Sensitivities of Monolayers and Spheroids of the Human Bladder Cancer Cell Line MGH-U1 to the Drugs Used for Intravesical Chemotherapy

Ruth Knuchel,1 Ferdinand Hofstadter, Wendy E. A. Jenkins, and John R. W. Masters


ABSTRACT

The in vitro cytotoxicities of the four drugs most frequently used for intravesical chemotherapy (Adriamycin, epirubicin, mitomycin C, Thiopeta) and epirubicin were compared using monolayers and multicellular tumor spheroids of the human bladder cancer cell line, MGH-U1. Adriamycin and epirubicin were most cytotoxic against monolayer cultures, whereas mitomycin C killed more cells in spheroids. Epirubicin was least cytotoxic against both two- and three-dimensional cultures. Thiopeta was the only drug more cytotoxic to three- than two-dimensional cultures. Topographic analysis of bromodeoxyuridine-stained nuclei using image analysis indicated that Adriamycin selectively removed or killed superficial cells in multicellular tumor spheroids, but had little effect on DNA synthesis within the spheroids. In contrast, Thiopeta killed cells throughout the spheroids. These in vitro data appear to reflect clinical experience using intravesical chemotherapy to treat superficial bladder cancer.

INTRODUCTION

Intravesical chemotherapy is used to treat superficial bladder tumors, either as an adjuvant to surgery to delay or prevent recurrence, or as definitive treatment for unresectable disease (1). It has the major advantages that high drug concentrations can be delivered directly to the tumor, but its effectiveness may be limited by the extent to which the drug used can penetrate the tumor. Three properties of a drug influence cellular uptake: its pKa; molecular weight; and lipophilicity (2).

Drug penetration and cellular uptake are difficult to study in vivo, and consequently a three-dimensional in vitro model, MCTS,2 has become popular for this purpose (3-8). In this study we used MCTS and monolayers of the human bladder cancer cell line MGH-U1 to compare the cytotoxicity and degree of inhibition of DNA synthesis of the four drugs most frequently used to treat superficial bladder cancer (Adriamycin, epodyl, mitomycin C, Thiopeta) and epirubicin.

MATERIALS AND METHODS

The cell line MGH-U1, a subline of T24 (9, 10), derived from a transitional cell carcinoma of the human bladder, was used throughout these studies. The cells were used over a restricted range of ten passages to minimize any changes resulting from long-term culture. The cells were maintained as monolayers in 25-cm2 culture flasks (Nunc, Gibco, Paisley, Scotland) in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine and 10% fetal calf serum. After a 1-h exposure the drug-containing medium was removed, and the cells were washed 3 times with medium and once with PBS. The cells were detached after a 10-min exposure to 0.2 ml of trypsin/versene solution, and the resulting single cell suspension was diluted if necessary and transferred to 5-cm plastic Petri dishes (Nunc, Gibco) at cell concentrations producing approximately 150 colonies per dish. After 8 days incubation, colonies were fixed in methanol (BDH) and stained with 10% Giemsa (BDH). Colonies consisting of more than 50 cells were counted using a dissecting microscope, and the colony-forming ability at each drug concentration was expressed as a percentage of that of the controls. Using the straight-line portions of the dose-response curves, IC50 and IC90 were calculated using linear regression analysis. All experiments were repeated at least 3 times.

For MCTS, spheroids with an average diameter of 0.7 mm (range, 0.65 to 0.75 mm) were transferred to 96-well microtest plates (Nunc, Gibco) and incubated for 48 h to allow exponential growth to resume. The medium was then removed and replaced by a range of drug concentrations (3 wells/concentration) or fresh medium (5 wells) containing the appropriate volume of solvent as controls. The drugs were diluted in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum. After a 1-h exposure the drug-containing medium was removed, and the cells were washed 3 times with medium and once with PBS. The cells were detached after a 10-min exposure to 0.2 ml of trypsin/versene solution, and the resulting single cell suspension was diluted if necessary and transferred to 5-cm plastic Petri dishes (Nunc, Gibco) at cell concentrations producing approximately 150 colonies per dish. After 8 days incubation, colonies were fixed in methanol (BDH) and stained with 10% Giemsa (BDH). Colonies consisting of more than 50 cells were counted using a dissecting microscope, and the colony-forming ability at each drug concentration was expressed as a percentage of that of the controls. Using the straight-line portions of the dose-response curves, IC50 and IC90 were calculated using linear regression analysis. All experiments were repeated at least 3 times.

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2 The abbreviations used are: MCTS, multicellular tumor spheroids; PBS phosphate-buffered saline; BrdUrd, bromodeoxyuridine; FCS, fetal calf serum; IC50, concentration reducing clonogenic cell survival by 50% (IC50, defined similarly).

1397
dilution of 1:75. After washing in PBS to remove excess antibody the sections were treated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, High Wycombe, United Kingdom) at a dilution of 1:100 for 30 min. Finally the sections were incubated with the substrate 3’3-diadamino benzidine tetrahydrochloride (Sigma, Poole, England) for 5 min.

Image Analysis. An image analysis system (Leitz TAS) was used to detect and automatically count BrdUrd- and Feulgen-stained nuclei as gray areas. An interactive computer program was developed (12) which permitted exclusion of staining artefacts and inclusion of weakly stained nuclei. The number of proliferating (BrdUrd positive) cells was compared with the total number (Feulgen positive) of cells using adjacent equatorial sections of MCTS.

RESULTS

MGH-U1 cells have a population doubling time during exponential growth in monolayer culture of 20 h and a colony-forming ability of 70% on plastic. The MCTS used in these experiments were cultured in medium containing 10% fetal calf serum and showed a linear increase in diameter for approximately 18 days (see Fig. 1). The spheroids were used after 12 to 13 days of growth, at an average diameter of 0.7 mm, each containing approximately 25,000 cells with little central necrosis. In contrast, in 5% FCS (see Fig. 1) the maximum diameter reached was 0.7 mm, and this took 20 to 22 days to achieve and was accompanied by extensive central necrosis.

Drug Sensitivities. Dose-response curves of monolayers and MCTS to the five drugs are shown in Fig. 2. The relative abilities of the drugs to kill cells in MCTS were compared using the ratio of the IC50 and IC90 in the two systems (see Table 1).

Adriamycin and epirubicin, the two anthracycline analogues, had similar cytotoxicities against both two- and three-dimensional cultures. In monolayer cultures these two drugs were the most cytotoxic agents, comparing equimolar concentrations. However, they were both approximately 20-fold less cytotoxic against three-dimensional cultures. Consequently mitomycin C, which was only 2-fold less cytotoxic against three- than two-dimensional cultures, killed most cells in spheroids, again comparing equimolar concentrations. Epoxyd was the least cytotoxic agent against both two- and three-dimensional cultures. However, its cytotoxicity was similar in both systems, and while it was more than 500-fold less cytotoxic than Adriamycin against monolayers, it was only approximately 30-fold less effective against MCTS. Thiotepa was the only drug to be more effective against three- than two-dimensional cultures. Also in contrast to the other drugs, whose dose-response curves diverged for the monolayers and spheroids such that the differences became larger with increasing concentration, the two curves were par-

![Fig. 2. Percentage of colony-forming abilities of MGH-U1 monolayers and spheroids following a 1-h exposure to a range of concentrations of Adriamycin, Epirubicin, Thiotepa, and mitomycin C. Bars, SE.](image)

Table 1 Comparison of IC50 and IC90 (µg/ml) in two- (monolayer) and three-dimensional (MCTS) cultures

<table>
<thead>
<tr>
<th>Drug</th>
<th>Monolayer IC50</th>
<th>MCTS IC50</th>
<th>Monolayer IC90</th>
<th>MCTS IC90</th>
<th>Ratio</th>
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<tr>
<td>Adriamycin</td>
<td>0.103</td>
<td>2.44</td>
<td>0.420</td>
<td>7.87</td>
<td>23.7</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>0.073</td>
<td>1.22</td>
<td>0.280</td>
<td>7.09</td>
<td>16.7</td>
</tr>
<tr>
<td>Epoxyd</td>
<td>56.700</td>
<td>70.80</td>
<td>93.65</td>
<td>187.55</td>
<td>2.53</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>0.500</td>
<td>1.02</td>
<td>0.93</td>
<td>2.03</td>
<td>2.04</td>
</tr>
<tr>
<td>Thiotepa</td>
<td>35.900</td>
<td>12.44</td>
<td>64.06</td>
<td>42.90</td>
<td>0.35</td>
</tr>
</tbody>
</table>

was more than 500-fold less cytotoxic than Adriamycin against monolayers, it was only approximately 30-fold less effective against MCTS. Thiotepa was the only drug to be more effective against three- than two-dimensional cultures. Also in contrast to the other drugs, whose dose-response curves diverged for the monolayers and spheroids such that the differences became larger with increasing concentration, the two curves were par-
parallel for Thiotepa (see Fig. 2). While Thiotepa was approximately 300-fold less cytotoxic against monolayers than Adriamycin, the difference was only 5- to 10-fold against spheroids.

DNA Synthesis. The results of the image analysis of the Feulgen- and BrdUrd-stained nuclei are summarized in Table 2. In the controls, the proportion of BrdUrd-stained nuclei nearly halved after an additional 2 days of culture in the spinner flasks, after a total of 15 to 16 days of culture. This finding was unexpected as the diameter of the spheroids increased linearly for up to 18 days of continuous culture under these conditions (see Fig. 1). Treatment with Adriamycin reduced the proportion of BrdUrd-stained nuclei 24 h after exposure. By 72 h after exposure the proportion was similar to that of the controls, but two-thirds of the cells had been lost, on the basis of the numbers of Feulgen-stained nuclei. Fewer cells were lost from the Thiotepa-treated MCTS, but the proportion of BrdUrd-stained nuclei was similar to that of the controls 24 h after exposure and remained at this high level after a further 2 days of culture.

The pattern of BrdUrd staining and cell loss differed between the Adriamycin- and Thiotepa-treated spheroids. Following treatment with Adriamycin the outer rim of proliferating cells (6 to 8 cell layers) was lost, although the pattern of BrdUrd staining in the central part of the MCTS was similar to that of the controls (see Fig. 3, upper and lower). In contrast, in Thiotepa-treated spheroids, BrdUrd-stained nuclei were abundant on the surface of the MCTS (Fig. 3, middle), and only 3 to 4 cell layers were lost.

**DISCUSSION**

Intravesical chemotherapy is used to delay recurrence and progression of superficial bladder cancer. While the clinical results are encouraging, there is uncertainty concerning the most effective drug and the optimal schedule of administration (1). In this study we compared the cytotoxities of the drugs used against two-dimensional monolayer and three-dimensional spheroid cultures. Thiotepa was the only drug more effective against spheroids than monolayers and is the only one of these drugs that frequently produces systemic toxicity following intravesical administration. Thus, the *in vitro* data appear to reflect clinical experience, indicating that this drug is readily able to penetrate tissues and cross the bladder into the systemic circulation. This property is thought to be associated with molecular weights of less than 200, Thiotepa having a molecular weight of 189 (1).

These studies confirm the general observation that a smaller proportion of cells are killed in three- than in two-dimensional culture (3, 7, 13, 14). Many factors could reduce the sensitivity of cells within spheroids, such as hypoxia, cell cycle differences, microenvironmental variation, drug penetration, pH, and metabolic rescue. Drug penetration is thought to be a major factor. Using fluorescence microscopy it was shown that little or no Adriamycin penetrated beyond the first 3 or 4 layers of cells within spheroids (15). Adriamycin killed only the outer 20 to 30% of cells in spheroids exposed to a concentration of 1 μg/ml for 1 h (16). Our data are compatible with these findings, in that following exposure to Adriamycin the superficial cells were lost, but there was little effect on DNA synthesis within MCTS. Another factor that may contribute to the lower sensitivity of the cells in MCTS is the "contact effect" described by Neder-

![Fig. 3. Image analysis pictures of Feulgen-stained (left) and BrdUrd-stained (right) nuclei in equatorial sections of spheroids, 24 h after a 1-h exposure to Thiotepa (middle), Adriamycin (lower), and untreated controls (upper). Prior to treatment all spheroids had been in continuous culture in spinner flasks for 12 to 13 days, and all had a diameter of between 0.65 and 0.75 mm. The number written beneath each photograph is the number of nuclei counted. Differences in the size of individual nuclei reflect relative staining intensity, and one gray area is counted as a nucleus irrespective of its size.](image-url)
man (5), resulting from metabolic cooperation between cells. Furthermore, it has been demonstrated that not only are the inner layers of spheroids more resistant to Adriamycin, but that the outer layer is also up to 6-fold more resistant than monolayers (17).

In contrast to Adriamycin, Thiotepa reduced the proportion of cells synthesizing DNA throughout the MCTS. This may be explained by the ability of Thiotepa to penetrate tumors, but some other explanation is required for the additional cytotoxicity. The microenvironment within a spheroid is more acidic than at the surface (18). As Thiotepa is much more toxic in acid medium (19), this probably accounts for the increased cell kill. Mitomycin C is also more cytotoxic in acid medium, whereas Adriamycin and epirubicin kill more cells in alkaline medium (19), and this partially explains why mitomycin C was the most cytotoxic compound in three-dimensional culture. Both Thiotepa and mitomycin C are more cytotoxic to the inner cells within spheroids, compared with the outer cells (16).

The topographical analysis of DNA synthesis within spheroids using image analysis complemented the cytotoxicity data obtained using colony-forming assays. This approach could be further refined by measuring the proportion of BrdUrd-staining nuclei at different levels within the spheroids by dividing the image into a series of concentric circles. A program is being developed to achieve this aim.

In the clinic, Adriamycin, mitomycin C, and Thiotepa are administered at similar doses, and there appears to be little to choose between them therapeutically, although Thiotepa has the disadvantage that it can produce systemic toxicity. Epirubicin is an analogue of Adriamycin and is the subject of clinical trials for superficial bladder cancer. However, the data from this study indicate that no additional benefit is likely to be gained by substituting epirubicin for Adriamycin for intravesical chemotherapy. In monolayer culture, Thiotepa appeared to be relatively ineffective, but in MCTS the differences between the cytotoxicities of the drugs were reduced. The toxicity data were supplemented by studies of the topographical distribution of cells synthesizing DNA and estimates of their numbers. The results with Adriamycin indicate that this drug causes a rapid loss of superficial cell layers, but has little effect at deeper levels. In contrast, Thiotepa kills cells throughout the spheroid. The results indicate that these two drugs could be synergistic in combination or in alternating schedules. Repeated applications of Adriamycin might successively strip a tumor, while Thiotepa might kill cells otherwise inaccessible. The results with epodyl indicate that higher concentrations are required to achieve the same level of cell kill. However, epodyl is used clinically at an approximately 10-fold higher concentration (1% solution) than these other agent, and thus the cytotoxicities in vitro are at a similar level.

Using monolayer cultures of MGH-U1 cells, Ehrlichman and coworkers (20) showed that drug concentration and period of exposure are equally important determinants of cytotoxicity for mitomycin C and Adriamycin. Similar findings were made for these two drugs and for Thiotepa and epodyl using the human bladder cancer cell line RT112 (21).

MGH-U1 spheroids have been used extensively as a three-dimensional in vitro model for studies on drug cytotoxicity (22, 23), cell growth (24), nutrient gradients (25, 26), and for the development of ultrasound backscatter microscopy (27). Data on the comparative cytotoxicities of Adriamycin against monolayers, spheroids, and xenografts of the MGH-U1 cell line have already been published (22), and our study yielded similar results. MGH-U1 was also used as two- and three-dimensional cultures to compare the cytotoxicities of cisplatin and cis-diammine-1,1-cyclobutane dicarboxylate (23). The concentration of each drug required to kill the same proportion of cells was similar in each system, but approximately 20-fold less cisplatin was needed to give the same cytotoxicity (23). We used spheroids of 0.7-mm diameter, when central necrosis was minimal. In contrast, central necrosis was observed at diameters of 0.4 mm and greater in an earlier study using this cell line (24). Spheroids are particularly sensitive to environmental conditions, and the system has to be standardized to obtain reproducible and comparable data. For example, in this study, halving the concentration of FCS to 5% markedly reduced viability and growth rate. A similar reduction in cell viability and growth rate was observed when glutamine levels were reduced below 0.2 mM (25).

In conclusion, MCTS derived from human bladder cancer cells appear to provide a useful model system for comparing the cytotoxicities of drugs proposed for intravesical use and a means of determining optimal conditions for their clinical administration.

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