported that HBO appears to have a stimulatory effect during the proliferative phase of carcinoma in hamster cheek pouch carcinogenesis.

**Table 2.** Histopathological Findings of Tumors in Groups 1 and 2

| Group | Number of examined tumors | Low-grade papilloma | High-grade papilloma | SCC | BCC | KA | Inflammation |
|-------|---------------------------|---------------------|----------------------|-----|-----|----|-------------|
|       |                           |                     |                      |     |     |    | Persistent  |
|       |                           |                     |                      |     |     |    | Active      |
| 1     | 13                        | 11                  | 1                    | 0   | 1   | 0  | 7           |
| 2     | 23                        | 6                   | 12                   | 4   | 0   | 1  | 8           |

*Significantly different in the 2×3 contingency table by χ²-test (p<0.05). ** See the text for details. SCC, squamous cell carcinomas; BCC, basal cell carcinoma; KA, keratoacanthoma.

**Table 3.** Ki-67 Labeling Indices of Tumors in Groups 1 and 2

| Group | Low-grade papilloma | High-grade papilloma | SCC | BCC | KA | Inflammation |
|-------|---------------------|----------------------|-----|-----|----|-------------|
|       |                     |                      |     |     |    | Persistent  |
|       |                     |                      |     |     |    | Active      |
| 1     | 15.27 ± 2.54 (n=11) | 25.00 (n=1)          | NA  | 17.55 (n=1) | NA |             |
| 2     | 29.67 ± 2.82 (n=6)  | 30.20 ± 3.91 (n=12) | 35.25 ± 4.56 (n=4) | NA | 19.51 (n=1) |   |

Mean ± SEM%. *Significantly different from group 2 (p<0.01). SCC, squamous cell carcinoma; BCC, basal cell carcinoma; KA, keratoacanthoma. NA=Not applicable (n=0).
esis, but it is complex to distinguish the DNA damage in lesions affected with HBO from those by DMBA and TPA used in this model. Further studies are needed.

In conclusion, we found that the HBO accelerated tumor development and enhanced tumor growth in a mouse skin chemical carcinogenesis model. Since there are several inconsistent reports regarding the effect of HBO, further investigations about the combined effect of HBO with radiotherapy or chemotherapy on tumor development are necessary.

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