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Abrogation of Taxol-induced G2-M Arrest and Apoptosis in Human Ovarian Cancer Cells Grown as Multicellular Tumor Spheroids

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Abstract

Tumor cells grown as multicellular spheroids are known to be intrinsically more resistant to a large and diverse array of anticancer chemotherapy drugs compared to the same cells grown as dispersed monolayer cell cultures. Some drugs, however, seem relatively insensitive to this multicellular drug resistance, e.g., cisplatinum. Whether the cytotoxic effects of Taxol, an anticancer drug of growing importance in the treatment of breast and ovarian carcinomas, are diminished by multicellular growth conditions is unknown. To study this question, we examined the relative sensitivity of a panel of four different human ovarian carcinoma cell lines to either Taxol or cisplatinum. Upon exposure to Taxol, all the cell lines manifested a relative drug-resistant phenotype when grown as multicellular tumor spheroids, compared to the same cells grown as sparse monolayer cultures. This multicellular-dependent drug-resistant phenotype was not observed when the same cells were exposed to cisplatinum for an equivalent length of time. Monolayer but not spheroid cultures exposed to Taxol demonstrated an accumulation of cells at G2-M and a sub-G1 apoptotic region. In addition, Taxol-induced apoptosis was detected in monolayer conditions but not in the spheroid cultures. The relative sensitivity of the monolayer cell cultures was associated with a decrease in bcxl2 protein levels after Taxol exposure, an effect not observed in drug-exposed spheroids. Taken together, these results suggest that some aspects of intrinsic Taxol resistance in ovarian carcinoma may be due to multicellular-dependent or -associated mechanisms. This raises the possibility of using adhesion agents to reverse multicellular-dependent Taxol resistance in certain circumstances as a potential means of increasing the initial efficiency of Taxol therapy against ovarian carcinoma.

Introduction

Patients whose tumors express resistance to chemotherapeutic drugs tend to do so in one of two ways, either intrinsically or after showing an initial response. The latter is usually referred to as acquired drug resistance. Approximately 60% of cases of drug resistance are of the intrinsic variety and include a broad spectrum of important tumors such as lung, bladder, prostate and renal carcinomas, and melanoma (1). There are a number of possible mechanisms that have been put forward to account for intrinsic drug resistance, and these include, among others, a lack of sufficient drug penetration into solid tumors, a low growth fraction, or an inherently reduced sensitivity to undergoing drug-induced apoptosis (2, 3).

Some of these aforementioned mechanisms may explain a long-known paradox involving drug resistance in cancer, namely that cell lines established from types of tumors known in general to be intrinsically drug resistant in vivo are frequently responsive to such chemotherapy drugs, especially when exposed in the form of exponentially growing monolayer cell cultures (3). This in vitro/vivo discrepancy can be resolved in many instances by growing tumor cell lines three-dimensionally as multicellular tumor spheroids (3–5). When grown in this way, there can be profound reductions in the proportion of proliferating tumor cells (3, 6). Because most anticancer drugs preferentially kill cycling cells, such a reduced growth fraction could serve to severely reduce the cytotoxic effects of such drugs. Moreover, some of these drugs might also have a limited ability to diffuse into the multicellular layers of tumor parenchyma, such as Adriamycin or methotrexate (7). Of some interest as well is the fact that we have recently uncovered cases in which acquired drug resistance is preferentially or exclusively expressed in multicellular tumor spheroid cultures (8).

The spectrum of drugs that tends to manifest such multicellular resistance is quite extensive and includes Adriamycin, cyclophosphamide, nitrogen mustards, methotrexate, vinblastine, and etoposide (3–5). There are some drugs, however, that generally seem to be equally effective against monolayer and spheroid tumor cell cultures, e.g., cisplatinum (9–11), and there are even some drugs that are actually preferentially cytotoxic in three-dimensional culture systems (12). These tend to be noncytotoxic produgs that can be activated to become toxic by the acidic or hypoxic environments found in the interior regions of tumor spheroids but not in monolayer cell cultures (12).

The taxane class of drugs, which includes Taxol (paclitaxel) and taxotere, has not yet been evaluated for its relative therapeutic (cytotoxic) effects in two- versus three-dimensional cell culture. Such drugs have become increasingly important in treating various cancers, especially breast and ovarian carcinomas (13–16). They function by inhibiting mitosis through enhancement of the polymerization of tubulin and consequent stabilization of microtubules (17, 18). The purpose of the present study was to evaluate whether multicellular spheroids of human ovarian carcinoma cells are more resistant to Taxol than monolayer cultures of the same cells, and if so, to determine in a preliminary way whether a differential sensitivity to undergoing apoptosis is involved. The rationale for undertaking such experiments is 2-fold: (a) the results obtained could provide new information relevant to gaining a better understanding of why some ovarian carcinomas may respond poorly to Taxol in the clinic; and (b) it could provide new directions for developing chemosensitization strategies designed to increase the effectiveness of Taxol therapy. For example, we have recently shown that intrinsic resistance to cyclophosphamide expressed by mouse mammary carcinoma cells grown as spheroids in vitro or as aggregated ascitic clumps in vivo can be reversed by the antiadhesive effects of hyaluronidase (6) or by exposure in vitro to antisense oligonucleotides to the cyclin-dependent kinase inhibitor p27kip1 (19). Similar strategies may be considered in the context of Taxol therapy if forms of multicellular resistance to
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TAXOL can be shown to exist. In this regard, our results support the concept that human ovarian carcinoma cells can indeed manifest increased resistance to TAXOL when exposed as intact three-dimensional multicellular aggregates.

Materials and Methods

Tumor Cell Lines and Cell Culture. The HEY human ovarian carcinoma cell line was obtained from Dr. R. Buick (Ontario Cancer Institute, Toronto, Canada), and the A2780 cell line was obtained from Dr. T. Hamilton (Fox Chase Center, Philadelphia, PA). The SKOV3 human ovarian carcinoma cell line was from the American Type Culture Collection, and the OVCA 429 cell lines were provided by Dr. Cinda Boyer (Duke University Medical Center, Durham, NC). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). The multicellular aggregates (spheroids) formed from the various cell lines were prepared by the liquid overlay technique, as described previously (6, 8).

Reagents. MTS³ was purchased from Promega. Phenazine methosulfate as well as cisplatin was from Sigma Chemical Co. [3H]Thymidine was supplied by Amersham. Taxol was supplied by Bristol-Myers Squibb.

In Vitro Colony Formation (Clonogenic Survival) Assay. Drug-treated monolayer cultures or three-dimensional cultures of cells that had been exposed to 10 µM Taxol for 48 h were compared to control cells by means of a colony formation assay as described previously (8). In this assay, cells were exposed to 10 µM Taxol for varying lengths of time (24 or 48 h) at 37°C, after which cells were washed three times with serum-free medium, trypsinized to obtain single-cell suspensions, and plated in triplicate in six-well plates at six different cell concentrations (5 x 10⁴, 5 x 10³, 5 x 10², 5 x 10¹, 5 x 10⁰ cells/well) in serum-containing medium. Seven to 14 days later, the plates were fixed with Carnoy's fixative (three parts methanol: one part glacial acetic acid) and stained with crystal violet. Colonies of more than 50 cells were counted.

MTS Staining. Cell viability was assessed essentially as described previously (20). Briefly, cells were incubated in 100 µl of media in 96-well plates, with additions as indicated. After incubation, 20 µl of the MTS/phenazine methosulfate solution was added to each well and incubated at 37°C for 6 h. MTS is bioreduced by cells into a formazan that is soluble in tissue culture medium. The relative cell viability was obtained by measuring the absorbance at 490 nm with an ELISA reader.

Measurement of Cell Proliferation. Cell proliferation was measured by tritiated thymidine incorporation. Briefly, cells were plated in 96-well plates as either spheroids or monolayer cultures for 24 h and then exposed to either 10 µM Taxol or 30 µM cisplatinum for 48 h. The cells were then incubated with [3H]thymidine for 6 h and harvested, and counts were read on a β-counter.

Monitoring Cell Cycle Perturbations after Drug Exposure. HEY cells and A2780 cells growing either in monolayer culture or three-dimensional culture for 24 h were exposed to 10 µM Taxol or 30 µM cisplatin for 48 h. For DNA analysis, cells were washed with PBS, trypsinized, fixed in 80% ethanol for at least 1 h, rinsed again with PBS and then rinsed with propidium iodide buffer (0.12% Triton X-100 and 0.12 mM EDTA), incubated for 45 min with 100 µg/ml RNase A, filtered through 30-µm mesh, and then stained with propidium iodide solution.

Flow Cytometry Methods for Detection of Apoptosis. Briefly, cells grown in monolayer or spheroid culture were incubated with 10 µM Taxol or 30 µM cisplatin for 48 h. Cells were collected and fixed with 1% paraformaldehyde for 15 min and fixed with 70% ethanol for 1 h. The Boehringer Mannheim in situ cell death detection kit was then used to study apoptosis. The assay is based on terminal deoxynucleotidyl transferase labeling of DNA strand breaks by the addition of fluorescein diUTP added onto free 3'-OH DNA ends. The cells were then analyzed by flow cytometry for detection of shifts in fluorescence that indicated increased DNA fragmentation.

Bcl-X₅ Detection by Western Blot Analysis. Cells grown in monolayer culture or three-dimensional culture were incubated with or without 10 µM Taxol or 30 µM cisplatin for 48 h. Cells were then lysed with lysis buffer supplemented with protease inhibitors (1 mM sodium-orthovanadate and 2 mM phenylmethylsulfonyl fluoride). The lysate was then centrifuged at 14,000 rpm for 15 min, and postnuclear supernatant was harvested and sampled for quantitation of protein concentration, using the Bradford dye. Thirty µg of the lysate were then mixed with 5 × SDS-PAGE sample buffer, boiled for 5 min, and subjected to electrophoresis in 12% SDS gels under reducing conditions. The separated proteins were then electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). After blocking by 5% nonfat dried milk in TBS with 0.125% Tween 20 for 1 h at room temperature, the membranes were blotted with a rabbit antihuman bcl-X₅ antibody (provided by Dr. Carlo Nalin, Novartis Pharma, East Hanover, NJ) at a concentration of 0.25 µg/ml. After washing in TBS with 0.125% Tween 20, the membrane was incubated with anti-rabbit immunoglobulin-horseradish peroxidase (1:1000 dilution) for 1 h. Protein bands were detected by enhanced chemiluminescence (Amersham).

Results

Attenuation of TAXOL-induced Toxicity in Spheroid Cultures. As can be seen in Fig. 1, there is a consistent shoulder on the viability curve when comparing the effects of TAXOL on monolayer versus spheroid cultures using a panel of ovarian cancer cell lines. Fig. 1A demonstrates the effect of the drug on HEY cells treated over a range of concentrations. At low concentrations (i.e., 0.5, 1.0, and 5 µM), there was no significant difference between the effects of the drug on the spheroid culture as compared to the monolayer culture. However, at concentrations of 10, 25, 50, and 100 µM, the spheroid culture demonstrated an apparent multicellular-dependent resistance. At a concentration of 25 µM, TAXOL exerted its maximum effects in both the spheroid and monolayer culture systems. After treatment for 48 h in monolayer culture, 23% of the cells remained viable, whereas in the spheroid culture under the same conditions, 46% of the cells remained viable. In monolayer culture, the LD₅₀ is 4.5 µM, whereas for spheroid culture, it is 16 µM. In contrast, the curves are virtually superimposable when both monolayer and spheroid cultures were treated with cisplatin (Fig. 1B).

TAXOL was also found to exert a greater effect on monolayer cultures compared to spheroid cultures of A2780 cells, another ovarian cancer cell line (Fig. 1C). At the 50 µM concentration, TAXOL exerted its maximum effect on spheroids, in contrast to 25 µM in monolayer cultures. The LD₅₀ is 8 µM for monolayer culture and 30 µM for spheroid culture. This relative drug resistance phenomenon was not observed when the same cells were exposed to cisplatin for the same period of time (Fig. 1D). TAXOL resistance in three-dimensional culture was also detected in the OVCA 429 ovarian carcinoma cell line (Fig. 1E). At concentrations as low as 0.5 µM TAXOL, there was a significant difference in the cell viability between spheroid and monolayer cultures. The LD₅₀ for the monolayer culture is 20 µM, whereas it is greater than 100 µM for multicellular spheroids. The fourth ovarian carcinoma cell line studied was SKOV3 (Fig. 1F). The LD₅₀ for monolayer culture is 18 µM, whereas it is 38 µM in the spheroid culture system. Only at concentrations of 25 µM was TAXOL able to induce cell death in the SKOV3 cells plated as spheroids; below this concentration, TAXOL did not have any effect. In contrast, at concentrations as low as 0.5 µM, TAXOL was able to kill 34.3% of the cells in monolayer. Thus the relative differential effect of TAXOL on monolayer and spheroid cultures is consistent in all four different ovarian carcinoma cell lines examined.

The results for the cell proliferation assay (Fig. 1, G and H) are similar to the MTS assay. At a concentration of 12.5 µM TAXOL, maximal effects were observed in which 9% of the A2780 monolayer cultures incorporated thymidine and thus were proliferating, whereas 100% of the spheroids incorporated thymidine at this concentration. Maximal effects on the spheroid cultures were observed at 50 µM.
Taxol. The effects of cisplatinum on cell proliferation in the monolayer and spheroid models were similar.

**CFE Is Greater in the Spheroid Culture after Taxol Treatment.** The relative resistance to Taxol of tumor cells grown as aggregates was also implicated by the enhanced CFE of the HEY cells in spheroid culture exposed to 10 μM Taxol for 24 and 48 h versus exposure in monolayer culture, as shown in Table 1. The percentage of CFE in the spheroids was similar in control and treated cultures, whereas there was a significant decrease in CFE when cells in monolayer cultures were exposed to the drug. At 24 h in the monolayer culture, CFE decreased to 1.26% from 9.26% after Taxol exposure, whereas in the spheroid culture, CFE was 7.5% compared to 9.86% in the controls. After 48 h of treatment, similar results were obtained. Taxol, therefore, does not significantly alter CFE in the spheroid culture under the conditions examined, in contrast to monolayer culture, in which CFE decreased to less than 10% of control after a 48-h incubation with the drug.

**Abrogation of Taxol-induced Ovarian Carcinoma G2-M Arrest in Spheroids.** The HEY and A2780 cell lines were plated in both spheroid and monolayer cultures and treated with 10 μM Taxol as well.

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Table 1 Percentage of CFE in HEY cells after 24- and 48-h treatments with 10 μM Taxol a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Monolayer (%)</th>
<th>±SD</th>
<th>Spheroid (%)</th>
<th>±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.26</td>
<td>±1.50</td>
<td>9.86</td>
<td>±0.92</td>
</tr>
<tr>
<td>Taxol</td>
<td>1.26</td>
<td>±0.20</td>
<td>7.50</td>
<td>±0.70</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.10</td>
<td>±1.19</td>
<td>7.16</td>
<td>±1.10</td>
</tr>
<tr>
<td>Taxol</td>
<td>1.43</td>
<td>±0.51</td>
<td>5.36</td>
<td>±0.11</td>
</tr>
</tbody>
</table>

a Each treatment was plated in triplicate, and each experiment was repeated twice.
Fig. 2. Abrogation of G2-M arrest in the spheroid culture after Taxol treatment. HEY cells (A) and A2780 cells (B) were plated in spheroid and monolayer cultures and treated with 10 μM Taxol for 48 h. At this time, cells were collected, and DNA profiles were analyzed by flow cytometry. ML, monolayer; Sph, spheroid; cont, control; tax, taxol-treated.

Attenuation of Taxol-induced Apoptosis in Cells Grown as Spheroids. When the HEY cells plated in monolayer culture were exposed to 10 μM Taxol for 48 h, 68.1% of the cells underwent apoptosis compared to 4.2% of the control cells (Fig. 3A). In contrast, when the cells were plated as spheroids (Fig. 3B), the Taxol-treated cells did not demonstrate a significant shift in fluorescence observed under identical conditions in the monolayer culture. Taxol exposure, therefore, does not induce DNA fragmentation indicative of apoptosis in the spheroid culture. This same assay performed with cisplatinum (Fig. 3, C and D) demonstrated that cisplatinum has the ability to induce apoptosis in both spheroid and monolayer cultures. In monolayer culture, 96.33% of the cisplatinum-treated cells underwent apoptosis versus 3.36% of the control cells. In spheroid culture, 87.46% of the cisplatinum-treated cells underwent apoptosis versus 11.34% of the control cells. Additional experiments implied that bcl-X₇ may be involved in this adhesion-dependent multicellular resistance. Thus, as shown in Fig. 3E, when HEY cells plated in both monolayer and spheroid cultures were treated with 10 μM Taxol for 48 h, the pattern of bcl-X₇ protein expression differed markedly. When the cells in monolayer culture were treated with Taxol, the levels of bcl-X₇ decreased significantly, and treatment with cisplatinum showed sim-
Taxol-treated cultures were exposed to Taxol, the levels of the bcl-XL protein remained elevated. In contrast, cisplatinum-induced apoptosis is similar in both types of cultures. HEY cells were plated in spheroid and monolayer cultures and treated with Taxol. We used cisplatinum as a control for these experiments, because this particular drug is one of the few anticancer cytotoxic drugs known to be equally cytotoxic to tumor cells grown as spheroids or monolayer cell cultures. Our results confirm this previous body of literature and thus strengthen the conclusion that Taxol is subject to multicellular-mediated resistance mechanisms, at least for human ovarian carcinoma cells.

As with other cytotoxic drugs that lose much of their potency when tested against tumor cell targets grown as multicellular spheroids, any one of several possible mechanisms could explain this type of resistance including: (a) a relative failure of the drug to penetrate into the interior of the spheroid; (b) a significantly reduced fraction of cycling (mitotic) cells in tumor spheroids; (c) a multicellular/adhesion-mediated survival mechanism (23-25) that renders cells less vulnerable to undergoing drug-induced apoptosis; and (d) changes in relevant drug-resistance-associated gene expression brought about by multicellular growth conditions that are known to alter gene expression (26). These possibilities are by no means mutually exclusive. For example, changes in the expression of genes known to modulate sensitivity to apoptosis, such as bcl-2, bcl-XL, bax, and so forth, could be altered in such a way as to result in a relative resistance to apoptosis. In this regard, it is of interest to note that the levels of bcl-XL protein detected in some of the human ovarian carcinoma cell lines grown as spheroids did not decline after Taxol exposure but did decline when exposed as monolayer cell cultures. This suggests, but obviously does not prove, a possible contribution of this antiapoptotic protein in the multicellular resistance we observed to Taxol. However, the expression of other proteins that are known to regulate apoptosis and that reside either within or outside the bcl-2 family will have to be examined before the contribution of bcl-XL to our results can be firmly established. In addition, the expression of other genes/proteins thought to contribute to aspects of either intrinsic or acquired Taxol resistance in human ovarian carcinomas such as P-glycoprotein (27) should be evaluated as well in monolayer versus spheroid culture, both before and after drug exposure. Such studies are in progress.

Regardless of the particular mechanisms involved, there is an important practical reason for determining whether ovarian carcinoma spheroids are less intrinsically sensitive to Taxol compared to single cells, namely the possibility of using antiadhesive agents as chemo-
sensitizers to minimize the expression of multacellular resistance and thereby increase initial drug sensitivity (26, 28). For example, we previously found that growth of EMT-6 mouse mammary carcinoma cells as compact multicellular spheroids renders the cells more intrinsically resistant in vitro to 4-hydroperoxy-cyclophosphamide than the same cells grown as monolayer cell cultures (6). Moreover, this effect could be duplicated in vivo using an ascites tumor model (6). Thus, when certain clones of EMT-6 cells were injected s.c. into BALB/c mice, a lethal malignant ascites developed that consisted primarily of cellular aggregates of varying sizes (6). Such aggregates (which are likely composed of both tumor and host cells) could be substantially disaggregated by i.p. injections of bovine testicular hyaluronidase (6). If cyclophosphamide was also cojected, the therapeutic effects of this drug could be enhanced by hyaluronidase, which itself had no therapeutic effect (6). It is of interest to note that ascites tumors in other preclinical models (e.g., the AHI36B rat hepatoma) also consist of islands of aggregated cells and that such three-dimensional structures can be disrupted by agents such as monoclonal antibodies to cell surface-associated adhesive factors (29). Moreover, the formation of peritoneal ascites tumors is a common clinical feature of advanced ovarian carcinoma, and, similar to the EMT-6/AH136B systems described above, the appearance of varying-sized cell aggregates has been observed in such cases (30). Cell aggregate formation is also a characteristic of malignant effusions in other types of tumors, e.g., colorectal carcinoma (31). Therefore, the possibility arises of using agents that have a disaggregating effect on ascitic human ovarian carcinoma cell clusters to render drugs such as Taxol or cyclophosphamide more effective against such tumors. Unfortunately, we have not found hyaluronidase to be an effective antiadhesive agent for human tumors, including ovarian carcinoma and are currently evaluating other types of agents for the possibility of mediating such a function.

In conclusion, human ovarian carcinoma cell lines grown as multicellular tumor spheroids express a significant intrinsic resistance to the cytotoxic effects of Taxol. This type of multacellular resistance could be a major factor in helping to reduce the initial effectiveness of Taxol against solid tumors commonly treated with this drug, such as breast and ovarian cancer. If so, the possibility arises of using non-toxic antiadhesive agents to reverse the multiclular resistance of tumors such as human ovarian cancer ascites or microscopic breast/ovarian cancer metastases.

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*References*


4 B. St. Croix, unpublished observations.
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