Influence of High-Pressure Oxygen and Chemotherapy on the AMel 4 Hamster Melanoma

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Summary

The effect of hyperbaric oxygen (HPO) on tumor growth and a possible synergism with chemotherapeutic agents (mechloretamine, amethopterin, and cyclophosphamide) were tested with the use of the AMel 4 hamster melanoma.

HPO alone prolonged survival ($P < 0.001$), but neither survival nor size of the primary tumor was modified by the addition of chemotherapy beyond that observed when these drugs were given alone. Hence the combined effects seem additive and not truly synergistic. Although weight loss was significantly greater in the HPO-treated animals, diet-restricted controls that lost more weight than the HPO groups had no increase in survival. Striking differences were evident in the gross and histologic appearance of pulmonary metastases, the extent of tumor being far less in the HPO-treated groups.

The experimental results indicate that HPO delays the death of animals having this tumor and suggest that this prolongation of survival is particularly a function of restraint of pulmonary metastases. HPO did not potentiate the effect of chemotherapy by the agents tested.

Introduction

The peculiar requirements of tumors for oxygen have long been apparent (28, 29). Several studies have shown that tumors have poor oxygenation compared with normal tissues, that the core of the tumor is particularly deficient to oxygen, and that oxygenation of the tumor is responsive to agents that alter blood flow and change the flow of transported oxygen. Investigations that incorporated direct tissue polarography have indicated that external HPO effectively increases tissue oxygen levels (8, 17, 21). With 4 atmospheres absolute of oxygen, a 12-fold rise in tumor $pO_2$ occurred with a 15- to 50-fold increase in the $pO_2$ of normal tissues (21).

The effective clinical application of this biologic evidence was prompted by Gray et al. (18), who collected the evidence of others and showed increased sensitivity of hyperoxygenated tissues to X-ray irradiation. Evidence has accumulated in clinical trials to indicate that supravoltage radiotherapy in a hyperbaric environment results in a response in excess of that expected from supravoltage therapy in comparable dosage at atmospheric pressure (9, 11, 26).

X-ray irradiation and the alkylating agents produce indistinguishable cytologic damage. Because there are similarities in clinical response, the alkylating agents have been referred to as "radiomimetic." Although both produce their effects by damaging DNA molecules, recent evidence suggests different methods of injury: radiation degrading DNA into smaller molecules by direct action and the nitrogen mustards internally cross-linking the DNA molecule (2). In contrast to X-ray irradiation, the action of alkylating agents appears unrelated to free radicals (4). However, general similarities in action have led several investigators to test the combined effect of the alkylating agents and HPO in animals and have led 2 groups to report somewhat equivocal results in tests on humans (1, 3, 4, 6, 22-24).

Our curiosity about the effect of HPO when used alone and in combination with cancer chemotherapy prompted the initiation of experiments to answer several questions. Would prolonged HPO alone affect survival in a rapidly metastasizing animal-tumor system? Would HPO provide an additive or synergistic effect to an alkylating agent (mechloretamine) that was not expected to change survival in this host tumor system or to another alkylating agent (cyclophosphamide) or a folic acid antagonist (amethopterin) that were expected to lengthen survival (25, 27)?

Methods

In each experiment 40 female Syrian golden hamsters (Mesocricetus auratus) of about 75 gm were weighed, labeled, anesthetized with sodium pentobarbital, and inoculated s.c. with approximately $4 \times 10^4$ viable cells of AMel 4 melanoma. The AMel 4 amelanotic melanoma is an aggressive, rapidly metastasizing tumor, 1st described by Fortner and associates (14, 15). The life-span of similarly inoculated animals has ranged from 7 to 10 days in our laboratory, where the tumor is transferred at weekly intervals. The 40 tumor-inoculated animals were then segregated randomly into 4 groups of 10 animals.

One group of 10 tumor-inoculated animals served as a control group at atmospheric pressure in each experiment (Tables 1, 2, 3). They received food and water ad libitum.

A 2nd group of 10 animals received only high-pressure oxygen, as follows: On the day of tumor inoculation, these hamsters were placed in an Emerson hyperbaric chamber. The chamber was flushed with oxygen, and then, with the use of 100% oxygen,
the internal pressure was raised to 15 psi. This pressure was maintained for 6 hr each day until death. The Emerson Hyperbaric Chamber, which has internal dimensions of 30 inches in diameter and 110 inches in length, readily accommodated the small animal cages. During hyperbaric pressure, an oxygen flow of 0.1–0.3 cu ft/min precluded accumulation of CO₂ in excess of 3%.

The hyperbaric environment of 15 psi for 6 hr each day was selected because, in pilot studies of 15–20 animals each at higher pressures or longer exposures, we found an increasing mortality from HPO alone. For example, continuous exposure of hamsters to HPO at 15 psi killed all animals in about 20 hr. At 30 psi, 100% of the hamsters died in about 3.5 hr; and at 45 psi, all died within 50 min. Postmortem examination showed histologic findings compatible with those described by van den Brenk (7).

The 3rd group of 10 animals received only cancer chemotherapy. In 2 separate experiments, mechlorethamine (nitrogen mustard, Mustargen) was injected i.p. on the 3rd day after tumor implantation. The mechlorethamine was administered as 1.0 mg/kg diluted in less than 1.0 ml of saline. In the next 3 experiments, amethopterin (methotrexate), as 2.0 mg/kg of body weight, was administered i.p. on the day of tumor implantation and daily for the subsequent 6 days (total dose, 14.0 mg/kg). In a single experiment, cyclophosphamide (Cytoxan) as 15 mg/kg of body weight, was initiated i.p. on the day of implantation and was continued daily for the ensuing 6 days (total dose, 105 mg/kg).

The 4th group of animals in each experiment received both HPO and chemotherapy. Pressure was administered at the same hyperbaricity and for the same duration as for the group receiving HPO alone, described above. The various drugs were given in the same dosage and timing as in each group that received chemotherapy alone. In the 2 experiments in which mechlorethamine was given, the interval from injection to obtaining the full treatment pressure ranged from about 45 min for the 1st hamster injected to about 10 min for the last.

Hence, each experiment had a minimum of 4 groups of tumor-inoculated hamsters: controls at atmospheric pressure; HPO alone; chemotherapy alone; and combined HPO and chemotherapy. Two separate experiments were carried out with mechlorethamine, 3 with amethopterin, and 1 with cyclophosphamide.

Four additional control groups were utilized. During 2 of the above experiments, diet-restricted controls were maintained for comparison with the HPO-treated animals; that is, tumor-bearing hamsters inoculated and randomized along with the basic 4 groups were kept at atmospheric pressure but had their food rations halved. They received water ad libitum. In another control group, to confirm absence of mortality from HPO alone, sets of 10 tumor-free hamsters were carried in HPO for the same intervals as those hamsters which had been inoculated with tumor. Control groups consisting of tumor-free animals given each drug in the identical manner used for the tumor-bearing animals allowed a check on any mortality from the agent alone. Finally, in 2 experiments, a group of 10 tumor-inoculated hamsters were paired with the tumor atmospheric pressure control group and treated with HPO alone exactly as the standard HPO tumor group. These animals were sacrificed at the time of death of their untreated mate to permit comparative gross and histologic studies of the extent of tumor growth.

All animals were followed with the recording of duration of survival, weight at death, size at the primary implant, and sites of metastases. The volume of the primary implant was calculated as an oblate spheroid, that is, \( \frac{4}{3} \pi ab \), where \( a \) and \( b \) are the major and minor semiaxes, respectively. Specimens from the primary tumor and all major organs were removed at necropsy and fixed in formalin for staining with hematoxylin and eosin. The data were analyzed by individual paired t tests and an F test for variances.

**Results**

**HPO Used Alone**

HPO used alone prolonged survival of the tumor-bearing animals significantly (\( P < 0.001 \)), compared with the tumor atmospheric pressure control groups (Tables 1, 2, 3). A significant weight loss occurred in the HPO-treated animals, whereas the control group had essentially no change in weight. Study of the 2 previously mentioned groups of diet-restricted, tumor-bearing animals at atmospheric pressure revealed that their survival was shortened, compared with both tumor-bearing atmospheric and HPO groups. The diet-restricted control group of 20 animals had a mean survival time of 6.32 days and a mean weight loss of 22.8 gm, whereas the paired HPO-treated groups had a mean survival of 8.2 days and an average weight loss of 10.2 gm. There were no deaths in the HPO- or drug-treated nontumor control groups.

Comparison of the autopsy findings in the HPO-treated and the control animals showed striking differences in the gross and histologic appearance of pulmonary metastases. On gross inspection we were unable to document by actual count a difference in the number of pulmonary metastases, but the total lung area involved by tumor was consistently less in the HPO animals. Microscopic examinations of the lungs (Figs. 2, 3) clearly confirmed this gross observation. In the control animals, pulmonary metastases were noted particularly adjacent to bronchi, bronchioles, and blood vessels. In the HPO-treated animals, metastases were diminished in these areas, and in some instances, it was difficult to demonstrate any tumor cells in the microscopic sections. These findings were noted in all HPO-treated groups whether or not chemotherapy was also administered. As previously mentioned, in 2 experiments extra groups of tumor-inoculated HPO-treated animals were used. At the same time as each member of the atmospheric tumor control group died, 1 of this group was sacrificed. Comparison of the lungs of these 2 groups showed even greater differences in the amount of gross and microscopic pulmonary involvement in favor of the HPO group (Fig. 1).

Comparison of the histology of the liver of control and HPO-treated animals showed some decrease in the amount of tumor in the HPO-treated group, but the difference was much less striking than that observed in the lungs.

When the volume of the primary tumor implant was compared

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3 Throughout this paper psi will be used to denote the lbs/sq inch gauge pressure; hence, 15 psi is approximately 2 atmospheres absolute.
in the 2 groups, the primary tumors tended to be smaller in the HPO-treated animals but were not significantly different. Examination of other tissues did not demonstrate any striking or consistent differences between the 2 groups. Diffuse multifocal metastases were observed in the lymph nodes, skin, breast, gastrointestinal tract, and brain.

**HPO Plus Cancer Chemotherapy**

Neither survival nor the size of the primary tumor was modified in the HPO-treated animals by addition of chemotherapy beyond that observed when the drugs were given alone or when HPO was administered alone (Tables 1, 2, 3). The combined effect seemed additive, not synergistic, with the cancer chemotherapeutic agents tested. Contrary to expectation, mechlorethamine alone resulted in a slight but significant (P < 0.025) prolongation of survival (Table 1). As was anticipated, amethopterin or cyclophosphamide alone resulted in a significant lengthening of survival (amethopterin, P < 0.001; cyclophosphamide, P < 0.05) (Tables 2, 3). When HPO was combined with either mechlorethamine, cyclophosphamide, or amethopterin, only an additive effect was observed; no synergism was obtained in the sense that the combined effect on survival did not exceed the sum of the individual effects on survival.

**Discussion**

The primary objective of these experiments was an evaluation of the effect of treatment with prolonged HPO on a rapidly metastasizing animal tumor system. Under the conditions of these studies, the evidence indicates that HPO resulted in a minor but significant improvement in survival. Since, either directly or indirectly, the HPO resulted in a significant weight loss, we wondered if the dietary restriction, or refusal to eat during therapy, was responsible for both weight loss and increase in survival in the HPO groups. However, a group of tumor-bearing, diet-restricted hamsters at atmospheric pressure had a greater weight loss, and their survival was shortened.

At postmortem examination of the 2 groups, the most impressive difference was in the lungs. Pulmonary metastases were distinctly less confluent in the HPO-treated group either with or without chemotherapeutic agents. On histologic examination, diminished metastases were noted particularly in the peribronchial and perivascular tissue of the lungs of the HPO-treated hamsters. Less impressive were slight differences in hepatic metastases and the volume of the primary flank tumor. Examination of other sites of metastases did not show any difference between the 2 groups. Although we cannot be certain how those treated animals died from the extensively metastasizing AMel 4 tumor, our impression is that the observed prolongation of survival with HPO resulted from preferential suppression of pulmonary metastases. The striking differences in pulmonary metastases, the less marked differences in hepatic metastases, and the minimal differences in the volume of primary tumors suggest that the effect of HPO on this animal tumor system is greater on the pulmonary metastases, or the reaction of lung tissue to the tumor, than it is by HPO acting to restrain the primary tumor and its ability to metastasize.

There is little doubt that HPO is toxic (7, 13, 19). Even at our relatively low pressures, it is reasonable to assume that both host and tumor were affected by HPO. In view of the known distortion of anerobic glycolysis in tumor cells, we are inclined to explain our results in part by postulating a "differential toxicity" of HPO, whereby the adaptive metabolic processes of the host are more efficient than those of the tumor. Another factor of probable importance is the gradient in oxygen tension between the fully oxygenated lungs and the substantially lower levels expected in the peripheral tissues (9). In addition, Dickens (10) has shown a differential sensitivity of normal tissues to high oxygen tension, the lungs being comparatively resistant. Perhaps our using the AMel 4 tumor, which rapidly produces massive, diffuse, pulmonary metastases, was a fortuitous circumstance to demonstrate a beneficial effect of HPO. It is possible that the results observed could be related to stress in the HPO-treated animals. However, exogenously administered cortisone augments rather than diminishes the severity of HPO toxicity (5). Also, there is no convincing evidence that cortisone increases survival or suppresses pulmonary metastases in such animal tumors.

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**TABLE 1**

Effect of High-Pressure Oxygen (HPO) and Mechlorethamine (HN2) on the AMel 4 Tumor

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>No. of Animals</th>
<th>Mean Survival Time</th>
<th>Mean Weight Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>7.3 ± 0.45</td>
<td>+1.8 ± 2.1</td>
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<tr>
<td>HPO alone</td>
<td>20</td>
<td>8.8 ± 0.6</td>
<td>−13.9 ± 7.3</td>
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<tr>
<td>HN2 alone</td>
<td>20</td>
<td>7.8 ± 0.8</td>
<td>−8.0 ± 5.1</td>
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<tr>
<td>HPO + HN2</td>
<td>20</td>
<td>9.0 ± 1.7</td>
<td>−14.9 ± 7.1</td>
</tr>
</tbody>
</table>

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**TABLE 2**

Effect of High-Pressure Oxygen (HPO) and Amethopterin (MTX) on the AMel 4 Tumor

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>No. of Animals</th>
<th>Mean Survival Time</th>
<th>Mean Weight Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>8.0 ± 0.7</td>
<td>+3.6 ± 6.3</td>
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<tr>
<td>HPO alone</td>
<td>29*</td>
<td>8.6 ± 1.1</td>
<td>−7.4 ± 5.3</td>
</tr>
<tr>
<td>MTX alone</td>
<td>26*</td>
<td>11.1 ± 1.9</td>
<td>+0.6 ± 11.3</td>
</tr>
<tr>
<td>HPO + MTX</td>
<td>28*</td>
<td>11.9 ± 1.7</td>
<td>−8.1 ± 4.8</td>
</tr>
</tbody>
</table>

* A few animals were lost in 1 experiment because they escaped from their cages.

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**TABLE 3**

Effect of High-Pressure Oxygen (HPO) and Cyclophosphamide (CPA) on the AMel 4 Tumor

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>No. of Animals</th>
<th>Mean Survival Time</th>
<th>Mean Weight Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>8.7 ± 0.4</td>
<td>−1.5 ± 6.0</td>
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<tr>
<td>HPO alone</td>
<td>10</td>
<td>9.3 ± 0.5</td>
<td>−4.2 ± 2.7</td>
</tr>
<tr>
<td>CPA alone</td>
<td>10</td>
<td>9.9 ± 0.2</td>
<td>+4.7 ± 3.0</td>
</tr>
<tr>
<td>HPO + CPA</td>
<td>10</td>
<td>10.5 ± 0.6</td>
<td>−2.6 ± 3.5</td>
</tr>
</tbody>
</table>
The results reported by Kluft and Boerema (22) with more prolonged periods of HPO are similar to our findings. The success of Antopol et al. (3) in modifying tumor growth with long-term use of compressed air (70 psi for 2-6 hr/day) raises the question of the responsible component; that is, is effect due to the high pressure itself or to the increased oxygen tension—a subject that has been examined in the literature (8, 21).

Mechloretamine alone was not expected to show any effect on the AMel 4 tumor, but a slight difference in survival was evident. Cyclophosphamide and amethopterin alone increased survival as expected. We were unable to demonstrate any synergism between HPO and mechloretamine, between HPO and cyclophosphamide, or between HPO and amethopterin. The absence of synergism in survival does not preclude the presence of a more obscure synergism in the biologic mechanism of action.

As was previously noted, we could not devise a practical method of administering mechloretamine to large numbers of hamsters while they were in a hyperbaric environment. For this reason we were concerned whether any mechloretamine would be active when hyperbaric conditions were reached. Work done in this laboratory on the disappearance rates of mechloretamine administered i.p. in vivo to mice with Sarcoma 180 ascites tumors, with the use of a nitrobenzyljirridine assay originally developed by Epstein et al. (12) and modified by Herbst et al. (20), revealed that at the end of 15 min, 51% of the injected dose had disappeared from the ascites fluid. Thirty min after injection, 63% had disappeared; and at the end of 60 min, 71% had disappeared. Since in our experiments the animals reached hyperbaric pressures within 10-45 min after injection of the agent, it is likely that some effect would have been evident if enhancement of the drug’s action had occurred, especially in the animals injected last and reaching a hyperbaric state about 10 min after injection. None was observed. In addition, we were unable to observe any synergistic effect between HPO and cyclophosphamide, a longer acting alkylating agent, which must be biotransformed to an active metabolite. There is, however, evidence that cyclophosphamide may act through a mechanism other than alkylation (16).

Acknowledgments

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References


Fig. 1. Lung of hamster dying from AMel 4 tumor on 8th day after tumor inoculation compared with that of simultaneously sacrificed HPO-treated animal inoculated with tumor at same time. Note relatively few metastatic areas in HPO lung and almost solid tumor in untreated control.

Fig. 2. A, Typical lung section of untreated hamster dying on 8th day after tumor inoculation. × 60. B, Same as Fig. 2A. × 350.

Fig. 3. A, Typical lung section of HPO-treated hamster dying on 9th day after tumor inoculation. × 60. Lungs were removed 18 hr after last HPO treatment. B, Same as Fig. 3A. × 350.


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