Activity of Mitomycin C for Aerobic and Hypoxic Cells in Vitro and
in Vivo^1

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ABSTRACT

We have observed the selective toxicity of mitomycin C toward hypoxic as compared to aerobic cells in vitro for three established cell lines (Chinese hamster ovary, Chinese hamster V-79, and human HeLa) and for cells from the transplantable KHT murine tumor. The magnitude of the selective toxicity was cell line dependent.

We have studied the in vivo effects of mitomycin C against aerobic and hypoxic cells of two transplantable murine tumors: the KHT fibrosarcoma and the 16/C mammary carcinoma. Either mitomycin C was given with radiation to kill most of the aerobic cells, or it was given alone. Endpoints of response were cell survival assessed by lung colony assay for the KHT tumor, and growth delay for the 16/C tumor. In some experiments, mitomycin C appeared more effective when used with radiation than when used alone, but the results of combined treatment fell just within the range of additivity as defined by isobologram analysis. The effects of combined treatment were independent of the order in which drug and radiation were given.

Mitomycin C was also used in combination with Adriamycin to treat the 16/C tumor, since we have found previously that Adriamycin spares hypoxic cells in this tumor. In three of four experiments, combined drug effects were slightly greater than predicted by an additive relationship. We conclude that mitomycin C is active against hypoxic cells in two murine tumors, but that it has at most minor specificity for hypoxic as compared to aerobic cells in vivo.

INTRODUCTION

Hypoxic cells in solid tumors are known to be resistant to treatment with radiation. Hypoxic cells may also be resistant to chemotherapy because of limited distribution of some drugs to them, and because hypoxic cells are often slowly proliferating, while most anticancer drugs are more active against rapidly proliferating cells. We have demonstrated previously that hypoxic cells of a solid tumor are resistant to Adriamycin (19), and there is evidence for limited distribution of Adriamycin and several other drugs to cells within solid tumors (5, 6, 10). Studies of penetration of several drugs into multicellular tumor spheroids also imply that limited drug distribution within solid tumor tissue may be a common source of treatment failure (9, 18, 23). Thus, there is considerable potential in combined modality treatment and in combination chemotherapy for drugs which are selectively toxic for hypoxic or poorly nourished cells.

Mitomycin C is an alkylating agent that is thought to require reduction of its quinone group for biological activity and has been reported to be selective for hypoxic cells in vitro (2, 8, 12, 13). In the present paper, we have confirmed that mitomycin C is more active against hypoxic cells from several cell lines and a transplantable tumor in culture. We also present a study of the response of aerobic and hypoxic cells to mitomycin C in 2 transplantable tumors in vivo.

MATERIALS AND METHODS

In Vitro Studies. CHO^3 and HeLa cells were routinely cultured in suspension in complete α-medium (15) supplemented with 10% fetal calf serum (v/v) (Flow Laboratories, Rockville, Md.) as described previously (20). Chinese hamster V-79 cells were grown as monolayers in the same medium and were thymosinized before use in the mitomycin C toxicity tests. Murine KHT tumor cells were prepared directly from tumors growing in vivo (22). The colony-forming ability of CHO, HeLa, and V-79 cells was determined by standard plating techniques (20). Colony-forming ability of KHT cells was determined by a soft agar assay (22).

Mitomycin C (Bristol Laboratories, Syracuse, N. Y.) was reconstituted with sterile 3-times-distilled water prior to dilution into growth medium to give the desired concentration for cell exposure. The stock drug was either made up immediately before use or stored at 500 µg/ml for no longer than 2 weeks at 4°C.

Toxicity tests were carried out in glass vials containing 10 ml of cells in stirred suspension at a concentration of 5 x 10^6/ml in α-medium plus 10% fetal cell serum using a method described in detail elsewhere (24). For aerobic experiments, 5% CO_2:95% nitrogen (oxygen content, <10 ppm) was passed over the cells. Cells were gassed for 45 min before drug addition; for the hypoxic experiments, this procedure results in radioactive gas outlet tube of the vial.

In Vivo Experiments with the KHT Fibrosarcoma. The KHT tumor was maintained by serial transplantation of tumor cells into the flanks of C3H mice, with reestablishment from a stock of frozen tumor cells at approximately 6-month intervals. For generation of tumors to be used in experiments, a cell suspension was made (22), and 0.1 ml of α-medium containing about 10^6 cells was injected s.c. into each flank of recipient mice. Mice were treated 8 to 10 days later when their tumors attained a mean diameter in the range of 9 to 12 mm.

Mice received treatment with mitomycin C and/or radiation. Mitomycin C was diluted in sterile water and injected i.p. in a volume of 0.01 ml/g body weight. Whole-body irradiation was delivered to nonanesthetized mice using ^137Cs γ-rays at a dose rate of 0.9 gray/min (4).

Survival of tumor cells was assessed at various intervals up to 24 hr after treatment by the lung colony assay (7). Mice were killed, their tumors were removed, and a cell suspension was prepared by a combination of mechanical and enzymatic methods (22). The total number of cells and dye-excluding cells was counted, and appropriate dilutions of

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the cell suspension in α-medium were admixed with $4 \times 10^6$ heavily irradiated KHT cells (100 gray) and 7.5 $\times 10^6$ microspheres (size, 15 ± 3 μM Company, St. Paul, Minn.), prior to i.v. injection into the tail veins of groups of 6 to 8 mice. The mice were killed 2 to 3 weeks later, the lungs were excised and fixed, and the number of lung colonies was counted with the aid of a dissecting microscope (7).

In Vivo Experiments with the 16/C Carcinoma. The 16/C tumor originated in a C3H mouse in Birmingham, Ala., and is sensitive to several anticancer drugs (1); it was obtained from the National Cancer Institute Tumor Bank, Worcester, Mass., and has been maintained by serial transplantation of tumor pieces into flanks of C3H mice, with establishment from a stock of frozen tumor cells at approximately 6-month intervals. We have been unable to generate a high-quality single-cell suspension or to develop a clonogenic assay for the 16/C tumor, and we have assessed the effects of treatment by using the endpoint of growth delay.

For generation of tumors, a cell suspension (containing small clumps) was prepared by mechanical means, and about $2 \times 10^6$ cells in 0.05 ml medium were injected i.m. into the left hind legs of mice. Palpable masses appeared 10 to 12 days later, and tumor diameter was estimated to the nearest 0.5 mm by passing the tumor-bearing leg through a series of graded holes drilled in Lucite. Mice were coded with ear tags and, when tumors attained a mean diameter of 9 mm (tumor weight, 0.3 g), the mice were randomized into groups of 7 to 8 animals for treatment. Mice were treated with mitomycin C, as described above, with or without local tumor irradiation. Irradiation was undertaken using a specially designed double-headed 250-kV X-ray machine at a dose rate of 11.4 gray/min (14), and was usually delivered to nonanesthetized mice that were restrained in a lucite container with the tumor-bearing leg taped within the field. In a few experiments, we irradiated the tumors under hypoxic conditions: hypoxia was achieved by applying a clamp to occlude blood supply to the tumor in anesthetized mice (tribromoethanol, 300 μg/g) for 5 min prior to and during irradiation (17).

For assessment of treatment on tumor growth, mice were reassigned to their original cages, and tumor diameter was estimated 3 times weekly by an observer who was unaware of their treatment history. Mice were killed humanely when their tumors attained a mean diameter of ∼15 mm. Tumor growth curves were constructed from a previously defined calibration curve relating tumor weight to mean tumor diameter. The endpoint used to determine response to treatment was mean delay in time to grow to 1 g (mean diameter, 12.5 mm) as compared to control animals.

RESULTS

In Vitro Studies. In initial studies, CHO cells were exposed to mitomycin C (1 μg/ml) under aerobic and hypoxic conditions (11). Hypoxic cells were more sensitive to mitomycin C than were aerobic cells (Chart 1a). Hypoxia alone had minimal effect on plating efficiency, and these control data are omitted for clarity. Previous results from this laboratory have indicated that the degree of selective toxicity for hypoxic cells of other drugs may be cell line dependent (20). Thus, human HeLa cells, Chinese hamster V-79 cells, and murine KHT tumor cells were also exposed to mitomycin C (1 μg/ml) under hypoxic and aerobic conditions. Results are shown in Chart 1, b, c, and d, respectively. All cells showed an increased sensitivity to mitomycin C under hypoxic as compared to aerobic conditions, but the magnitude of the difference varied among the 4 cell lines tested. The 16/C carcinoma was not studied in vitro because of the lack of an in vitro colony assay for this tumor. Our results indicated that the selective toxicity of mitomycin C for hypoxic cells is a general phenomenon in vitro which might be exploitable in an in vivo model tumor system.

Experiments with the KHT Fibrosarcoma. The design of initial experiments to compare toxicity of mitomycin C for aerobic and hypoxic cells in vivo is shown schematically at the top of Chart 2. Groups of KHT tumor-bearing mice received graded doses of mitomycin C either alone or immediately after 20 gray irradiation. Animals were killed 24 hr later, and tumors were removed and assayed for cell survival. Mitomycin C showed some toxicity to unirradiated, predominantly aerobic cells, but there was an apparent increase in toxicity toward cells that survived 20 gray radiation; these cells are presumed to be hypoxic at the time of drug injection (Chart 2).

To study the above effects in more detail, groups of mice were given mitomycin C (8 μg/g) at 2, 1, or 0.5 hr before 20 gray irradiation, and cell survival was assayed immediately after irradiation. Other groups of animals received 20 gray irradiation followed immediately by mitomycin C (8 μg/g), and tumors were left for 0.5, 1, 2, or 24 hr before the animals were killed and tumor cell survival was assessed. Other animals received mitomycin C at similar times prior to assay but without irradiation. Results of these experiments are shown in Chart 3. Survival was the same for unirradiated KHT cells (predominantly aerobic) as for irradiated cells (predominantly hypoxic) from 0 to 2 hr, and was independent of whether mitomycin C was given before or after irradiation. In contrast, there was an apparent decrease in survival for the irradiated plus mitomycin C group (as compared to mitomycin C alone) when the survival assay was delayed until 24 hr after treatment, thus confirming the results of Chart 2.

The results of Charts 2 and 3 express cell survival as a percentage of the cells recovered at the time of the assay. In
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Chart 2. Survival of cells (percentage of cells recovered) in the KHT tumor following treatment with graded doses of mitomycin C (Mit C) alone (Y, A, ●, ○) or given immediately after 20-gray radiation (Y, A, Δ, C). Survival was assessed by lung colony assay at 24 hr after treatment. Different symbols indicate different experiments; symbols with arrows, upper limit of cell survival.

Chart 4. Total cell recovery from KHT tumors at different times after treatment with mitomycin C (Mit C) alone or with 20-gray irradiation. Experimental conditions were the same as for Chart 2.

Peckham (16) have shown that limits of additivity for 2 agents can be determined only by isobologram analysis derived from complete dose-response curves for each agent used alone. The dose-response curve for the 16/C tumor treated with radiation has been published previously (19); in Table 1, we use these data and the dose-response curve for mitomycin C (2.5 or 5.0 μg/g) and radiation (15 gray), with limits of additivity as defined by isobologram analysis. The interaction of mitomycin C and radiation falls at the upper end of the expected range of additivity (Table 1).
Mitomycin C and Hypoxia

We conclude that mitomycin C has at most minor selectivity for hypoxic cells of the 16/C tumor.

Interaction of Mitomycin C and Adriamycin. We have shown previously that Adriamycin tends to spare hypoxic cells of the 16/C tumor, probably because the drug has limited diffusion from tumor blood vessels (19). If mitomycin C were selective for hypoxic cells in vivo, then the combination of Adriamycin and mitomycin C might be therapeutically beneficial due to the 2 drugs acting on different populations of tumor cells. We have, therefore, studied this interaction.

The effect of combining different doses of the 2 drugs on growth delay is shown in Table 3; drug doses were chosen to achieve acceptable levels of host toxicity that would allow antitumor effects to be assessed by regrowth delay. The expected range of values of growth delay, based on an additive interaction derived by isobologram analysis of the dose-response curves for mitomycin C (Chart 6) and Adriamycin [published previously (19)], is also included in Table 3. In 3 of 4 experiments, the interaction appeared to be superadditive, although we also found considerable toxicity. This result might, of course, be due to pharmacological interactions other than drug effects on different tumor cell populations and, in the absence of normal tissue assays, does not indicate therapeutic benefit.

<table>
<thead>
<tr>
<th>Dose of mitomycin C (μg/g)</th>
<th>No. of tumors</th>
<th>Delay in growth to 1 g (days)</th>
</tr>
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<tbody>
<tr>
<td>2.5</td>
<td>21</td>
<td>12.0 ± 1.5 (8.5–11.4)</td>
</tr>
<tr>
<td>5.0</td>
<td>22</td>
<td>21.1 ± 1.6 (14.1–20.5)</td>
</tr>
</tbody>
</table>

Table 1. Mean delay in growth to 1 g for the 16/C tumor following treatment with mitomycin C and 15-gray radiation

<table>
<thead>
<tr>
<th>Dose of mitomycin C (μg/g)</th>
<th>Difference in aerobic-hypoxic growth delay (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.4 ± 1.8</td>
</tr>
<tr>
<td>2.5</td>
<td>5.2 ± 2.2</td>
</tr>
<tr>
<td>5.0</td>
<td>3.8 ± 5.4</td>
</tr>
</tbody>
</table>

Table 2. Difference in time for tumors to grow to 1 g following radiation treatment given under aerobic as compared to hypoxic conditions, at 0.5 hr after mitomycin C

To further analyze the interaction of mitomycin C with aerobic and hypoxic cells of the 16/C tumor, we administered the drug at 0.5 hr before 15-gray radiation given under either aerobic or hypoxic conditions (Table 2). The difference in growth delay for radiation alone given under aerobic and hypoxic conditions depends on the proportion of aerobic cells in the tumor that can be protected by application of a clamp to occlude the blood supply (17, 19). A drug that is selectively toxic for aerobic cells would be expected to decrease this difference when given prior to irradiation, whereas a drug that is selectively toxic for hypoxic cells would be expected to increase it. Although there was some tendency for mitomycin C to increase the separation between regrowth curves following subsequent radiation under aerobic and hypoxic conditions, this effect was not significant (Table 2).

<table>
<thead>
<tr>
<th>Dose of drugs (μg/g)</th>
<th>Mitomycin C</th>
<th>Adriamycin</th>
<th>No. of spontaneous deaths</th>
<th>Delay in growth to 1 g (days)</th>
</tr>
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<tbody>
<tr>
<td>2.0</td>
<td>6.0</td>
<td>1/8</td>
<td>7</td>
<td>14.0 ± 2.0 (4.4–7.3)</td>
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<tr>
<td>2.5</td>
<td>7.5</td>
<td>2/7</td>
<td>7</td>
<td>11.9 ± 1.1 (6.2–9.6)</td>
</tr>
<tr>
<td>2.5</td>
<td>5.0</td>
<td>5/16</td>
<td>13</td>
<td>8.6 ± 1.2 (3.9–7.3)</td>
</tr>
<tr>
<td>4.0</td>
<td>4.0</td>
<td>1/8</td>
<td>6</td>
<td>5.8 ± 1.5 (6.5–9.5)</td>
</tr>
</tbody>
</table>

Table 3. Mean delay in growth to 1 g for the 16/C tumor following treatment with mitomycin C and Adriamycin

a Drugs were administered within a 0.5-hr period.
b Animals that died after their tumors grew to 1 g are included in analysis of growth delay.
c One mouse achieved permanent local control in each of these groups and was excluded from calculation of mean delay in growth.
d Mean ± S.E.
e Numbers in parentheses, expected range, calculated on the basis of additive interaction using isobologram analysis.
DISCUSSION

We have confirmed that mitomycin C has selective toxicity for hypoxic as compared to aerobic cells in vitro using several different cell lines and the KHT tumor. The reasons for the differences in the relative sensitivity of the cell lines to mitomycin C under hypoxic and aerobic conditions are not known and are currently under study. The present results are in qualitative agreement with those previously reported by Kennedy et al. (8) and Teicher et al. (21) for murine EMT6 cells in vitro. However, in those studies and in earlier work (13), prolonged (>4 hrs) hypoxic incubation was necessary before selective toxicity could be seen, and no differential toxicity was observed if the drug was added at the same time as the gas phase above the cultures was changed from aerobic to hypoxic. In the present work, cells were incubated for 45 min under hypoxia before drug was added. Further work will be necessary to study the effect of this hypoxic preincubation on drug sensitivity. Such preincubation is necessary for oxygen to be effectively removed and might lead to biochemical alterations within the cell that influence cellular sensitivity to mitomycin C.

We have shown that mitomycin C is active for cells which survive irradiation in 2 experimental tumors (and Adriamycin treatment of the 16/C tumor), and these cells are known to be hypoxic at short intervals after treatment with radiation or Adriamycin. Mitomycin C was additive with radiation, as has been reported for studies with cells in culture (12). However, we found little evidence for selective effects of mitomycin C against hypoxic cells in vivo. Similar conclusions may be drawn from an earlier study using EMT6 tumors (13).

The activity of drugs against cells within a solid tumor depends both on cellular sensitivity to the drug and on the diffusion of the drug to clonogenic cells. Cells in solid tumors might be hypoxic because they are situated at some distance from tumor blood vessels, or because of acute stasis in neighboring vessels. Drugs such as Adriamycin are known to have poor diffusion characteristics into solid tumors and spheroids (10, 18), and this effect probably leads to sparing of hypoxic cells that are distant from blood vessels (19). There are no data on penetration of mitomycin C into tissue, but this drug may also be expected to show a decreasing concentration with increasing distance from tumor blood vessels. The lack of specificity of mitomycin C for hypoxic cells in vivo might be due, therefore, to 2 opposing effects: a greater cellular sensitivity, but a lower drug concentration in the neighborhood of hypoxic cells. Alternatively, the cells which survive radiation in these tumors might be acutely rather than chronically hypoxic, whereas selective drug effects may require more prolonged exposure of cells under hypoxic conditions. A limited life span of hypoxic cells in vivo, and reoxygenation of initially hypoxic cells after treatment with radiation or Adriamycin (19), might also limit the effective contact time between mitomycin C and hypoxic cells and, hence, their killing by the drug.

Despite clinical interest in the combination of mitomycin C and radiation (3), our results do not imply a therapeutically beneficial interaction. The drug is toxic to both animals and humans, and its toxicity is often unpredictable. Improvements in the results of combined modality treatment might occur with drugs that have selective toxicity for hypoxic cells, and we believe that research into the development of such drugs is important. Our current results suggest the need for agents that: (a) have greater selectivity for hypoxic cells than does mitomycin C; (b) require fairly short exposure time for cell killing; and (c) have diffusion characteristics that enable a high concentration to be achieved in the proximity of hypoxic cells in vivo.

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