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Acquired multicellular-mediated resistance to alkylating agents in cancer

(drug resistance/chemotherapeutic drugs/tumor spheroids)

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ABSTRACT **EMT-6 murine mammary tumor sublines highly resistant to cyclophosphamide, *cis*-diamminedichloroplatinum(II), or *N,N',N"*-triethylenethiophosphoramide were generated *in vivo* by sequential treatment of tumor-bearing mice with the respective drugs. Previous studies demonstrated the drug-resistant phenotypes of the sublines were not expressed *in vitro* when the cells were grown as monolayer cultures. We now show that expression of drug resistance—including patterns of cross-drug resistance observed *in vivo*—can be fully recapitulated *in vitro* when the cells are grown under *in vivo*-like, three-dimensional conditions—namely, as multicellular tumor spheroids. Moreover, the spheroids generated from all of the drug-resistant sublines manifested a much more compact structure. Immediate drug-sensitivity testing of single cells released by trypsin treatment from compact drug-resistant spheroids revealed that such cells lost much of their drug-resistant properties. The results suggest a possible mechanism of acquired drug resistance in tumors based on the response of a cell population (i.e., multicellular or tissue resistance) as opposed to classic (uni)cellular resistance mechanisms.**

Expression of resistance to the toxic effects of anticancer drugs—whether *de novo* or acquired—remains one of the most significant obstacles to improving cancer therapy. Elucidation of the physiological, cellular, and molecular mechanisms of drug resistance in cancer is therefore essential to devising strategies to overcome the problem. An illustrative paradigm in this respect is pharmacologic inactivation of P-glycoprotein—an energy-dependent drug efflux pump—by drugs such as cyclosporin or verapamil (1). This can lead to a functional reversal of multidrug resistance to diverse lipophilic natural chemotherapeutic compounds (1).

Proposed mechanisms of drug resistance in cancer are based largely on the study of drug-resistant variants isolated from tumor cell lines exposed to various classes of drug in monolayer tissue culture (2, 3). Such studies have revealed a number of biochemical mechanisms of drug resistance operative at the cellular level (2, 3). They involve, for example, reduced drug uptake, increased drug efflux, increased drug inactivation, increased DNA repair, and altered molecular expression of drug targets within cells (1–3). However, the impact of such (uni)cellular mechanisms of resistance to clinical drug resistance remains largely speculative (3). This method of analysis also tends to put little or no emphasis on physiologic mechanisms of drug resistance operative at the level of whole tissues. An example of this is the relative inability of certain drugs to penetrate deeply into solid tumor masses as a result of unfavorable interstitial pressure gradients and nonuniform blood supply (4, 5). This is one of the proposed reasons to help explain why tumor cells grown in monolayer culture are frequently much more sensitive intrinsically to certain cytotoxic drugs than when grown as three-dimensional multicellular spheroids (6, 7) or native state tissues (8), although other unknown mechanisms, not involving reduced drug uptake, have been implicated as well (8, 9).

Some of these problems might be circumvented by studying drug-resistant tumor variants isolated *in vivo*. In this regard, Teicher *et al.* (10) recently derived a series of alkylating agent-resistant variants of the EMT-6 mouse mammary tumor by *in vivo* drug administration to syngeneic BALB/c tumor-bearing mice. Mice were exposed to cyclophosphamide (CTX), *cis*-diamminedichloroplatinum(II) (cisplatin) (CDDP), *N,N',N"*-triethylenethiophosphoramide (thiotepa), or carboplatin and a total of 10 sequential passages over a 6-month period was involved (10). Although this generated stable alkylating agent-resistant sublines, their drug resistance properties were only manifested *in vivo*: surprisingly, the cells plated in (monolayer) tissue culture were no more resistant than the parental EMT-6 cell line (10). Reinjection of the cultured cells into mice resulted in reexpression of their drug resistance properties *in vivo* (10). The results suggested a mechanism of resistance to alkylating agents operative only *in vivo* (10).

The purpose of the present study was to investigate the basis of this newly discovered mechanism of acquired drug resistance. As a first step, we decided to investigate whether culture of the *in vivo*-derived drug-resistant sublines as three-dimensional multicellular spheroids would rescue their drug-resistant phenotypes *in vitro*. The results obtained raise the intriguing possibility that some forms of acquired drug resistance operate at the multicellular—i.e., whole population (tissue)—level.

MATERIALS AND METHODS

Tumor Cell Lines. The cell lines used in these studies included the EMT-6 mammary tumor and three alkylating agent-resistant variants, as described (10). Three alkylating agent-resistant lines have been established by serial treatment of female BALB/c mice bearing tumors with CTX (300 mg/kg), CDDP (20 mg/kg), or thiotepa (15 mg/kg), injected intraperitoneally (i.p.) 24 hr before repassage of each tumor line into new host animals. The parent tumor line (EMT-6/P) was passaged in the same manner but in the absence of drug treatment. After 10 rounds of drug treatment and passages

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Abbreviations: CTX, cyclophosphamide; CDDP, *cis*-diamminedichloroplatinum(II); thiotepa, *N,N',N"*-triethylenethiophosphoramide; ECM, extracellular matrix.

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over a 6-month period, the alkylating agent-resistant sublines were used for study and designated EMT-6/CTX, EMT-6/CDDP, or EMT-6/Thio, which were resistant to CTX, CDDP, or thiotepa, respectively. *In vitro* monolayer cultures from each tumor were maintained in Waymouth's MB 752/1 medium (GIBCO), supplemented with 10% fetal bovine serum, at 37°C in 5% CO_2/95% air.

Quantitative Assay for *in Vivo* Drug Resistance. As described (10), 2×10^6 tumor cells were inoculated intramuscularly into the hind legs or subcutaneously (s.c.) into the flank of 8- to 10-week-old female BALB/c mice. When the tumors were ≈100 mm^3 in volume (day 8 after tumor cell implantation), the animals were given i.p. injections of the drug. Twenty-four hours later, a single cell suspension of each treatment group was then prepared from four tumors excised under sterile conditions (11) and was plated at three different cell concentrations in duplicate for the colony-forming assay. No significant difference was observed in total cell yield from the four pooled tumors in any treatment group. One week later, the plates were stained with crystal violet and colonies of >50 cells were counted. The plating efficiencies of the EMT-6 parent and resistant tumor lines were similar (10–16%).

Clonogenic Assay for *in Vitro* Drug Resistance. Exponentially growing cells in monolayer cultures were exposed to various concentrations of each drug for 1 hr at 37°C as described (10). 4-Hydroperoxycyclophosphamide (4-HO_2-CTX) was used *in vitro* as an activated form of CTX. Non-drug-treated controls were handled identically. Serial dilutions of alkylating agents were prepared in medium without serum immediately before use and added to the dishes. After treatment, the medium was removed and the cultures were washed twice with serum-free medium and a single-cell suspension was prepared using 0.05% trypsin with 0.02% EDTA. Known numbers of cells were plated at three different cell concentrations in triplicate for colony-forming assays, as outlined above.

In the case of multicellular tumor aggregates, 10^5 tumor cells were seeded onto 1% agarose-coated 24-well plates (see below). Three to 5 days later, cell aggregates were exposed to drugs for 1 hr. After being washed twice, cell aggregates were treated with trypsin using the same trypsin/EDTA solution to prepare the single-cell suspensions and then checked for their ability to form colonies as described above. In the experiment with cells obtained from dissociated spheroids (Fig. 3*F*), a known number of EMT-6/CTX spheroids treated with trypsin for 5–10 min at 37°C and disrupted by pipetting to ensure release of single cells. The cells were washed and then resuspended in complete medium. Immediately after plating in 35-mm tissue culture dishes, cells were exposed to 4-HO_2-CTX for 1 hr. The dissociated cells were reharvested by trypsinization and checked for their colony-forming abilities in comparison with the cells grown in monolayers or spheroids of EMT-6/CTX, which were similarly treated with the drug. In all cases of clonogenic assays, no significant difference was observed among the cell viability (>90%) of the parental and resistant tumor lines after 1 hr of drug exposure.

Production of Multicellular Tumor Aggregates or Spheroids. The liquid overlay system was used to generate spheroids (12). Agarose (SeaPlaque) was diluted to 1% with serum-free Waymouth's MB 752/1 medium and each well of 24-well plates was coated with a thin layer (0.25 ml) of this solution. Tumor cells (10^5 in 1 ml of complete medium) were plated in each well and incubated at 37°C under 5% CO_2/95% air. Under these conditions, the cells could not attach to the tissue culture plates, which were agitated gently once a day for the initial 2 days. All the cell lines were treated similarly. In this culture system, a single aggregate of cells was evident in each well.

Cell Viability Assay for *in Vitro* Drug Resistance. Tumor cells (10^5) were seeded into six-well plates (monolayer culture) or 1% agarose-coated 24-well plates (three-dimensional culture). Three to 5 days later, serial dilutions of drugs were added to the plates. After 48 hr of incubation, all cell lines were exposed to the same trypsin treatment and checked for viability by trypan blue exclusion testing.

Histologic Analysis of Multicellular Aggregates on Tumor Spheroids. Drug-resistant spheroids were fixed with 10% buffered formalin containing 0.2% methylene blue for ≈15 min, after which the fixative was replaced with plain 10% buffered formalin. Spheroids were then wrapped in onionskin paper, placed in a tissue processing cassette, fixed for a further 24 hr in formalin, dehydrated, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin and photographed.

Due to their fragility, parental EMT-6 cell aggregates were individually fixed with 10% buffered formalin containing 0.2% methylene blue for ≈30 min in the agarose-coated wells on which they were grown. Formalin was then removed and the aggregates were overlaid with 0.5 ml of a solution of 1% agarose in phosphate-buffered saline at 37°C. After the agarose solidified, a further 2–3 ml of formalin was added to the wells and left for 3 days in order for the agarose to harden to a consistency that would allow easy manipulation of the cell aggregates. Cell aggregates embedded in agarose were then scooped out of the wells with a small spatula and processed as indicated above.

RESULTS

Drug-Resistance Profiles of EMT-6 Sublines *in Vivo*. Fig. 1 *A–C* shows the sensitivity of each tumor line to the *in vivo* drug exposure assayed by the ability to form colonies *in vitro* (10). The resistance ratio, calculated from the surviving fraction of the resistant lines divided by the surviving fraction of the parental line at each drug dose, indicated that the CTX-resistant tumor line (EMT-6/CTX) was 7.3, 52, and 140 times more resistant, respectively, to 100, 300, and 500 mg of CTX per kg than the parental tumor line (EMT-6/P). The CDDP-resistant line (EMT-6/CDDP) was 2.7, 23, and 79 times more resistant to CDDP at 10, 20, and 30 mg/kg, respectively. The thiotepa-resistant line (EMT-6/Thio) was 4.5 and 31 times more resistant to thiotepa at 15 and 30 mg/kg, respectively.

Drug-Resistance Profiles of EMT-6 Sublines *in Vitro*. In spite of these significant levels of *in vivo* drug resistance, each of the three alkylating agent-resistant tumor lines showed only a slightly higher *in vitro* survival as estimated by surviving fraction analysis, compared to that of the parental line, after exposure for 1 hr to the drugs in monolayer culture: thus, the resistance ratios never exceeded 2.1-fold in any kind of drug exposure with the concentration of 12.5–500 μM (Fig. 1 *C–E*). These results are compatible with previously reported data (10) and suggest that the mechanism of drug resistance in each line might be developed through some mechanism expressed only *in vivo*. In subsequent experiments, we tried to resurrect the drug-resistance properties of the variants *in vitro* by growth on Matrigel, a reconstituted basement membrane extract, or laminin, an extracellular matrix (ECM) component, or by coculture with normal stromal cells such as murine dermal fibroblasts. These procedures did not result in expression of drug resistance (unpublished observations).

Drug Resistance *in Vitro* Is Expressed by EMT-6 Sublines When Grown as Multicellular Spheroids. The use of cell lines grown in conventional monolayer tissue culture is a mainstay of cancer biology research including studies in drug resistance. However, solid tumors grow as three-dimensional structures, and for many types of *in vitro* investigations it is

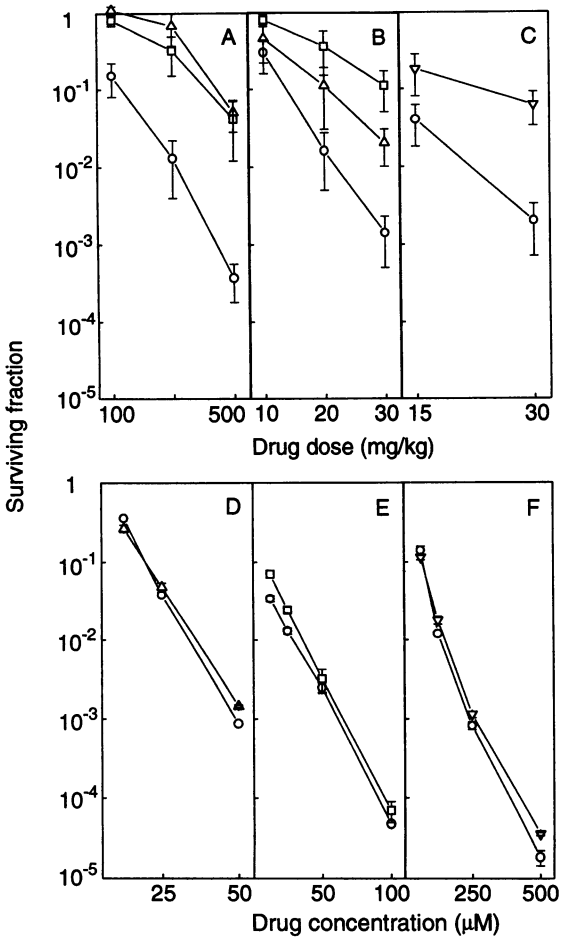

FIG. 1. Colony-forming assay using EMT-6/P (○), EMT-6/CTX (△), EMT-6/CDDP (□), and EMT-6/Thio (▽) tumor cells treated *in vivo* (*A–C*) or *in vitro* (*D–F*) with various concentrations of alkylating agents. (*A–C*) When the tumors were ≈100 mm³ in volume, the animals were given i.p. injections of CTX (*A*), CDDP (*B*), or thiotepa (*C*). Twenty-four hours later, a single-cell suspension was prepared from excised tumors and plated for colony-forming assay. Points are means of three independent experiments and are expressed as the surviving fraction ± SEM of cells from treated groups, compared with untreated control groups. (*D–F*) After 1 hr of exposure to 12.5–50 μM 4-HO_2-CTX (*D*), 12.5–100 μM CDDP (*E*), or 62.5–500 μM thiotepa (*F*) in monolayer culture, a colony-forming assay was performed. All assays were done in triplicate with consistent results.

necessary to recapitulate this type of growth (7, 8, 13). For example, sensitivity of tumor cells to radiotherapy can be strongly affected by hypoxic regions (5, 7). Similarly, the ability of certain cytotoxic drugs or antibodies (or drug–antibody conjugates) to kill tumor cells can be limited because of the necessity of having to penetrate deeply into tumor nodules (5, 7). Thus, performing chemosensitivity or radiosensitivity assays on tumor cells grown only in monolayer culture can sometimes provide misleading information with respect to the situation *in vivo* (7, 8, 13). There are several methods that can be used to obtain three-dimensional growth of carcinoma cell lines that normally grow as attached monolayers (7, 13)—e.g., by growth in spinner flasks or on plastic surfaces coated with a thin layer of 1% agarose where attachment to the plastic surface is prevented (7, 13). These procedures can result in the formation of large (e.g., 1- to 2-mm-diameter) multicellular tumor spheroids (see below).

The next experiments were therefore designed to examine drug resistance in three-dimensional tissue culture using the multicellular tumor spheroid model. When the tumor cell lines were cultured on 1% agarose-coated plates, they could be grown as multicellular aggregates—i.e., tumor spheroids, a form of prototissue. The first thing we noted was that the appearance of such aggregates obviously differed between the parental tumor line and alkylating agent-resistant tumor sublines: the parental line made a loose, grape-like cellular aggregate, somewhat ellipsoid in shape (Fig. 2*A*), while every resistant tumor subline formed a highly compact spheroid under the same conditions (Fig. 2 *B–D*). Each of the subline cellular aggregates was exposed to various drugs for 48 hr and checked for cell viability by trypan blue exclusion testing. Although the survival of cells from monolayer cultures exposed to the agents was not very different between the parental EMT-6/P tumor line and resistant tumor lines, the survival of cells from spheroids of resistant tumor lines was much greater than that of cells obtained from cellular aggregates of parental tumor line (Fig. 3 *A* and *B*). The emergence of a large difference in drug sensitivity between parental tumor line and resistant tumor lines in three-dimensional culture was also repeatedly confirmed by the ability to form viable colonies. When the resistance ratio was compared between them after 1 hr of exposure to various concentrations of alkylating agents, the EMT-6/CTX tumor line was 8.1, 250, and 4900 times more resistant, respectively, to 25, 50, and 100 μM 4-HO_2-CTX than the EMT-6/P parental tumor line (Fig. 3*C*). The EMT-6/CDDP line was 18, 15, and 58 times more resistant to CDDP at 25, 50, and 100 μM, respectively (Fig. 3*D*). The EMT-6/Thio line was 3.6, 12, and 65 times more resistant to thiotepa at 125, 250, and 500 μM, respectively (Fig. 3*E*). As shown in Fig. 1 *A* and *B*, the EMT-6/CDDP tumor line was as resistant to CTX as was the EMT-6/CTX line after *in vivo* drug treatment. The EMT-6/CTX tumor was somewhat resistant to CDDP, especially at high doses of the drug, compared with the EMT-6/P parental line. A similar pattern of cross-drug resistance of EMT-6/CTX and EMT-6/CDDP against CDDP or 4-HO_2-CTX, respectively, was reproduced *in vitro* by both the cell viability assay and the colony-forming assay using three-dimensional culture systems (Fig. 3 *A–D*).

Partial Drug Resistance Manifested in Single Cells Derived from Disaggregated Spheroids. To evaluate the possible importance of drug penetration as the cause of a resistance of the cells in spheroids, clonogenic survival of single cells freshly derived from spheroids was compared with those of exponentially growing monolayers and spheroids using EMT-6/CTX cell line (Fig. 3*F*). When the resistance ratio was compared among three conditions of EMT-6/CTX line, the spheroids were 59 and 7000 times more resistant while the disaggregated spheroids were 5.0 and 210 times more resis-

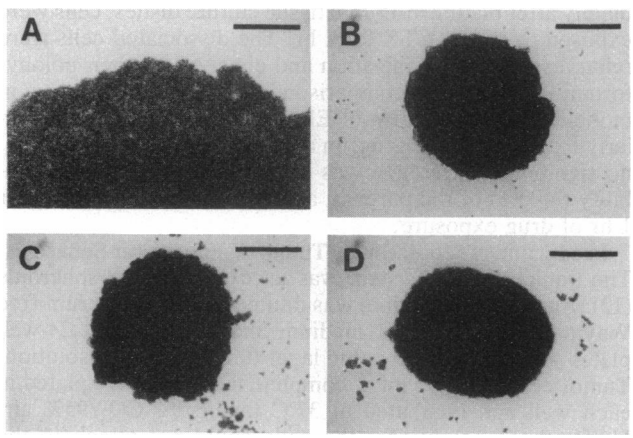

FIG. 2. Morphological appearance of multicellular aggregates of each tumor line 5 days after the cell plating onto 1% agarose-coated 24-well plates. (*A*) EMT-6/P. (*B*) EMT-6/CTX. (*C*) EMT-6/CDDP. (*D*) EMT-6/Thio. (Bars = 0.5 mm.)

tant, respectively, to 50 and 100 μM 4-HO_2-CTX than the EMT-6/P parental tumor line grown as monolayer. Therefore, EMT-6/CTX tumor cells composing a compact spheroid manifested significant levels of drug resistance (albeit at a greatly reduced level) even if they were exposed to the drug immediately after being separated from each other. The rate at which this remaining resistance is lost in monolayer culture remains to be analyzed.

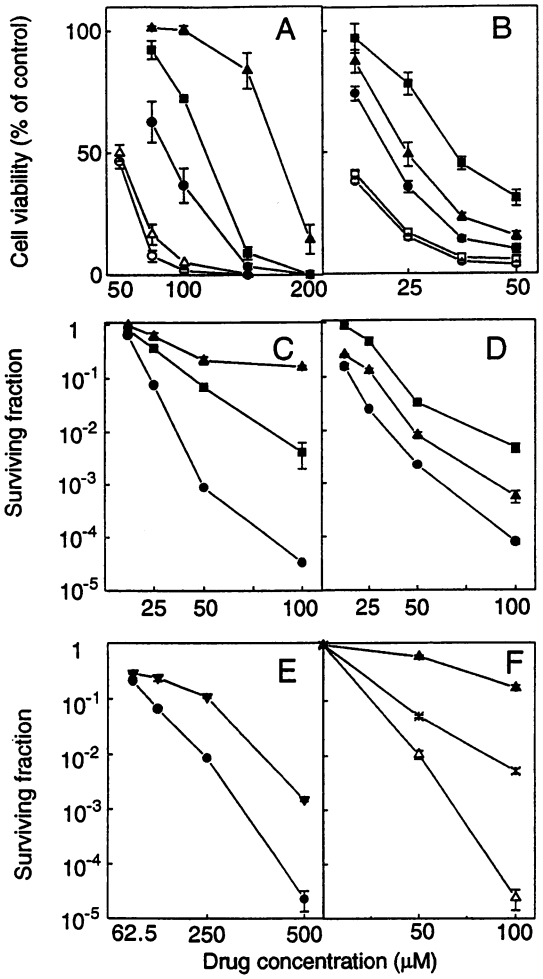

FIG. 3. (*A* and *B*) Cell viability after drug exposure in two- or three-dimensional culture systems. Tumor cells were seeded in six-well plates (two-dimensional culture) or onto 1% agarose-coated 24-well plates (three-dimensional culture) and incubated for 3–5 days. After 48 hr of drug exposure to 50–200 μM 4-HO_2-CTX (*A*) or 12.5–50 μM CDDP (*B*), all cell lines were checked for viability by trypan blue exclusion testing. Two-dimensional culture: ○, EMT-6/P; △, EMT-6/CTX; □, EMT-6/CDDP. Three-dimensional culture: ●, EMT-6/P; ▲, EMT-6/CTX; ■, EMT-6/CDDP. Results are expressed as viability of cells from treated groups compared to untreated control groups. Reported means ± SEM are calculated from quadruplicate samples. (*C–E*) Colony-forming assay after drug exposure in three-dimensional culture systems. After multicellular aggregates of each tumor line were exposed to drug for 1 hr, a single-cell suspension was prepared from each aggregate and plated for the colony-forming assay. (*C* and *D*) EMT-6/P (●), EMT-6/CTX (▲), and EMT-6/CDDP (■) were exposed to 12.5–100 μM 4-HO_2-CTX (*C*) or CDDP (*D*). (*E*) EMT-6/P (●) and EMT-6/Thio (▼) were exposed to 62.5–500 μM thiotepa. Results are expressed as surviving fractions ± SEM of cells from treated groups compared with untreated control groups. All assays were performed three times with comparable results. (*F*) Colony-forming assay after drug exposure of EMT-6/CTX in monolayers, spheroids, and disaggregated spheroids. Cells in monolayers (△) and spheroids (▲) were exposed to 4-HO_2-CTX as described. Single-cell suspension (×) was obtained by treating spheroids with trypsin and then exposing them to the drug for 1 hr in tissue culture dishes. A single-cell suspension was reprepared from each dish and plated for the colony-forming assay.

Histologic Appearance of Multicellular Aggregates or Spheroids. As shown in Fig. 4 *A* and *B*, the drug-resistant EMT-6 lines were capable of forming compact spheroids with diameters ranging from 0.78 (EMT-6/CTX) to 1.22 (EMT-6/Thio) mm 8 days after plating 10^5 cells on agarose-coated plates. All the spheroids had a necrotic center surrounded by a rim of viable cells. The thickness of the viable rims ranged from 120 (EMT-6/CDDP) to 150 (EMT-6/Thio) μm. As described by Sutherland and Durand (14) for most spheroids, the rims of viable cells in the present spheroids had three morphologically distinct zones: the cells near the periphery appeared to be more loosely attached to each other, whereas the intermediate zone in the center of the rims was more tightly packed; the zone near the necrotic center had a large proportion of cells with pycnotic nuclei, a characteristic of degenerating tissue.

In contrast to the drug-resistant variants, the parental EMT-6/P cells failed to produce compact spheroids but formed large (3- to 4-mm diameter), rather loose, cellular aggregates (Fig. 4 *C* and *D*). Cells in these aggregates maintained a rounded morphology and had condensed nuclei. Interestingly, interspersed within loosely attached cells were small aggregates (≈100-μm diameter) with tightly packed cells, somewhat resembling miniature versions of the drug-resistant spheroids except that they lacked the necrotic core.

DISCUSSION

Our results clearly demonstrate that the EMT-6 sublines selected for acquired resistance to various alkylating agents

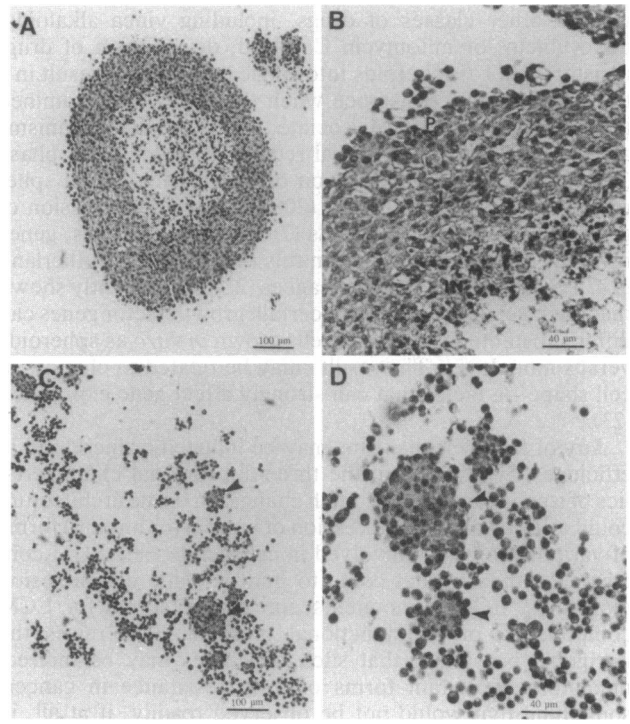

FIG. 4. Sections showing morphology of EMT-6/CTX (*A*) and EMT-6/CDDP (*B*) spheroids as well as parental EMT-6/P cell aggregates (*C* and *D*) at the light microscopic level. As shown in *A*, a distinct necrotic core was present in the drug-resistant spheroids. Various zones of the rim of viable cells are shown in *B*. P, peripheral zone; I, intermediate zone; IN, inner zone. Note that cells in the peripheral zone are loosely attached to each other and a high proportion of cells in the inner zone have pycnotic nuclei. Parental EMT-6 cells formed mainly loose cellular aggregates (*C* and *D*) with a few compact small aggregates or minispheroids (arrowheads).

in vivo manifest their resistance in tissue culture only when grown three-dimensionally, in this case as multicellular tumor spheroids. Since the same sublines manifested only a slight significant degree of resistance when grown as two-dimensional monolayer cell cultures, the results strongly suggest this is not a form of (uni)cellular resistance. Hence, we have coined the terms acquired multicellular resistance (or acquired tissue resistance) to describe this type of drug resistance.

Many studies over the past decade have shown that when tumor cells not previously selected for drug resistance are grown in the form of multicellular spheroids, the cells are often much more spontaneously resistant to toxic drugs than when grown in monolayer culture. This is particularly true for vinca alkaloids and antimetabolites (6, 15–17). In some cases, the results can be explained by limited drug access—i.e., the drugs cannot reach many of the cells encased within the spheroids (7). Thus, if tumor cells are released from spheroids by disaggregating these structures and the cells are plated into monolayer culture, their relative sensitivity to drugs can be restored (7). This observation immediately suggests that three-dimensional tumor masses may be able to increase their relative resistance to toxic drugs by alteration of tissue architecture and, in so doing, bring about a heightened state of group protection of the individual tumor cells within the tumor mass. While this could conceivably explain our drug-resistance results, we feel that reduced drug penetration is unlikely to be involved as either the predominant or the only mechanism. First, alkylating agents seem to be as effective in killing nonselected drug-sensitive tumor cells grown within spheroids as in monolayer cultures (18, 19). Second, B.A.T. (unpublished data) have found the EMT-6 alkylating agent-resistant sublines are not cross-resistant *in vivo* to other classes of drugs, including vinca alkaloids, doxorubicin, or mitomycin C. Third, dissociation of drug-resistant EMT-6 spheroids into single cells did not result in a total loss of drug resistance when the cells were examined immediately after being dissociated. Thus, other mechanisms are involved—e.g., numerical reduction of cells in S phase (7), altered DNA conformation of tumor cells within spheroids [i.e., the contact effect (20)], or altered expression of genes of cells within spheroids (7) including, perhaps, genes involved in drug resistance. In this respect, both Sutherland *et al.* (21) and Kerbel and colleagues (22) have recently shown that the level of expression of certain growth factor genes can differ substantially in tumor cells grown *in vitro* as spheroids versus monolayer. The results may be related to changes in cell shape—a factor that can strongly affect gene expression (23).

Any of these mechanisms may be induced or increased in efficiency by changes in the three-dimensional characteristics of a solid tumor mass. Such changes in tissue architecture could arise by altered expression of normal (or mutant) forms of various molecules involved in cell–cell or cell–ECM contacts. Candidates that come to mind include cell adhesion molecules, ECM components, integrin receptors for ECM components, or gap junctional proteins. This raises the intriguing possibility that such molecules may be indirect mediators of certain forms of drug resistance in cancer, something that would not be observed readily, if at all, in conventional monolayer cell culture systems used to assay cellular drug resistance. In this regard, it is interesting to note that Teicher *et al.* (10) observed an abundance of collagen filbrils *in situ* within the ECM of the drug-resistant EMT-6 sublines, a characteristic that was absent in the EMT-6 parental tumors.

Acquired drug resistance in cancer is generally thought to be a manifestation of resistance mechanisms at the single-cell level (1–3). Our results point to an additional possibility in which such resistance is manifested at the multicellular/tissue level. In this respect, a recent interesting precedent for the concept of multicellular drug resistance was reported with prokaryotic cells (24). It was shown that one particular oxidant-defense enzyme (catalase) does not protect individual *Escherichia coli* against bulk-phase hydrogen peroxide (H_2O_2), whereas catalase-positive *E. coli* grown as dense colonies are resistant to external H_2O_2. Ma and Eaton (24) speculate that colonial growth offers group protection through safety in numbers. Our results suggest that the same may be true for eukaryotic (tumor) cells.

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