Effects of Hyperoxia on Growth Characteristics of Metastatic Murine Tumors in the Lung

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ABSTRACT

Suspensions of an oxygen-sensitive (MT-7) and of an oxygen-insensitive (M109) tumor cell line were injected i.v. into BALB/c mice. Exposure to 100% O2 after injection of the cells did not modify the initial arrest of either cell line in the lung. Exposure of animals given injections of MT-7 cells for 60 h to 100% oxygen decreased the number of lung colonies formed even when onset of oxygen exposure was delayed up to 10 days after injection of the cell suspension. Cell cycle time and growth fraction in lung colonies growing in vivo were estimated from an analysis of the percentage of mitoses labeled. In lung colonies formed by MT-7 cells, hyperoxia produced a mitotic delay and a 30 to 40% reduction in the growth fraction. In M109-derived colonies, oxygen did not change cell cycle times or reduce growth fraction. In earlier experiments done in vitro and reported by others it had been found that, in tumor cell lines other than the ones used in the present study, a prolongation of the early prophase was the most oxygen-sensitive event. The present data show that in vivo oxygen inhibits lung colony formation in MT-7 cells by a similar mechanism.

INTRODUCTION

Following i.v. injection of murine tumor cell suspensions into syngeneic mice, multiple nodules develop in various organs, particularly in the lung. We have recently found that the development of such artificial lung colonies can be modified by hyperoxia (1, 2). If BALB/c mice are exposed for 48 h to 100% oxygen prior to the injection of a tumor cell suspension, formation of lung colonies is greatly enhanced, regardless of what tumor cell line is injected. If the animals are exposed repeatedly for 48 h to 100% oxygen (or for longer periods to an atmosphere of 70% oxygen) following injection of the tumor cells, a different picture develops. The growth of some tumor cell lines, such as line MT-7 (derived from a mammary carcinoma) line 1 or line 498 (derived from a lung tumor) is severely reduced by hyperoxia. On the other hand, exposure of mice given injections of the lung tumor-derived cell line M109 to oxygen will not affect the development of tumor nodules in the lung (1, 2). Therefore, it became of interest to search for mechanisms underlying the toxicity of oxygen in the susceptible tumor cell line. We found that in vivo oxygen produced a mitotic delay and a decrease in the growth fraction in tumor nodules derived from the sensitive cell line MT-7. No such changes were seen in the oxygen-resistant cell line M109.

MATERIALS AND METHODS

Cell Cultures. Line M109, originally derived from a lung adenocarcinoma in BALB/c mice, was obtained from Dr. S. Kennel, Biology Division, Oak Ridge National Laboratory. MT-7, a mammary tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in BALB/c mice, was obtained from Dr. R. L. Ulrich, Biology Division, Oak Ridge.

Line M109 was maintained in McCoy's Medium 5A with 10% fetal bovine serum and antibiotics (1100 units/ml of penicillin and 100 μg/ml of streptomycin; Gibco, Grand Island, NY). MT-7 cells were maintained in Ham's F-12 medium (K. C. Biologicals, Lenexa, KS) supplemented with 4 μg/ml of bovine pancreatic insulin (Schwarz-Mann, Orangeburg, NY), fetal bovine serum, and antibiotics. The cells were grown in a humidified incubator with a 5% CO2 atmosphere at 37°C.

Animals. Male BALB/c mice, 7 to 10 wk of age, were bred in our own facility. They were kept 10 to 12 cages on hardwood bedding. The animal quarters are temperature (20-22°C) and humidity (40 to 60%, relative) controlled with a 12-h light/dark cycle. Throughout the experiments, mice were given hyperchlorinated water and fed a conventional laboratory diet (Rodent Laboratory Chow 5001; Ralston Purina Co., St. Louis, MO) ad libitum.

Production of Lung Colonies. Lung colonies were produced and scored essentially as described before (1, 2). Briefly, actively growing cell cultures were harvested by mild trypsinization (60 s with 0.25% trypsin-0.2 g/liter of EDTA; GIBCO), washed in medium, and suspended in cold phosphate-buffered saline for injection at the desired number of viable cells (25,000 to 150,000 cells per mouse, as indicated in the tables). All cells were injected into a tail vein in a volume of 0.2 ml. For any given experiment, injections were completed within 1 to 2 h and all animals were assigned at random to the different experimental group, after injection of the cells.

Oxygen Exposure and Scoring of Lung Tumors. Cages containing the experimental animals were placed inside 450-liter plastic chambers for exposure to 100% oxygen or a mixture of air and oxygen. At the end of the experiment, the animals were killed, and the lung surface tumors were counted. Details of procedures have been given before (2, 3).

Retention of Radiolabeled Cells in the Lung. The fate of i.v. injected tumor cells was determined essentially as described by Fidler (4). MT-7 or M109 cells were exposed in their culture medium for 24 h to radiolabeled iododeoxyuridine, 0.4 μC/ml, in tissue culture medium (5'-[3H]iodo-2-deoxyuridine; specific activity, approximately 5 Ci/mg; Amersham, Arlington Heights, IL). After incubation, the cells were thoroughly washed and injected i.v. into mice (75,000 MT-7 cells or 150,000 M109 cells per mouse). One group of animals was kept in air throughout the experiment, and 4 to 6 animals were killed 2 min and 2, 6, 24, 48, and 72 h later. A second group of animals was placed immediately after injection of the cells into 100% oxygen; animals were killed at 6, 24, and 48 h. A third group of animals was placed into 100% oxygen 24 h after tumor cell injection; these animals were killed at 48 and 72 h.

After removal from the thoracic cavity, each lung was rinsed in water and dropped into 10 ml of 70% ethanol. The ethanol was changed 3 times, and the entire lung was counted in a well-type iodine crystal scintillation counter. The radioactivity in each lung was calculated as a percentage of the initially injected radioactivity.

Construction of Labeled Mitoses Curve. For construction of a labeled mitoses curve, 140 to 160 mice were given injections i.v. of 75,000 MT-7 or 150,000 M109 cells per mouse. For any given experiment, injections were completed within 1 to 2 h and all animals were assigned at random to Treatment Groups A, B, and C. The treatment given each group is described in "Results." Sixteen days after injection of the tumor cells, all animals received an i.p. injection of tritiated thymidine; the injection was given at 8:00 a.m. (methyl-[3H]-thymidine; specific activity, 2 μCi/mmol; 50 μCi/animal; purchased from Amersham Corporation). One h later, six animals from each experimental group were killed by CO2 asphyxiation. Two to three
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animals per group were then killed 2, 3, 4, 5, 7, 8, 9, 10, 12, 15, 18, 21, 24, 27, 30, 33, and 36 h after injection of the thymidine. The lungs were inflated with neutral buffered formalin and immersed in the same fixative. Lung tissue containing tumors was embedded in paraffin, cut at 4 to 5 μm, mounted on glass slides, and dipped in NTB-2 photographic emulsion (Eastman Kodak Co.). Two wk later the slides were developed and stained with hematoxylin-eosin. The percentage of labeled mitoses at each time point was scored by counting 50 to 100 mitotic figures. The data were analyzed with a computer program according to the method developed by Steel and Hanes (5). In a second experiment, 30 mice were each given injections of MT-7 or M109 cells. Eighteen days later, half of the animals were placed at 4 p.m. into 100% oxygen. All animals received [3H]thymidine i.p. at 8 a.m. the next morning and were killed at hourly intervals until 6 h later. The percentage of labeled mitoses was scored as described before.

Statistical Analysis. Data were analyzed with Student’s t test, analysis of variance, or by a 2 x 2 contingency table for independent variables. A 95% confidence interval for a normal distribution was used for a significance level.

RESULTS

First, we examined whether hyperoxia would interfere with the arrest of i.v. injected tumor cells in the lung. Cultures of MT-7 and M109 cells were labeled in vitro by exposing them for 24 h to [125I]iododeoxyuridine. After removal of nonincorporated label by several washes, cells were injected i.v. into mice. Some of the animals were placed immediately after injection of the cells into 100% oxygen, whereas a second group was placed into oxygen 24 h after i.v. injection of the labeled cell suspension. The radioactivity in the total lung was measured at selected intervals. The data are presented in Figs. 1 and 2. It can be seen that, of the oxygen-resistant cell line M109, about 10 times as many cells were retained in the lung as were of the oxygen-sensitive MT-7 cells, although only twice as many cells were injected. Exposure to 100% oxygen beginning immediately following or 24 h after cell injection had no significant effect on retention of radiolabel in the lung in either cell line. Percentages of radioactivity retained remained essentially the same regardless of treatment.

In previous experiments we had found that continuous exposure to 70% O2 for 3 wk or intermittent exposure to 100% O2 (48 h per wk during 3 consecutive wk) significantly diminished the number of lung colonies formed by MT-7 cells. The same treatments did not decrease the number of lung colonies formed by M109 cells (1). To further characterize the effects of hyperoxia on the oxygen-sensitive cell line, mice were given injections of MT-7 cells and placed either immediately or after 24 h for 3 wk into 80% oxygen; all animals survived. The data are shown in Table 1: the same number of tumors developed regardless of treatment.

In a second experiment, animals were given injections of MT-7 cells and exposed for 60 h to 100% oxygen beginning 1 day or beginning 5, 7, or even 10 days after injection of the cell suspension. After the 60 h in 100% oxygen, the animals were returned to air, and all animals were killed 3 wk after injection of the tumor cells. Hyperoxia reduced the number of lung colonies regardless of how late oxygen exposure was begun, although in animals exposed after 10 days, the difference to the controls was statistically no longer significant (Table 2). It can be concluded that oxygen may affect the development of lung colonies even when they are already well established in the lung.

We also observed that oxygen not only reduced the number...
of lung colonies formed by MT-7 cells, but influenced their size. The diameter of the lung tumors was measured under a magnifying glass. In a typical experiment, we found that the average diameter of lung colonies allowed to develop in air was, 21 days after i.v. injection, 1.22 ± 0.06 mm, whereas in animals kept for 3 wk in 70% oxygen, the average diameter of the tumor nodules was only 0.81 ± 0.04 mm. It should be noted that, as in previous experiments, a 3-wk exposure of mice to 70% or 80% O2 did not produce mortality or gross lung damage (2, 3).

The affect of oxygen on the tumors while they were growing in the lung was characterized by measuring cell cycle times and growth fractions in the tumor nodules. Mice were given injections of either MT-7 or M109 cells. For each of the two cell lines, the animals after injection of the cells were assigned at random to 3 experimental groups (Groups A, B, and C).

Group A served as control group. Sixteen days after the cell suspension had been injected, at a time when it could be expected that a suitable number of tumor nodules was present in each lung, each mouse received an i.p. injection of tritiated thymidine. At selected intervals later, animals were killed, lung tissue was prepared for autoradiography, and the percentage of mitoses labeled was determined for each time point. Experimental Group B received the labeled thymidine at the same time as did Group A. Immediately after the injection, the animals were placed in a chamber ventilated with 100% O2; the mice were only removed from the O2 when being killed, at the same time points as Group A.

Group C was treated in a different manner; 60 h before receiving the thymidine injection, the animals were placed into 100% oxygen. After injection of the radiolabel, which was done at the same time as for Groups A and B, the animals were removed from the O2 and kept in air until they were killed.

Figs. 3 and 4 show the curves fitted to the data, and Table 3 gives the estimates on cell cycle times and growth fraction. The data may be summarized as follows. In the oxygen-sensitive line MT-7, oxygen clearly affected several cell cycle parameters. In animals exposed to oxygen immediately following injection of thymidine (Group B), the duration of S phase was prolonged, and the growth fraction was reduced to two-thirds of that found in controls. In animals preexposed to oxygen (Group C), there was a pronounced extension of G2 and an even somewhat bigger reduction of the growth fraction. Also, the maximum percentage of mitoses labeled was only 80%, compared with 100% in the other two groups.

The observations made with M109 cell-derived lung colonies were in sharp contrast to what was seen with MT-7 cells. During exposure to oxygen (Group B), there was again a prolongation of the S phase. However, the growth fraction was only slightly reduced compared to control animals. Moreover, in animals preexposed to oxygen (Group C), there was only a slight increase in length of G2, and the growth fraction was larger than in the two other groups.

Thus a decrease in the growth fraction and a division delay appeared to be the main effect of oxygen in the sensitive cell line, not seen in the resistant cell line. To verify the observation, we examined in an additional experiment whether or not we could confirm the division delay. Animals given injections of the two tumor cell lines were, 15 days later, placed into an atmosphere of 100% oxygen; an equal number of animals was kept in air as controls. Sixteen h later, all animals received an injection of 3H-labeled thymidine. The animals preexposed to O2 were kept in O2, and animals were killed at hourly intervals up to 6 h after injection of the radiolabel.

The data are shown in Figs. 5 and 6. In MT-7 cells, at all time points (except at 5 h), the percentage of mitoses labeled was significantly lower in the animals kept in 100% oxygen compared to controls. The data confirm the conclusions drawn from the previous experiment: in the MT-7 cells, oxygen causes a mitotic delay by slowing down the cells in G2. Oxygen also prevents a substantial number of the cells from going into mitosis altogether. No such effect was observed in the M109 cells; the labeled mitoses curved were very similar.

DISCUSSION

We established before that oxygen inhibits the development of chemically induced tumors in the lungs of mice and of rats (2, 3). Oxygen also prevents the development of lung colonies following i.v. injection of tumor cells. In the same mouse strain,
The BALB/c mouse, one cell line was oxygen sensitive, whereas a second cell line was not affected in vivo under hyperoxic conditions (1). This observation suggests that the effects of oxygen depend upon some inherent characteristics of the injected tumor cell line and appear not to be host dependent.

One explanation of the inhibitory effect of hyperoxia on the development of lung colonies could be that oxygen prevents initial arrest or produces a detachment and loss of arrested tumor cells from the pulmonary capillary bed. Another possibility might be that oxygen acts as a cytotoxic agent and destroys cells as soon as they become arrested in the lung. Data presented in this paper make these possibilities rather unlikely. Hyperoxia inhibits lung colony development regardless of whether oxygen exposure is begun immediately after injection of the cells or 24 h later. If oxygen affects arrest of cells or kills them outright, one might expect a bigger reduction in the number of lung colonies in the lungs of animals exposed immediately after injection of the cells; this was not the case. The experiment with labeled cells confirms the conclusion. There was no difference in the amount of label retained in the lung regardless of when exposure to 100% oxygen was begun. With the technical procedure used, label retained in the lung can be assumed to be associated with the DNA of the prelabeled tumor cells (4). Dislodgement of cells or even frank cytotoxicity by oxygen could be expected to produce a substantial loss of radiolabel over the one seen in control animals, an observation that was not made. We, therefore, conclude that oxygen, if administered after injection of a cell suspension, does not prevent the initial settlement of cells in the lung. This observation must be distinguished from the observation that oxygen, in a different experimental protocol, enhances formation of lung colonies. If animals are exposed to hyperoxia for a few days before tumor cells are injected, more lung colonies develop (1, 6). Damage to the pulmonary capillary endothelium appears to be the responsible mechanism (6, 7).

It was reasonable to expect that oxygen would interfere with lung colony formation by inhibiting DNA synthesis. In vivo experiments have documented that hyperoxia greatly diminishes the incorporation of radiolabeled precursors into pulmonary DNA, usually a sign of decreased synthesis of DNA (8–10). However, in the present experiments, the in vivo effect of oxygen on the sensitive tumor cell line appears not to be a simple inhibition of DNA synthesis. In animals exposed to oxygen (Group B) or preexposed to O2 (Group C), the tumor-labeling index, a measure of DNA synthesis, was only slightly decreased. The effect of oxygen was to interfere with a specific step of the cell cycle; it produced a mitotic delay. From the estimated cell cycle times it could be calculated that the growth fraction of the tumors was reduced. One explanation of the inhibitory effect of hyperoxia on the development of lung colonies could be that oxygen reduces the growth fraction. The finding is limited to the oxygen-sensitive cell line. In lung colonies formed by the resistant cell line, oxygen did not decrease the growth fraction.

**Table 3** Cell kinetic parameters in MT-7 and M109 lung colonies

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Groupa</th>
<th>T\textsubscript{G1} (median no. of h)</th>
<th>T\textsubscript{S} (median no. of h)</th>
<th>T\textsubscript{G2} (median no. of h)</th>
<th>Labeling index (%)</th>
<th>Growth fraction (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-7</td>
<td>A</td>
<td>11.3 (0.6)</td>
<td>8.2 (0.5)</td>
<td>1.6 (0.3)</td>
<td>19.7</td>
<td>12.6 ± 1.4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9.2 (1.2)</td>
<td>10.0 (0.4)</td>
<td>1.9 (0.5)</td>
<td>18.6</td>
<td>10.6 ± 0.9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6.7 (2.0)</td>
<td>7.8 (0.4)</td>
<td>3.9 (0.8)</td>
<td>15.1</td>
<td>9.3 ± 0.9</td>
<td>5</td>
</tr>
<tr>
<td>M109</td>
<td>A</td>
<td>7.8 (1.4)</td>
<td>13.0 (0.5)</td>
<td>1.7 (0.7)</td>
<td>20.1</td>
<td>21.9 ± 1.8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7.4 (1.5)</td>
<td>15.1 (0.4)</td>
<td>2.0 (1.3)</td>
<td>22.0</td>
<td>22.6 ± 1.8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10.1 (1.1)</td>
<td>13.1 (0.6)</td>
<td>2.5 (0.1)</td>
<td>22.9</td>
<td>25.7 ± 1.1</td>
<td>6</td>
</tr>
</tbody>
</table>

* Group A, air controls; Group B, exposed to 100% O\textsubscript{2} during measurement of cell cycle times; Group C, preexposed for 60 h to 100% O\textsubscript{2}.

* TC|, duration of d; Ts, duration of S; TG2, duration of G2; Tc, cell cycle time.

* Numbers in brackets, number of animals.

* Numbers in parentheses, coefficient of variation.

* Mean ± SEM.

* Mean ± SEM.

* Numbers in brackets, number of animals.
The observations made on the in vivo effects of oxygen confirm earlier in vitro studies made with different cell lines (11–16). For example, Drew et al. showed that in vitro exposure of HeLa cells to 95% oxygen would prolong the duration of the G2 phase by about 1 h and produce a delay in the appearance of labeled mitotic figures (12). Different cell lines have been found to have different sensitivity to hyperoxic conditions (13). Balin et al. (14) studied the effect of hyperoxia on human diploid WI-38 cells. They reported that the event most sensitive to oxygen was the early prophase (G2). Higher oxygen concentrations were needed to inhibit DNA synthesis. Our own data confirm that these conclusions also apply to in vivo conditions. Exposure of MT-7 cell-derived tumors to oxygen prior to and after injection of thymidine produced a mitotic delay and prevented a certain portion of the cells from entering mitosis altogether. No such observations were made in the resistant cell line M109.

Why one cell line is resistant and the other is sensitive remains unclear. Van der Valk and coworkers developed oxygen-tolerant HeLa or Chinese hamster ovary cells (17, 18). Antioxidant defense mechanisms (activities of superoxide dismutase, glutathione peroxidase, and catalase) were higher compared to normal cells only in the oxygen-adapted Chinese hamster ovary cells, but not in the oxygen-adapted HeLa cells. In previous experiments we found no difference in levels of antioxidant defense mechanisms between MT-7 and M109 cells (1), although the two cell lines differed in their capability to respond to oxygen when injected into animals (1).

In the present study we have identified a possible target site for oxygen toxicity in dividing tumor cells, the G2 phase of the cell cycle. It is generally assumed that oxygen exerts its toxicity via a free-radical mechanism (19). A similar mechanism has been postulated for radiation damage, and it has been stated that both oxygen and X-rays act in a similar way (20). A recent study argued convincingly that the target for radiation-induced delay was not the DNA itself, but rather a cellular structure which came in contact with DNA during G2 or early mitosis; possible candidates for such a structure would be the nuclear envelope (21).

We found a measurable effect of oxygen in the sensitive MT-7 cell line 17 days after injection of the tumor cell suspension. At this time, most tumor nodules are well developed and of a size recognizable to the naked eye. Even at this stage, it was possible to reduce the growth fraction with oxygen treatment by 30 to 40%. It is interesting to note that some recent data obtained with both animal and human material suggest that cancer cells arising from other sites than lung appear to be sensitive to the toxic action of oxygen (22–25).

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