Cytotoxicity of Adriamycin in MGH-U1 Cells Grown as Monolayer Cultures, Spheroids, and Xenografts in Immune-deprived Mice

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ABSTRACT

The cytotoxic activity of Adriamycin was examined in the MGH-U1 human bladder carcinoma line, grown as monolayer culture, as spheroids, and as xenografts in immune-deprived mice. The MGH-U1 cells grown as spheroids were much more resistant to Adriamycin (concentration of drug resulting in 37% cell survival, 4.5 µg/ml) than when treated as monolayer cultures (concentration of drug resulting in 37% cell survival, 0.9 µg/ml). Adriamycin fluorescence was demonstrated only in the outer two layers of cells forming the spheroids, suggesting that limited drug penetration is an important factor in the resistance of spheroids to Adriamycin. Sequential trypsinization of spheroids 750 µm in diameter allowed us to determine the cytotoxic effects of Adriamycin in MGH-U1 cells derived from different depths of the spheroid. We found that cells near the surface of the spheroid had a survival similar to those of exponentially growing monolayer cells treated with Adriamycin. Cells located in the middle of the viable rim were more resistant to Adriamycin, and those found near the necrotic center were least resistant to Adriamycin. The effects of Adriamycin treatment on spheroid growth delay were determined also. In spite of a small cytotoxic effect on the clonogenic fraction of cells in MGH-U1 spheroids, the growth delay effect of Adriamycin in intact spheroids was marked. This observation is consistent with Adriamycin killing primarily the cells in the outer layers of the spheroid, where most of the proliferation in the spheroid occurs. In vivo treatment of MGH-U1 xenografts with Adriamycin followed by assessment of cell survival in vitro showed minimal evidence of cytotoxicity, consistent with the poor drug penetration observed in the spheroid model. These studies suggest that: (a) Adriamycin penetrates poorly into solid tissues; (b) in vitro clonogenic survival following Adriamycin exposure of a cell suspension may predict falsely for drug sensitivity to chemotherapy; (c) a small decrease in clonogenic survival can be translated into a long growth delay but, ultimately, the tumor regrows because some clonogenic cells are spared; and (d) for Adriamycin, the spheroid model more closely parallels the in vivo effects than does monolayer culture. The use of the spheroid model for the study of Adriamycin cytotoxicity gives further insight into the action of this drug in solid tumors.

INTRODUCTION

Diminished access of antineoplastic agents to some clonogenic cells in solid tumors may be due to poor drug penetration or limited vascularization in tumor nodules. Support for this comes from the results of Goldacre and Sylven (5), who found that blood-borne dyes did not diffuse to some regions of solid tumors based on studies in experimental murine models. Limited drug access into a tumor may be a major cause of "drug resistance" which has been largely overlooked. In order to study drug penetration, we have utilized the multicellular tumor spheroid system developed by Sutherland et al. (11) as an in vitro model for the growth of solid tumors. The spheroid is a good model for solid tumors, because it shares many of their characteristics, including: (a) 3-dimensional, intercellular contact; (b) zones containing cells with differing nutritional status; (c) cell cycle heterogeneity; and (d) central necrosis with dependence on diffusion of metabolites for cell viability. A major advantage of this in vitro model is the ability to grow and maintain these spheroids with precise control of nutrients and drugs and without the technical difficulties of variations in blood flow, levels of nutrients, and drug concentrations which occur in vivo.

Human bladder cancer is increasing in incidence and metastasizes frequently and, in common with other solid tumors in humans, it has a definite but limited response rate to chemotherapy (1). We have developed spheroids from the MGH-U1 human bladder cancer cell line as a model for studying drug effects and toxicity in a human solid tumor. This cell line was derived from a Grade 4 bladder tumor in 1972 and has been characterized extensively by others. We elected to assess the effects of Adriamycin using this cell line because this agent is clinically active in patients with bladder cancer (1) but is incapable of completely eradicating the solid tumor. Failure of therapy might be due in part to limited accessibility of the agent to all clonogenic tumor cells. In the studies reported here, effects of Adriamycin were determined when the MGH-U1 cells were grown exponentially as monolayer cultures in which drug penetration is not limiting, as spheroids in which penetration may be important, and as xenografts in immune-deprived mice in which drug pharmacokinetics is an additional contributing factor. Cytotoxicity was assessed by a clonogenic assay in each case. In addition, the end point of clonogenicity was compared to the growth delay end point in the spheroids.

MATERIALS AND METHODS

Materials. MGH-U1 cells were obtained from Dr. G. Prout and his colleagues (Boston, MA). Male CBA/CAJ mice were purchased from The Jackson Laboratory. Adriamycin was kindly supplied by Adria Laboratories, Inc. (Dublin, OH). Trypsin and Nobile agar were purchased from Difco (Detroit, MI). All other chemicals were reagent grade. Solution A, a calcium-free PBS, was prepared as follows: 8 g of sodium chloride, 0.2

1 This work was supported in part by a grant from the National Cancer Institute of Canada.

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Received December 19, 1983; accepted August 9, 1984.
counted, and survival in drug-treated dishes was expressed as a fraction for 10 min, and stained with a 1:1 (v/v) dilution of Giemsa (Fisher Scientific dishes (Falcon Plastics, Oxnard, CA) at varying dilutions in replicates of fresh medium was added. The cells were counted, diluted, and plated in equal volumes of PBS and 1 volume of Solution A. Spheroids were then transferred to 17- x 100-mm plastic tubes (Falcon), and the medium was removed from the wheel, and the spheroids were allowed to settle. Drug-containing medium was added to the appropriate concentrations in a-MEM with 10% (v/v) PCS for the desired time period.

Drug-containing medium was removed, and the monolayers were washed twice with equal volumes of PBS and then with Solution A. A single-cell suspension was prepared using 0.05% trypsin with 0.02% EDTA in Solution A. Cells were inspected microscopically to ensure a monodispersed population and counted using a Model-F Coulter Counter. The cells were then diluted appropriately and plated in 60-mm Petri dishes (Falcon Plastics, Oxnard, CA) at varying dilutions in replicates of 6 for controls and 3 for drug treated cells. The dishes were incubated for 12 days at 37°, being rotated constantly in a roller wheel. The tubes were then removed. Fresh medium containing drug at the appropriate concentration in a-MEM with 10% (v/v) PCS was added to each tube, and the cell was disaggregated completely.

Spheroid Cytotoxicity Studies. MGH-U1 spheroids were initiated according to a modification of the method described by Yuhas et al. (17) and maintained in 250-ml spinner flasks (John Scientific, Toronto, Ontario, Canada) containing a-MEM with 10 nm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10% (v/v) FCS at 37°. Cultures were stirred at 130 rpm. Details of the growth and characteristics of MGH-U1 spheroids are described elsewhere. Spheroids were sized using a calibrated ocular micrometer on an inverted microscope. The longest spheroid diameter, a, and the perpendicular diameter, b, were measured, and the mean diameter was calculated using the formula (a x b)^0.5. To determine the cytotoxic effects of the chemotherapeutic agents, spheroids were transferred to 17- x 100-mm plastic tubes (Falcon), and the medium was removed. Fresh medium containing drug at the appropriate concentrations was added to yield a final spheroid concentration of approximately 2 spheroids/ml. The tubes were incubated for the appropriate time at 37°, being rotated constantly in a roller wheel. The tubes were then removed from the wheel, and the spheroids were allowed to settle. Drug-containing medium was removed, and the spheroid was washed with 2 equal volumes of PBS and 1 volume of Solution A. Spheroids were then trypsinized using the trypsin:EDTA solution for 10 min and mechanically disrupted to ensure a single-cell suspension. Then, an equal volume of a-MEM plus 10% (v/v) FCS was added to each tube, and the cell suspension was centrifuged at 1000 rpm for 5 min. The supernatant was decanted, and fresh medium was added. The cells were counted, diluted, and plated in a manner similar to that described for monolayer cells. They were incubated for 12 days at 37° and 5% CO2 fixed with methanol and stained with Giemsa, and colonies were counted as described previously. Survival of drug-treated dishes was expressed as a fraction relative to the survival of the subpopulations from control spheroids. The results for each of the subpopulations were normalized to a survival of 1.0 for control cells.

Spheroid Growth Delay. Growth delay of MGH-U1 spheroids was determined by performing spheroids measuring approximately 400 μm in diameter with specified concentrations of Adriamycin for 1 hr. The spheroids were then placed into multwell dishes (Costar). Between 6 and 12 spheroids/treatment group and control were utilized for each experiment. Between 3 and 5 experiments were performed for each drug treatment. Spheroids were sized daily for the first 4 days after treatment and then every second day. One ml of medium from the total 2 ml in the multwell dishes was changed every second day. Growth delay was determined by measuring the time required for the spheroid volume to increase by a factor of 10 for control and drug-treated spheroids. The growth delay was defined as the difference in time between drug-treated and control spheroids to reach this 10-fold increase in volume. We chose 10-fold increases in volume because our initial studies had shown that the growth curves at this point were generally parallel to each other. The most commonly used value of a 4 times increase in volume (14) resulted in measurements being made when the growth curves were not parallel (Chart 4).

MGH-U1 Xenograft Studies. MGH-U1 cells were grown as xenografts in CBA/CAJ immune-deprived mice which were prepared according to the method of Steel et al. (9). Briefly, 4- to 6-week-old CBA/CAJ mice were thymectomized and, 2 weeks later, mice were treated with 1-β-D-arabinofuranosylaunomycin (200 mg/kg), followed 48 hr later by 850 rads of whole-body irradiation. Between 1 and 5 × 10^6 MGH-U1 cells were injected in each flank of a single mouse 1 week after the irradiation, and this resulted in a 100% incidence of progressively growing tumors with a doubling time of 14 days (6). When the tumor measured 0.5 to 0.75 g (approximately 1 month after injection), the animals were treated i.p. with Adriamycin (10 or 20 mg/kg). The 10% lethal dose on Day 14 after treatment was determined to be less than Adriamycin (7.5 mg/kg) administered i.p. We also studied the possibility that xenografting might lead to changes in the intrinsic drug sensitivity of MGH-U1 cells. MGH-U1 cells derived from xenograft tumors were placed in tissue culture and treated with drug 2 days later when growing exponentially. Survival was compared to that of cells which have been maintained in tissue culture.

Histological Sectioning of Fluorescence Microscopy. In order to minimize Adriamycin diffusion, spheroids treated with Adriamycin were removed from the drug-containing medium, placed on a cryostat block, embedded in OCT compound (Tissue Tech, CanLab, Toronto, Ontario, Canada) and frozen rapidly in liquid nitrogen. Frozen sections of 5 μm were prepared using an Amers Lab Tech Cryostat. Sections were then examined for Adriamycin fluorescence using a Zeiss fluorescence microscope. The excitation wavelength was 450 to 490 nm with a barrier filter of 550 to 560 nm. We had determined that exposure of single cells to Adriamycin (0.1 μg/ml) for 1 hr resulted in detectable intracellular fluorescence with this microscopic arrangement.

RESULTS

The doubling time of MGH-U1 cells growing exponentially in monolayer was 20 hr. The plating efficiency of these cells was 80%, whereas that of cells derived from trypsinized spheroids was 65 to 70%. The plating efficiency for cells derived from in vivo tumors was 50%. The MGH-U1 spheroid diameter increased by 109 μm/day between the size of 200 and 800 μm. MGH-U1 cells growing exponentially or spheroids with a diameter of 750 μm were exposed to varying concentrations of Adriamycin for 60 min. Spheroids measuring 750 μm in diameter...
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were disaggregated with the trypsin-EDTA solution, and the single-cell suspension was also treated with Adriamycin for 1 hr. The survival curves for treatment under these 3 conditions are shown in Chart 1. Clonogenic survival of monolayer cells was related exponentially to concentration of drug but exhibited a small shoulder on the curve. The survival curve of the cells derived from drug-treated spheroids was exponential, with a very shallow slope. The $D_0$ value for the monolayer was 0.9 $\mu$g/ml, while that of the spheroid curve was 4.5 $\mu$g/ml. Concentrations of 5 $\mu$g/ml for 1 hr killed only 65% of the clonogenic cells. The survival curve of single cells derived from spheroids and then treated with Adriamycin was biexponential. The initial part of the curve had a steep slope parallel to that of the exponential monolayer, with a $D_0$ of 0.3 $\mu$g/ml. The terminal part of the curve has a more shallow slope, with a $D_0$ of 3.3 $\mu$g/ml, and it represents 13% of the clonogenic cells in the spheroid. When spheroids were exposed to Adriamycin (4 $\mu$g/ml) for varying periods of time, survival decreased steadily but, even after a 2-hr exposure, 20% of the clonogenic cells survived (Chart 2).

To assess the importance of drug penetration as the cause of a resistance of the cells in spheroids, fluorescence microscopy of rapidly frozen spheroids was undertaken. Fig. 1 illustrates the fluorescence observed in a control spheroid and a spheroid treated with Adriamycin (10 $\mu$g/ml) for 1 hr. The concentration of 10 $\mu$g/ml was necessary in order to detect the fluorescence. Little fluorescence was seen in the control sample but, in the drug-treated spheroid, green fluorescence characteristic of Adriamycin was recognized but was limited to about 2 outer cell layers of the spheroid.

Spheroids measuring 750 $\mu$m in diameter were treated with Adriamycin (1, 2, or 3 $\mu$g/ml) for 1 hr, and the spheroids were dissociated by sequential trypsinization in order to assess the effect of Adriamycin on the survival of cells from different depths in the spheroid. The spheroids could be dissociated into 3 fractions designated Fractions A, B, and C (Chart 3). Fraction A, representing the outer layer of cells, contained approximately 25% of the total spheroid cell yield; Fraction B contained another 25% of the total cell yield; and Fraction C contained 50% of the cells. It should be noted that spheroids at 750 $\mu$m in diameter would be expected to have a necrotic center measuring approximately 250 to 300 $\mu$m in diameter under the conditions in which we normally grow and treat these spheroids. The survival curves for Fractions A, B, and C, which are shown in Chart 3, demonstrate a graded sensitivity of these cells. The $D_0$ is 1.2 $\mu$g/ml for Fraction A, 1.7 $\mu$g/ml for Fraction B, and 3.3 $\mu$g/ml for Fraction C. The fourth survival curve, which is unlabeled, is that of exponentially growing monolayer cells. This curve is very similar to that of cells derived from the outer layer of the spheroid.

Spheroids measuring approximately 400 $\mu$m in diameter were exposed to Adriamycin at concentrations of 1, 2, 3, and 4 $\mu$g/ml for 1 hr. Typical growth curves for a representative experiment are shown in Chart 4. All volumes have been normalized such that the volume on the treatment day is 1. The control spheroids increase in volume exponentially, whereas the drug-treated spheroids demonstrate an initial growth rate similar to the controls and then a delay in growth which is dependent on drug treatment.

Chart 1. Survival curves for MGH-U1 cells treated with increasing concentrations of Adriamycin for 1 hr, when cells were growing as exponential monolayers (C), spheroids (B), and single cells derived from disaggregated spheroids (X). Mean spheroid diameter was 750 $\mu$m. Points, mean of 3 to 5 experiments; bars, S.E.

Chart 2. Survival of MGH-U1 growing as spheroids and treated with Adriamycin (4 $\mu$g/ml) for varying periods of time. Each point represents the mean of 3 experiments; bars, S.E.
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Charts. Survival of MGH-U1 cells derived from spheroids treated with Adriamycin for 1 hr and then sequentially trypsinized. Curve A represents cells from the outer layer of the spheroid; Curve B represents cells from the middle of the spheroid; Curve C represents cells from the inner part of the spheroid. The curve through the x represents survival of monolayer.

Ultimately, spheroids recover from the effects of drug treatment and resume their growth at a rate which is similar to that of the control spheroids. The growth delay for spheroids treated with Adriamycin (1, 2, 3, and 4 μg/ml) was 2.8, 4.7, 5.4, and 8.6 days, respectively, to reach 10 times their initial volume. The linear relationship between growth delay and treatment is shown in Chart 5a. A small decrease in surviving fraction to 0.43 by an Adriamycin treatment of 4 μg/ml is translated into a growth delay of almost 9 days in the spheroids. Chart 5b depicts the relationship between growth delay and clonogenic survival. There is a very shallow slope on this curve.

To compare these in vitro effects observed with MGH-U1 cells grown in monolayer, and as spheroids to those in vivo, xenografts of this tumor were treated in vivo at doses of Adriamycin (10 and 20 mg/kg) i.p. These doses of Adriamycin were above the 10% lethal dose for the CBA/CAJ mice but did not lead to death in the 18-hr interval between drug administration and assay. The clonogenic survival of the cells derived from the excised tumors was unaffected at a dose of 10 mg/kg and decreased only slightly at 20 mg/kg (Table 1). We assessed the effect of the drug treatment on clonogenic survival 2 hr after drug administration to determine whether repair of potential lethal damage might explain the 18-hr results. As shown in Table 1, no significant difference in survival of clonogenic cells was observed at 2 hr as compared to 18 hr. The cells derived from xenografted MGH-U1 tumors treated as monolayers with Adriamycin were assessed for clonogenic survival. The survival curves observed were identical to those seen with the monolayer cells maintained in vitro (Chart 6).

DISCUSSION

Our initial studies with Adriamycin raise the possibility that drug penetration into solid tumors may be limited for selected agents. We have observed a marked resistance of MGH-U1 cells treated when growing as spheroids in comparison to monolayer cultures. The fluorescence microscopy studies demonstrate that little Adriamycin reaches cells distant from the spheroid surface, and this is in agreement with the results obtained with spheroids of different origins (3, 10). The limited penetration is probably due to the avid binding of Adriamycin by the outer layer of cells, thereby shielding the inner cells from exposure to significant concentrations of Adriamycin. Thus, the limited cytotoxic effects seen in spheroids treated with Adriamycin occur because very few clonogenic cells are exposed to adequate concentrations of the drug. The sequential trypsinization experiments demonstrate that the cells at the surface of the spheroid which are exposed to concentrations of Adriamycin similar to that of monolayer cells have a survival curve that is essentially identical to treatment in monolayer culture. Cells located more deeply in the spheroid exhibited less sensitivity to Adriamycin, thus supporting the contention that drug penetration can be a problem for this agent in solid tumors.
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1.0
0.1
0.01
0.0
2
4
6
8
10
Growth delay (days)

Adriamycin (µg/ml)

Chart 5. a, linear relationship between growth delay to 10 times initial volume of Adriamycin. b, plot of clonogenic survival versus growth delay to 10 times initial survival of MGH-U1 spheroids. Line, least square fit of data (r² = 0.96); bars, S.E.

Table 1

<table>
<thead>
<tr>
<th>Dose of Adriamycin (mg/kg)</th>
<th>Surviving fraction at 2 hr</th>
<th>Surviving fraction at 18 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.96 ± 0.13b</td>
<td>0.97 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>0.83 ± 0.12</td>
<td>0.78 ± 0.15</td>
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* Time of sacrifice after treatment.

** Mean ± S.E. of 4 experiments.

The survival curve of single cells treated with Adriamycin after disaggregation from spheroids is biexponential (Chart 1), suggesting there are 2 populations of cells in the spheroid, one having a sensitivity similar to the monolayer cells and one more resistant. This is consistent with the conclusion that some cells are easily accessible to drug and others are not when taken in conjunction with the fluorescent microscopy studies.

The experiments performed using in vitro growth delay of spheroids as an end point for drug treatment demonstrate that we can use an end point used commonly in in vivo studies in this in vitro system and compare it to clonogenic survival. The growth delay is related linearly to Adriamycin concentration at a rate of 2.0 days/µg ml⁻¹ for a 1-hr treatment (Chart 5a). Small decreases in clonogenic survival caused by Adriamycin treatment of the spheroid results in a marked growth delay, which is depicted graphically in Chart 5b, in which surviving fraction is plotted as a function of growth delay. The marked growth delay associated with little cell kill is consistent with drug killing rapidly proliferating cells in the spheroid. We have observed that the rapidly dividing population of cells in MGH-U1 spheroids is located near the surface, i.e., those cells primarily killed by Adriamycin. Although we cannot rule out that Adriamycin causes significant delay of cell cycle progression, our results suggest that spheroid growth is primarily dependent on the outermost layer of cells.

The in vivo experiments show little cytotoxic effects of i.p. Adriamycin treatment. These findings are consistent with a problem of drug penetration into the tumor. We cannot, however, rule out that the pharmacokinetics of the drug in the mouse are such that the area under the curve for in vivo exposure is much less than that which we achieved in vitro, either in the monolayer or in the spheroid system. Our studies determining the clonogenic survival at 2 hr showed no difference when compared to those 18 hr after treatment. Therefore, repair of potential lethal damage is unlikely to be an important factor in explaining the resistance which the in vivo tumor has demonstrated. Furthermore, the similar sensitivity of xenografted tumor cells treated in vitro to the cell line maintained in culture rules out the possibility that a resistant subpopulation of cells was somehow selected by the xenografting procedure.

Based on these studies with Adriamycin, we proposed that a mechanism of drug resistance in solid tumors which has been largely overlooked is the limited penetration of antitumor drugs into these tumor nodules. Our results with Adriamycin and MGH-U1 spheroids concur with those of Sutherland et al. (10) and Durand (3) using EMT/6 and V79 spheroids, respectively. Bleomycin (13), methotrexate (15), and amsacrine (16) have been reported by others as having decreased efficacy due to limited drug penetration also. 5-Fluorouracil (8), on the other hand, may encounter little hindrance in passage from blood vessels into solid tumors. Factors such as avid drug binding by intervening
cells in the case of Adriamycin, degree of ionization, molecular size, and lipid solubility may all bear on the ability of a drug to penetrate solid tumors. In some cases, this might be overcome by drug infusion, resulting in a prolonged gradient between tumor and intravascular drug. The prolonged exposures used in the spheroid experiments with Adriamycin did show some further killing of clonogenic cells but did not make a major impact on the cytotoxic effects of the drug. In other instances, changing the pH of the surroundings, altering lipid solubility of the agent, or combining a drug which penetrates poorly with one that has good penetration may be necessary to decrease clonogenic survival significantly. The implications of limited drug penetration is that growth inhibition or even tumor regression may occur with chemotherapy, but some clonogenic cells may be unaffected by drug treatment. These factors may contribute to Adriamycin and other drug resistance as well as the limitations of drug penetration. It is difficult to isolate the mechanism of resistance in spheroids, but all of these complex factors are likely to apply in solid tumors. We have attempted to separate these potential causes of drug resistance in solid tumors by designing experiments to assess clonogenic survival after drug treatment in combination with those to determine the location of the drug in the spheroid.

Finally, our studies raise another question regarding the validity of drug sensitivity testing in vitro for solid tumors and the prediction of in vitro drug sensitivity using a clonogenic assay. Solid tumor specimens are usually dispensed to allow good access of drug to individual cells, and then survival is determined. This would be expected to result in better cell kill in vitro than might occur in vivo and an overestimate of drug efficacy. Our data with Adriamycin illustrate this point clearly, since monolayer cells were sensitive to the drug, but the same cells in spheroids responded to Adriamycin in a manner more closely to that observed in vivo. Therefore, caution must be exercised when extrapolating in vitro sensitivity studies in the treatment of human solid tumors.

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Fig. 1. Fluorescence microscopy of MGH-U1 spheroids exposed to: (A) No Adriamycin (x100); (B) Adriamycin (10 g/ml) for 1 hr (x100); and (C) Adriamycin (10 g/ml) for 1 hr (x400). Spheroid preparation is described in “Materials and Methods.”
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*Cancer Res* 1984;44:5369-5375.

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