Use of the Agar Diffusion Chamber for the Exposure of Human Tumor Cells to Drugs

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ABSTRACT

Human melanoma xenografts in immune-deprived mice have been used to assess the value of the agar diffusion chamber for chemosensitivity testing. Tumor cells were treated with melphalan, Adriamycin, or methyl trans-1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea either as solid tumors growing in mice or as suspensions in agar in i.p. diffusion chambers. Survival of clonogenic human tumor cells was measured by the agar diffusion chamber assay in both cases. Cell survival curves were log-linear for treatment of tumor cells in vivo or in the chambers. For melphalan the slopes of survival curves were significantly greater for treatment in the chambers than as solid tumors in vivo, but for methyl trans-1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea or Adriamycin, they were indistinguishable.

Experiments with [14C]melphalan showed that the levels of drug achieved were less inside the diffusion chambers than in the tumors in vivo so that the sensitivity of tumor cells to melphalan was much greater when they were treated in chambers.

The differences in drug exposure and in cellular chemosensitivity between chambers and tumors suggest caution in the interpretation of drug testing using this system, but the log-linear nature of the dose-response curves is an important feature which may be useful in the eventual development of optimal chemosensitivity testing systems.

INTRODUCTION

The development of methods which allow the direct measurement of the chemosensitivity of human tumor cells is an important area of current research. However, difficulties have been encountered both in the choice of suitable conditions for the exposure of cells to drugs and in the choice of an end point to measure drug effect. Growth of human tumor cell colonies in semisolid media allows the measurement of the survival of clonogenic cells after cytotoxic treatment (4, 11), and this end point may have advantages over alternatives for measuring chemosensitivity (9, 10). However, the exposure of cells to drugs in vitro is highly artificial and restricts the use of drugs which require metabolic activation. It is doubtful whether in vitro drug exposure can accurately reflect in vivo pharmacokinetics.

The ADC3 incubated in the peritoneal cavity of an irradiated mouse provides a means of growing colonies from cell suspensions of human bone marrow cells (6), human tumor xenografts in immune-deprived mice (16), and human tumors taken directly from patients (3). Plating efficiencies of tumor cells with this assay are higher than those reported for some in vitro assays (10, 18), but a direct comparison with the in vitro method devised by Courtenay (4) failed to show any significant difference in plating efficiencies (3). The ADC assay has been used to measure clonogenic cell survival after treatment of bone marrow cells (5), a series of human tumor xenografts (12, 16, 17), cryopreserved human tumors (13), and human tumors taken directly from patients (7).

The ADC assay may be used to grow colonies from tumor cells treated with drugs in vivo as xenografts (Chart 1). In this context, it has proven to be linear, robust, and reliable, but it is time consuming and expensive. However, the assay has the unique feature that cells may also be exposed to drugs by treatment of the ADC-bearing mice ("in ADC" drug exposure; Chart 1). In this treatment system, the drug pharmacokinetics is in vivo, and it has been advocated as a method for chemosensitivity testing in a clinical setting (3, 7, 8).

In the present study, we have continued our use of the human tumor xenograft model to investigate and evaluate methods for chemosensitivity testing in human tumors. The in ADC exposure of human melanoma cells to drugs was compared to in vivo treatment of the same cells as xenografts (Chart 1), and drug penetration into the ADC and xenograft tumors was measured using radiolabeled melphalan. The details of the xenografting method, the characteristics of the tumors, and the in vivo survival curves have been published previously (12, 14).

MATERIALS AND METHODS

Melanoma xenografts were established and evaluated as described previously (14). Three xenograft lines, HX34, 41, and 47 were used for the present study. The 3 xenografts retained their histological appearance, human antigens, and human chromosomes throughout the experiments. HX47 significantly decreased its volume-doubling time with serial passage, but HX34 and 41 did not. HX34 lost melanin pigment progressively with serial passage but retained the dopa oxidase enzyme system (14).

The ADC assay for clonogenic human tumor cells was described by Smith et al. (16). In brief, cell suspensions were prepared by mechanical disaggregation without enzymatic degradation and counted in a hemocytometer using lissamine green under phase contrast. Bright cells which exclude dye were regarded as viable and were scored. Nontumorous cells were excluded by size and morphology only. Cell suspensions in Ham's Medium F-12 with 20% Special Bobby Calf Serum (Gibco Bio-Cult, Paisley, Scotland) were mixed with agar to a final concentration of 0.3% and introduced into a Millipore 0.35-ml diffusion chamber. Chambers contained between 5 x 10^6 and 2 x 10^6 cells, adjusted according to previously found plating efficiencies. For
each drug dose used, a range of cell concentrations was used to allow accurate estimation of the proportion of surviving cells. Chambers were incubated in the peritoneal cavities of preirradiated (900 R) male C57B mice (one chamber per mouse). Mice were treated, 2 days before irradiation, with 1-β-o-arabinofuranosylcytosine with (200 mg/kg), which prevents the lethal effects of this irradiation dose (16). Colonies of more than 50 cells were scored approximately 3 weeks later by direct vision under a dissecting microscope. Plating efficiency was calculated as colonies per viable cell plated. Surviving fractions of colony-forming cells were calculated as the ratio of the plating efficiencies of treated cells to that of control, untreated cells.

Chart 1 shows the alternative methods of drug treatment. For in vivo treatment, immune-deprived mice bearing tumors approximately 1 cm in diameter were treated by i.p. injection with melphalan, Adriamycin, or methyl CCNU. Methods of drug preparation have been described (12) and fresh solutions were always used. One or 2 mice (2 or 4 tumors) were used for each dose level in each experiment. Cell suspensions were prepared 18 hr later and assayed for colony forming cells by plating in 4 to 6 ADCs. Experiments were repeated at least 3 times each with melphalan and methyl CCNU, but usually twice with Adriamycin where no cell kill was observed. The 18-hr delay before preparation of cell suspensions is required to allow completion of drug action but is sufficiently short to avoid artifacts due to repopulation or removal of dead cells (12).

For "in ADC" treatment, a cell suspension was prepared from a xenograft tumor and plated in agar in chambers precisely as described for colony growth. Chambers were then implanted into the peritoneal cavity of a normal male C57B mouse (2 chambers/mouse) under brief ether anesthesia. Under the same anesthetic, the mouse was given an injection by tail vein of the drug under test. Eighteen hr later, the mouse was killed, and each chamber was transferred into a preirradiated mouse (1 chamber/mouse, 6 chambers/dosage level of drug) for incubation, and colonies were scored 3 weeks later.

The penetration of melphalan into ADC was measured using [14C]-melphalan. The application of this method to the measurement of drug penetration into xenograft tumors of different sizes has been described (15). Approximately 7.5 μCi of labeled melphalan (specific activity, 3.8 mCi/mmol; Radiochemical Centre, Amersham, U. K.) were added to unlabelled melphalan (Alkeran; Wellcome, Beckenham, Kent, U. K.) to a total dose of 15 mg melphalan per kg for each mouse. The drug solution was administered i.v. into male CBA/lac mice bearing xenograft tumors or male C57B mice bearing ADC. The xenograft tumor HX32, an undifferentiated adenocarcinoma which has been described previously (see Ref. 15 for details), was used for this aspect of the study. Mice were killed, and samples of blood, whole tumors, and ADC contents were collected at specific times thereafter and immediately frozen at −20°. Test samples, samples containing known quantities of the labeled drug, and untreated control samples were subsequently completely oxidized using an Oxymat electric furnace (Intertechique Ltd., London, U. K.), and the 14CO2 was collected in alkaline scintillant and counted in an automated scintillation counter (Intertechique, Ltd.). The results were expressed as the content of melphalan plus metabolites per g of material, derived by comparison of the content of the radioactivity in the sample to the radioactivity in the standard melphalan solution.

RESULTS

Experiments were performed on HX41 and HX47 in their first five serial passages. HX34 was initially studied in late passage (passages 12 to 17) but was reestablished from cryopreserved material and studied in the first 2 passages.

Cell suspensions were easily prepared by mechanical means and more than 10⁶ viable tumor cells were obtained per g of wet tumor in all experiments. There was no detectable decrease in cell yield from treated tumors or differences in the proportion of viable cells between treated and control tumors. Plating efficiencies were 10 to 50% for HX34, 10 to 75% for HX41, and 3.5 to 18% for HX47. Both HX41 and HX47 showed a significant increase in plating efficiency with passage (see Ref. 12 for further details). There was a close linear relationship between cells plated and colonies scored in this system.

Clonogenic cell survival curves for three different human xenografts treated in ADC or in vivo with melphalan, methyl CCNU, and Adriamycin are shown in Charts 2, 3, and 4. The highest doses indicated on the horizontal axes for the data are related to the approximate 10% lethal dose for each drug in our mice (melphalan, 15 mg/kg; methyl CCNU, 30 to 35 mg/kg; Adriamycin, 12 to 15 mg/kg) so that the slopes of the lines give an impression of the relative effectiveness of equitoxic doses of each drug. Parameters of the curves derived by logarithmic linear regression analysis are shown in Table 1. The differences between slopes of the regression lines were tested for statistical significance at the 5% level using a t test.

The dose-response curves for treatment of the tumors in ADC or in vivo with melphalan were log-linear over the dose range tested and down to surviving fractions of 10⁻³. The results of treatment in ADC and in vivo were similar for HX34,
but for HX41 and HX47 the slopes of the regression lines ($D_{10}$) were significantly greater for treatment in ADC than for treatment in vivo. There was a negative shoulder on the dose-response curve for HX41 treated in ADC which was greater than that observed for treatment in vivo. If the tumors were ranked for sensitivity to melphalan, and only significant differences were considered, then the in vivo results would suggest a ranking $41 < 34 = 47$, while in ADC results suggest $41 = 34 < 47$.

The cell survival curves for treatment with methyl CCNU of HX34 and HX47 in vivo and in ADC were log-linear and there was no significant difference between them. The data for HX41 were scattered in the first decade of cell kill for both treatment systems so that no curve shape could be defined. There was no detectable clonogenic cell kill in any of the melanoma xenografts by treatment with Adriamycin in vivo or in ADC.

Chart 5 shows levels of melphalan plus metabolites achieved after injection of 15 mg/kg i.v., in blood, HX32 xenograft tumors, and ADCs. The in vivo tumor levels approached blood levels after 60 min and fell similarly until 120 min. Levels in the chambers were generally less than half of those in these tumors and never reached blood levels in the course of the experiments. The drug levels achieved in tumors using the i.v. route of administration (Chart 5) were similar to those achieved by...
Drug Treatment in Agar Diffusion Chambers

Table 1
Parameters of cell survival curves

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Drug exposure phase</th>
<th>%D10 (mg)</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan HX34</td>
<td>In vivo</td>
<td>6.5</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>In ADC</td>
<td>6.7</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td>HX41</td>
<td>In vivo</td>
<td>20.6</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>In ADC</td>
<td>9.8</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.0</td>
</tr>
<tr>
<td>HX47</td>
<td>In vivo</td>
<td>6.2</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>In ADC</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>Methyl CCNU HX34</td>
<td>In vivo</td>
<td>4.4</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>In ADC</td>
<td>4.1</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.3</td>
</tr>
<tr>
<td>HX47</td>
<td>In vivo</td>
<td>8.9</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>In ADC</td>
<td>5.3</td>
<td>4.1</td>
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<td></td>
<td>7.5</td>
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</tbody>
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The in ADC drug exposure system produced cell survival curves of similar exponential shape to those observed for treatment in vivo. In this respect, it differs from drug treatment of tumor cells in vitro where survival curves have generally not been observed to be exponential for human tumors (1, 11) or human tumor xenografts (2, 18), even when the same tumor xenografts show exponential cell survival curves when treated in vivo (2). Cryopreserved, noncultured human tumor cells also appear to demonstrate exponential cell killing by cyclophosphamide and Adriamycin when treated in ADC (13). It therefore appears that the nonexponential character of survival curves after drug treatment in vitro does not reflect the presence of resistant subpopulations of tumor cells but may be an explained artifact of the in vitro systems.

Gordon and Blackett (5) investigated the exposure of murine and human normal bone marrow cells to drugs in ADC. The dose-response curves were exponential for cyclophosphamide and 5-fluorouracil in all cases. Dose-response curves for methotrexate and vinblastine were exponential when mouse marrow cells were treated in ADC but showed plateaus when treated in vivo and subsequently plated in agar. The plateaus were observed when the cells were exposed to drugs in agar-free chambers, harvested, and then replated in agar-containing chambers. It was concluded that the presence of agar substantially altered the response of cells to the drugs, perhaps by altering drug access and clearance. It appears, therefore, that the in ADC exposure method for treating cells also may produce artifactual survival curves for some drugs in some circumstances.

In the present study, the slopes of the cell survival curves for treatment in ADC differed significantly from those obtained for treatment in vivo with melphalan but not with methyl CCNU. The amount of the differences appears to vary between the tumors. The differences between the in ADC results and the results in vivo lead us to be cautious about advocating the use of the ADC exposure system for clinical chemosensitivity testing, although in vitro chemosensitivity testing could have similar or even greater errors. The labor-intensive and expensive nature of the ADC method dictates that it should only be preferred to an in vitro method when special advantages can be demonstrated. The exponential shapes of the dose-response curves may be such an advantage but further work will be needed to clarify the reason for this difference from the in vitro techniques.

We suspected that the greater sensitivity of tumor cells to in ADC treatment with melphalan might be due to better drug penetration into the chambers since earlier work had shown relatively poor penetration by melphalan into the centers of s.c. growing xenografts (15). However, the radiotracer experiments do not show this. Drug levels in the chambers were less than those in the tumors. The data do not allow conclusions to be drawn about the duration of exposure to melphalan because significant levels of drug were still present after 2 hr. The presence of agar may prolong the exposure to melphalan. We have not excluded the possibility that the melphalan in the agar diffusion chambers is held selectively inside cells rather than in the agar matrix which might explain the low average melphalan

DISCUSSION

The treatment of tumor cells in the chambers in mice (in ADC) may be useful as an alternative method of chemosensitivity testing for human tumor biopsies. In this study, we have attempted to evaluate the validity of this method by comparing the results obtained for treatment of tumor cells in ADC with the results obtained for treatment of the same cells growing as xenografts in immune-deprived mice (in vivo). In both cases, the survival of clonogenic tumor cells after treatment was measured by using growth in soft agar as the end point.

The i.p. injection in a separate series of experiments (data given in Ref. 15) suggesting that differences between treatment in vivo and in ADC did not arise from different drug levels achieved by the two injection routes.

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concentration for chamber contents. It should also be noted that we have studied only a single xenograft line, and there may be variation between individual lines. However, it does seem likely that the melphalan concentration x time (C x t) product for tumors in vivo is greater than for chambers. It is surprising that cell kill by melphalan in ADC may be greater than in vivo for some tumors, and this obviously implies that cells are very much more sensitive to melphalan when suspended in agar. This difference might be due to changes in the cell proliferation kinetics of tumors when plated in chambers. This explanation would be compatible with the known proliferation dependence on the cytotoxic effect of melphalan but apparent absence of proliferation dependence of nitrosoureas (19).

The ADC assay and the treatment of cells in ADC have been useful in the investigation of colony growth and chemosensitivity of human tumor cells. However, the data presented here demonstrate that the exposure of cells to drugs may be different in chambers from tumors and that cellular chemosensitivity may also differ when the cells are placed in the chambers. Attempts at clinical application for this technique would therefore seem inappropriate at this time, but it may have a place, together with in vitro cloning assays and human tumor xenografts, in work to develop an optimal system for the growth of clonogenic human tumor cells and the measurement of their chemosensitivity.

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