Tumor Size Dependency in the Radiation Response of the Lewis Lung Carcinoma

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SUMMARY

Tumor cell survival characteristics were assessed following 60Co γ-irradiation of the Lewis lung carcinoma as 500-cu mm s.c. tumors or as 0.5-cu mm (1 mm in diameter) pulmonary metastases. Cells in the small pulmonary tumors were markedly more radiosensitive ($D_0 = 106$ rads; hypoxic fraction < 0.005) than were those in large s.c. tumors (final $D_0, 315$ rads; hypoxic fraction, 0.36). When pulmonary metastases were excised and irradiated intact under well-oxygenated conditions in vitro, the hypoxic fraction rose to 0.30. This implies that, under normal in situ conditions, these nodules contain a microvascular system that achieves adequate oxygen supply to the great majority of tumor cells. Thus, the tumor cells within these small metastatic implants were more sensitive to irradiation, largely due to better oxygenation, and may be more sensitive to chemotherapy, due to better drug availability.

INTRODUCTION

Although irradiation oncologists have long recognized the importance of tumor size as a determinant of therapeutic response (1, 6, 7, 22), relatively few radiobiological studies have evaluated this association (12, 20, 21). The dependence on tumor size of tumor cell sensitivity to cytotoxic therapy is of critical importance to the rationale of developing improved irradiation and chemotherapeutic treatment regimes for patients presenting with advanced stages of many tumors. In many such advanced solid tumors, treatment failures result from uncontrolled growth of undetected regional or blood-borne metastases rather than from failure to control the primary tumor with irradiation or surgery (18). Therapeutic improvements require that these microscopic or subclinical metastatic deposits be sterilized, either by the use of irradiation or systemic chemotherapy. This may be achieved with lower doses than are required to control larger primary tumors, partly because fewer cells must be inactivated but perhaps also because the cells in these small deposits are intrinsically more sensitive to cytotoxic therapy. This latter possibility formed the basis of the present study.

MATERIALS AND METHODS

Tumor System. The Lewis lung carcinoma, a transplantable, poorly differentiated epidermoid carcinoma that arose spontaneously in the lung of a C57BL mouse in 1951 (17) and has been studied in this department over the last 3 years, was used throughout this study. The i.m. TD$_{50}$ of our subline was approximately 3 cells. Tumors were passaged and grown in 2- to 3-month-old female C57BL mice of the Institute of Cancer Research colony by the s.c. injection into both flanks of inocula of $10^4$ to $10^5$ viable tumor cells. The tumors used in each experiment all came from the same passage, which had been implanted 12 to 14 days earlier. Before treatment, s.c. tumors were selected according to volume by in situ measurement of 3 diameters using vernier calipers. For each experiment on s.c. tumors, mice bearing tumors were allocated into treated and control groups so that each group contained 4 tumors, the mean volumes of which differed by less than 5%. The mean volume of tumors varied slightly from experiment to experiment in the range of 450 to 600 cu mm.

Pulmonary tumors were prepared as "artificial metastases" by the i.v. injection of 0.20 ml of a suspension containing $10^4$ viable tumor cells, $10^5$ heavily irradiated tumor cells, and $10^6$ 15-μm (in diameter) plastic microspheres (Minnesota Mining and Manufacturing Co., Minneapolis, Minn.). This technique, developed previously for the lung colony assay for these tumor cells, yielded about 25 pulmonary tumors/animal with a mean diameter of 1.0 mm and a mean tumor volume of 0.5 cu mm after 13 or 14 days of growth (14).

Irradiations. All irradiations in situ were given as whole-body doses to unanesthetized mice at a 60Co γ-ray dose rate of 350 to 380 rads/min as previously described (14). For irradiation in hypoxic conditions, the tumor-bearing mice were sacrificed by nitrogen asphyxiation 15 min before treatment. For hypoxic treatment of pulmonary tumors, tracheal compression was applied during the 15-min interval and during irradiation to prevent intrabronchial diffusion of oxygen.

In vitro γ-irradiations were given at 300 to 350 rads/min

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1 The work contained in this report was partly supported by National Cancer Institute Contract NCI-CM-23717.

2 Work in this report done while on a Moseley Fellowship from Harvard Medical School, Boston, Mass. To whom reprint requests should be addressed, at Department of Radiation Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Mass. 02114.

Received December 12, 1974; accepted May 21, 1975.
to suspensions of single tumor cells (10⁶ cells/ml) or to intact excised pulmonary tumors in Ham's F-12 medium (Biocult Laboratories, Glasgow, Scotland) in equilibrium with 5% CO₂ in air at 37°C. In vitro irradiation of excised pulmonary nodules was performed in containers containing 20 tumors in 10 ml of medium. These were incubated in 5% CO₂ in air for 20 min before irradiation in order to allow the oxygen tension within each nodule to reach a steady state level.

**Preparation of Cell Suspension.** The s.c. tumors were excised and minced with scissors into fragments not exceeding 2 mm in diameter, and a suspension of single cells was prepared by a combined mechanical and enzymatic technique as previously described (14). A minor modification of this technique was used to prepare single cell suspension from the small pulmonary tumors. Immediately following treatment, the lungs containing the pulmonary tumors were removed from mice in both the control and the treated groups (3 mice/group). The tumor-bearing lungs were washed in Dulbecco's phosphate-buffered Saline Solution A (Oxoid Ltd., London, England). Approximately 20 pulmonary tumors, carefully selected to be 1.0 to 1.2 mm in diameter, were excised intact and nearly free of adjacent lung parenchyma. These tumors were then minced individually in order to prevent preferential selection of cells from the tumor periphery and were incubated for 20 min at 37°C in Dulbecco's phosphate-buffered Saline Solution A containing 0.15% trypsin (Bacto-trypsin, Difco Laboratories, Detroit, Mich.) and 0.1 mg of crude beef pancreatic DNase per ml. All subsequent procedures were the same for cells from both pulmonary and s.c. tumors. The technique resulted in clean suspensions of single tumor cells of which at least 90% excluded the dye erythrosin B. The cell yield from both pulmonary and s.c. tumors, either treated or untreated, was approximately 2 × 10⁶ viable (nonstained) cells/g of tumor excised.

**Cell Survival Assays.** Two techniques were used to assay, by colony-forming ability, the survival of the tumor cells. The ability of the cells to form lung colonies in recipient mice was measured by the technique of Hill and Bush (9). With this technique, a linear relationship between tumor yield and the number of viable Lewis lung carcinoma cells injected was observed for lung colony counts ranging from 1.5 to 60 pulmonary tumors/animal. The mean cloning efficiency (i.e., the proportion of cells producing lung colonies in the injected suspension) for untreated tumor cells was between 0.20 and 0.30%. This assay, due to its low cloning efficiency, was not satisfactory to evaluate the survival of cells from the 0.5-cu mm pulmonary tumors because it required more cells than could be recovered from these small tumors. The 2nd technique was an in vitro colony-forming assay developed for this subline of Lewis lung carcinoma (V. D. Courtenay. An in Vitro Colony Assay in Soft Agar for the Lewis Lung Tumor and the B16 Melanoma Taken Directly from the Mouse, in preparation). As previously described (14), this technique of cell growth in a double layer of soft agar yielded tight spherical colonies containing an average of more than 500 cells after 10 to 12 days of incubation. Plating efficiencies ranged between 20 and 50%. Good agreement has been observed between the results obtained with the soft agar assay and the lung colony assay (Chart 2; Ref. 14).

For the cells from s.c. tumors that were irradiated in situ, surviving fractions were calculated by the fractional reduction in the yield of colonies per tumor in the treated tumor groups compared to the untreated group (14). For cells irradiated in vitro and those from the 0.5-cu mm pulmonary tumors, the surviving fractions were calculated as the ratio of the cloning efficiencies of the irradiated tumor cells to the untreated tumor cells. Since in the present experiments the time between in situ irradiation and tumor excision was short, these 2 methods gave similar results. The standard errors of the mean are shown for all surviving fractions larger than the symbol except where the error bars for points at the same dose level might cause confusion. Survival curve characteristics ($D_{0}$, $D_{10}$, and $n$) and their 95% confidence limits were computed by linear regression analysis on all results at dose levels yielding surviving fractions below 0.10.

**RESULTS**

In air-breathing unanesthetized mice, the cells from 0.5-cu mm pulmonary tumors were markedly more sensitive to γ-irradiation than were those from 500-cu mm s.c. tumor (Chart 1). As will be shown presently, the major reason for this difference is that only the large Lewis lung tumors contained a substantial proportion of cells that were hypoxic under normal air-breathing conditions. This subpopulation of hypoxic cells resulted in the radioresistant terminal portion of the biphasic survival curve for cells from large s.c. tumors ($D_{0} = 315$ rads). Chart 2 and Table 1 showed that this slope was similar to that for tumors in which all cells were made severely hypoxic before in situ γ-irradiation by nitrogen asphyxiation ($D_{0} = 307$ rads). The fraction of hypoxic cells, calculated from the vertical displacement of
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Chart 2. Co γ-ray survival curves of cells from 500-cu mm s.c. tumors treated: in vitro as well-oxygenated cell suspensions (●, D0 = 111 rads, D0 = 342 rads, n = 22); in situ in air-breathing, unanesthetized mice (●) and A, D0 = 315 rads; and in situ in nitrogen-asphyxiated mice (○ and Δ, D0 = 307 rads, D0 = 647 rads, n = 8). ○, ○, and ●, in vitro soft agar assay; Δ and A, lung colony assay.

Table 1

<table>
<thead>
<tr>
<th>Conditions of irradiation</th>
<th>D0(rads)</th>
<th>D0(rads)</th>
<th>n</th>
<th>Hypoxic fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c. tumors, 500 cu mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro, single cells</td>
<td>111 (99–127)*</td>
<td>342</td>
<td>22 (8–60)</td>
<td>0.00</td>
</tr>
<tr>
<td>In situ, air breathing</td>
<td>315 (290–347)</td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>In situ, hypoxic</td>
<td>307 (270–357)</td>
<td>647</td>
<td>8 (3–22)</td>
<td>1.00</td>
</tr>
<tr>
<td>Pulmonary tumors, 0.5 cu mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro, single cells</td>
<td>89 (63–148)</td>
<td>267</td>
<td>20 (0.5–900)</td>
<td>0.00</td>
</tr>
<tr>
<td>In vitro, excised and intact tumors</td>
<td>250 (206–314)</td>
<td></td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>In situ, air breathing</td>
<td>106 (72–198)</td>
<td>318</td>
<td>20 (0.4–1100)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>In situ, hypoxic</td>
<td>270 (240–308)</td>
<td>985</td>
<td>39 (13–111)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, 95% confidence limits.
suspension of single cells (D, = 89 rads, D90 = 267 rads, n = 20); in vitro of the Lewis lung carcinoma treated: in vitro as a well-oxygenated Colony-forming ability was assayed in vitro in soft agar. as excised intact 0.5-cu mm tumors (D, = 250 rads); and in situ in nitrogen-asphyxiated mice (D, = 270 rads, D90 = 985 rads, n = 39).

in situ in tumors were sufficiently well perfused in situ by an intrinsic microvascular supply to prevent the appearance of detectable regions of hypoxia. An alternative explanation, that the cell-suspending technique only removed cells from the well-oxygenated periphery of these small tumors, was guarded against by carefully mincing the tumors before trypsinization.

Other investigators have also seen a reduced hypoxic fraction in small tumors. Suit and Maeda (19), using a C3H mouse mammary carcinoma assayed by local tumor control probability, reported that 0.6-cu mm tumors grown subdermally in the ear had a hypoxic fraction of 0.001, while 250-cu mm tumors grown in the flank had a hypoxic fraction of about 0.15. Fu et al. (8) have performed experiments similar to those reported here using the EMT6 tumor and have also found a hypoxic fraction of about 0.3 for approximately 1-g s.c. tumors but a very low hypoxic fraction in 0.5 to 2 mm (in diameter) lung nodules.

The initial portion of the γ-ray survival curve of cells from 500-cu mm tumors irradiated in situ in air-breathing mice (Chart I) was drawn with the shape predicted theoretically for a mixed population with this Lewis lung carcinoma of 64% oxygenated and 36% severely hypoxic cells, as previously reported (14). The absence of a significant inflection or “break” point prior to the radioresistant hypoxic terminal portion is probably the combined result of the high extrapolation number of the oxygenated cell population and the relatively high hypoxic fraction (14). However, the surviving fractions observed after low doses of γ-irradiation (200 to 500 rads) for cells treated in situ from both 0.5-cu mm and 500-cu mm tumors were lower than were predicted by the multitarget, single-hit survival curve model that has evolved from in vitro single cell survival curve studies (5). While this consistent discrepancy is not statistically significant, it may reflect that a portion of cells within these solid tumors sustain irreparable irradiation damage and have an irradiation survival curve with an initial negative slope, as suggested by Dutreix et al. (3).

The data in Table 1 show that cells taken from pulmonary tumors had a lower D90 than did cells from s.c. tumors under 2 different test situations in which the oxygen concentrations were similar: cells removed and irradiated in vitro cells and cells treated in situ in dead mice (hypoxic irradiation). These differences were not large and their significance could only be established reliably by multiple repeat experiments, but if they are real they could reflect differences in the age density distributions (4) for tumors of different size. There is, however, little support for this hypothesis in the available cell kinetic data on this tumor. The pulmonary tumors have been found to have a thymidine labeling index of 50% (R. P. Hill and J. A. Stanley, Pulmonary Metastases of the Lewis Lung Carcinoma: Cell Kinetics and Response to Cyclophosphamide at Different Sizes, in preparation), but in paraffin sections of the s.c. tumors we have found a labeling index of 45%. Cell suspensions from tumors labeled with thymidine in vivo and removed 2 hr later had a labeling index of 36%. The growth fraction of 14-day-old pulmonary tumors was estimated at 0.85 (R. P. Hill and J. A. Stanley, Pulmonary Metastases of the Lewis Lung Carcinoma: Cell Kinetics and Response to Cyclophosphamide at Different Sizes, in preparation), compared with a value of 0.90 for 21-day-old s.c. tumors, as reported by Simpson-Herren et al. (15).

Durand and Sutherland (2), who studied V79 Chinese hamster cells grown in vitro as “spheroids,” reported that intercellular contact increased the shoulder of the survival curve. Their studies suggested that tumor cells in situ might, because of intercellular contact, accumulate and repair considerably more sublethal irradiation damage than was predicted by studies on tumor cell lines grown in tissue culture. Such a phenomenon should be revealed in our data by a higher extrapolation number or D90 value in the curve for 0.5-cu mm tumors irradiated in situ compared with cells from these tumors irradiated in suspension. Unfortunately,
the scatter in the data and the resulting wide confidence limits on the \( n \) values prevent us from detecting even quite large differences in \( n \) value. Thus, the fact that the best-fit extrapolation number was 20 in each case must be held as possibly being a coincidence. However the data suggest that in situ intercellular contact does not increase the shoulder of the irradiation survival curve for Lewis lung carcinoma cells.

In concurrent experiments, Steel and Adams have shown an apparent size dependence of therapeutic response to cyclophosphamide of this subline of Lewis lung carcinoma (16). They have shown, using both the in vitro agar colony and the end-point dilution assays, that the surviving fraction of tumor stem cells following a dose of cyclophosphamide was lower by a factor of approximately 10 in small pulmonary tumors than in larger i.m. tumors. Whether this was the result of better drug availability or increased intrinsic cellular sensitivity in the pulmonary tumors has not yet been established. However, the results of Steel and Adams and the data in the present report suggest the probability that tumor cells within small metastatic implants are more sensitive to chemotherapy than are those in larger tumors, due to, at least in part, better drug delivery to the tumor cells from a better tumor microvasculature. This possibility has not as yet been fully considered in the oncological literature as an explanation for the apparent success in treating micrometastases with systemic chemotherapy (13).

The major finding of this study, that the sensitivity of \( \gamma \)-irradiation of cells within small pulmonary implants is considerably higher than those in larger tumors, may in part explain the success in the control of some subclinical lymph node deposits (6, 7, 22) and, more recently, of presumed subclinical pulmonary metastases from osteogenic carcinoma (11). However, while our results showed micrometastases to have greater tumor cell radiosensitivity at all dose levels (Chart 1) compared to that of the large tumors, clearly the conventional fraction schedules that are used clinically were not evaluated in this study. Early reports of similar successes in controlling subclinical pulmonary metastases from osteogenic sarcoma by tolerated doses of methotrexate may also have been due to improved drug delivery to well-perfused small pulmonary implants (10). Thus these results encourage prophylactic organ-specific irradiation or systemic chemotherapy in patients having a high probability of undetected metastatic disease, because tumor cells in these small implants may be highly sensitive to cytotoxic therapy. It will be necessary in such treatments to stay well short of the dose levels that are known to have a significant complication rate, but if the human tumor micrometastases have a microvasculature that resembles that of the experimental tumor studied here, such treatment should nevertheless be expected to benefit the patient.

ACKNOWLEDGMENTS

We gratefully acknowledge the helpful advice and criticism of Dr. O. C. A. Scott, Professor M. J. Peckman, and Professor L. F. Lamerton throughout the project and during the preparation of the manuscript and the valuable assistance of Dr. N. M. Blackett in statistical analysis.

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Cancer Res 1975;35:2488-2493.

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