

# Growth of Human Tumor Cells in Macroporous Microcarriers Results in p53-Independent, Decreased Cisplatin Sensitivity Relative to Monolayers

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## ABSTRACT

Multicellular contact has been shown to influence the in vitro sensitivity of cells to drug treatment. We investigated the use of macroporous gelatin microcarriers, CultiSpher-G, as a convenient laboratory system for the molecular analysis of this "contact effect". We determined that human A549 cells can be grown in CultiSphers with growth and cell cycle parameters similar to those of monolayers. In addition, cells in CultiSphers express less p27/kip1, an indicator of cell cycle arrest, than equivalent cells in monolayers. When treated with drugs, A549 cells grown in CultiSphers or monolayers accumulate equivalent amounts of platinum-DNA adducts and similar amounts of doxorubicin. Moreover, A549 and KB-3-1 cells in CultiSphers have significantly decreased sensitivity to *cis*-platinum(II)diammine dichloride (cisplatin), 4-hydroperoxycyclophosphamide,

doxorubicin, and paclitaxel (taxol) compared with cells in monolayers when assayed by clonogenic survival. Cisplatin treatment in monolayers or CultiSphers did not result in apoptotic cell death. In contrast, paclitaxel caused a significant amount of sub-G<sub>1</sub> DNA, an indicator of apoptosis, which was diminished when cells were grown in CultiSphers compared with monolayers. When grown in CultiSphers, cells with abrogated p53 function (A549/16E6 and NCI-H1299) were less sensitive to cisplatin than the corresponding monolayer cells, indicating that the decrease in sensitivity is p53 independent. Taken together, the data suggest that CultiSpher-G microcarriers are a useful in vitro system to examine the effects of three-dimensional cell contact on drug sensitivity of human tumor cells.

Three-dimensional intercellular contact in vitro influences the relative sensitivity of cells to both radiation and drug treatment, a phenomenon generally known as the "contact effect" (Sutherland et al., 1979, 1980; Rasey, 1984; Olive and Durand, 1994; Sakata et al., 1994). Several three-dimensional in vitro culture systems have been used to investigate the effect of intercellular contact on drug sensitivity (Vescio et al., 1991; Hoffman, 1993; Casciari et al., 1997), with most studies using multicellular spheroids (Twentyman, 1980; Sutherland, 1988; Olive et al., 1993; St. Croix et al., 1996b).

Although this "contact effect" has been extensively studied over the last 20 years, the molecular determinants of this effect have just begun to be identified. This can be attributed, in part, to the difficulty of separating the effects of cell-cell contact from gradients of drug penetration, nutrients, oxygen, pH, and cell cycling (Durand, 1990; Erlichman and Wu, 1992; Ramachandran et al., 1995).

Recently, we characterized the use of CultiSpher-G (HyClone Laboratories, Logan, UT), macroporous porcine gelatin microcarriers, as a model cell culture system for drug sensitivity studies. These gelatin "sponges" with their variegated surface and large internal channels allow cell growth on the surface and within the bead. Cells growing in CultiSphers histologically demonstrate significant three-dimensional contact without central necrosis, lack edge-to-center gradients of proliferation by [<sup>3</sup>H]thymidine autoradiography, and give no indication of a hypoxic fraction on radiation-response curves (Rasey et al., 1996). We also documented that in optimal

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**ABBREVIATIONS:** 4-HC, 4-hydroperoxycyclophosphamide; cisplatin, *cis*-platinum(II)diammine dichloride; Pt-DNA, platinum-DNA; TBST, 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Tween 20, p27, p27/kip1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium.

culture conditions, CultiSphers can be used to produce populations of cells with extensive cell-cell contact whose cell cycling profiles and doubling times are similar to those of monolayers of the same cell type (Rasey et al., 1996).

To determine whether a "contact effect" might be elicited by growth of tumor cells in CultiSphers, we used four common chemotherapeutic agents eliciting their cytotoxicity through different mechanisms of action: 4-hydroperoxycyclophosphamide (4-HC), the activated analog of cyclophosphamide, paclitaxel (taxol), doxorubicin, and *cis*-platinum(II)diammine dichloride (cisplatin). Cisplatin has the advantage of producing readily detectable platinum-DNA (Pt-DNA) adducts, and doxorubicin is epifluorescent, characteristics that can be used to determine the equivalency of drug treatments. In the current study, we examined the drug sensitivity of isogenic cells, differing only by growth in CultiSphers or in monolayers on porcine gelatin-coated plastic. We report that growth of the A549 human lung tumor cell line in CultiSphers caused a decreased sensitivity to cisplatin, 4-HC, paclitaxel, and doxorubicin by clonogenic survival assay compared with monolayer controls. This was observed under conditions in which cells in both growth modes had similar cell cycle profiles and accumulated similar amounts of drug. In addition, we report that growth of two other independently derived tumor cell lines (KB-3-1 and NCI-H1299) in CultiSphers decreased their sensitivity to cisplatin. Finally, we report that decreased sensitivity to cisplatin of A549 and NCI-H1299 cells grown in CultiSphers was p53 independent.

## Experimental Procedures

**Materials.** All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Culture media was prepared in-house from powder (Gibco-BRL, Gaithersburg, MD) and media additives were from Gibco-BRL or Atlanta Biologicals (Norcross, GA), except serum, which was from HyClone. CultiSpher-G gelatin microcarriers (mean diameter = 220  $\mu$ m, range 170–270  $\mu$ m), for cell culture were obtained from HyClone Laboratories. 4-HC was a gift of S. Rowley, Fred Hutchinson Cancer Research Center (FHCRC) (Seattle, WA). Enhanced chemiluminescence Western blotting detection reagents were from Amersham (Arlington Heights, IL). Antisera to human p27 were a generous gift of Jim Roberts (FHCRC; Polyak et al., 1994). Quantitative protein assay was by Bio-Rad (Hercules, CA). Puregene DNA isolation kits were from Gentra Systems (Minneapolis, MN).

**Cell Lines and Culture Conditions.** A549 cells, a p53 wild-type human lung carcinoma cell line (ATCC CCL-185; American Tissue Culture Collection, Rockville, MD) and transfected derivatives were maintained in monolayer culture in F/DV medium plus 10% bovine calf serum (growth medium). A549 derivatives A549/LXSN and A549/16E6 contain the stably transfected LXSN retroviral vector or vector cDNA encoding the E6 protein of plus human papilloma virus strain 16, respectively (Russell et al., 1995). KB-3-1 cells, a human epithelial carcinoma derivative (ATCC CCL-17) (Akiyama et al., 1992) were maintained in monolayer culture in Dulbecco's modified Eagles medium plus 5% bovine calf serum. NCI-H1299 cells (ATCC CRL-5803) were maintained in RPMI-1640 medium with 10% fetal bovine serum. All media were supplemented with penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) and 2 mM glutamine. Tissue culture plates were gelatin coated by treating each dish with 8 ml of 5 mg/ml gelatin (type A, porcine skin) in 0.05 N HCl for 1 h followed by rinsing three times with sterile water. Monolayer cells were plated at  $4 \times 10^5$  cells per 10 cm<sup>2</sup> on gelatin-coated plates and allowed to grow for 48 h before drug treatment. CultiSpher-G gelatin microcarriers were seeded by addition of a single cell suspension ( $5 \times$

$10^6$  log phase cells) to 30 ml medium per 0.1 g CultiSphers in a siliconized spinner flask, agitated for 5 min, and then allowed to settle undisturbed for 2 h. The spinner was reagitated for an additional 5 min and allowed to resettle for 2 h. CultiSphers were removed, washed three times with growth medium to remove unattached cells, and replaced in the flask with 50 ml growth medium per 0.1 g CultiSphers. The following day an additional 50 ml growth medium was added per 0.1 g CultiSphers. Each day thereafter, the beads were allowed to settle briefly, then half the medium was removed and replaced. CultiSpher occupancy was monitored by staining an aliquot of beads with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 500  $\mu$ g/ml in growth medium, at 37°C, for 30 min before observation. Cell number per bead was quantitated by counting the number of beads in an aliquot in a gridded Petri dish, recovering and disaggregating the CultiSphers as described below, and counting cells in a hemacytometer. CultiSphers were treated with drugs when they reached 200 to 500 cells/bead, a density at which cells remained in log phase growth, with no evidence of non-cycling cells by flow cytometry, doubling time, and Ki67 analyses (data not shown). Cells in monolayers and CultiSphers have similar cell cycle profiles under these conditions (Rasey et al., 1996).

**Histology.** For histological analysis, A549 cells were seeded and grown in CultiSphers to an average of ~1000 cells/bead. CultiSphers were fixed in neutral buffered formalin, embedded in paraffin, sectioned and stained with H&E as previously described (Rasey et al., 1996).

**Drug Treatment Conditions.** Monolayers or CultiSphers were rinsed once with growth medium to remove detached cells and drug-containing medium was added at 37°C for one hour except as noted. Care was taken to ensure equivalent drug exposure and harvesting conditions with cells grown as monolayers and in CultiSphers. Five milliliters of drug-containing medium was used per 10 cm dish ( $\sim 1.6 \times 10^6$  cells). Larger volumes of CultiSphers were treated in 100- to 250-ml spinner flasks with a similar total drug-medium volume-to-cell ratio. For treatment of small aliquots, washed CultiSphers were placed in drug-containing medium at similar drug-to-cell ratios in 6-cm<sup>2</sup> bacteriological dishes, so that cells did not attach, for 1 h with gentle agitation every 10 min. Both monolayer and CultiSpher cells were then harvested using collagenase as per the clonogenic survival assay below. Cisplatin was freshly prepared as a 1 mg/ml stock in PBS, sterile filtered, and appropriate dilutions made in growth medium at 37°C immediately before use. 4-HC stock (100 mg/ml) was freshly prepared in growth medium, sterile filtered, and diluted in medium for immediate use. Doxorubicin was prepared as a 5-mM stock in water and paclitaxel prepared as a 5-mM stock in dimethyl sulfoxide and further diluted in growth medium for immediate use. Cells were then drug treated, washed three to four times in growth medium to remove drug, and then resuspended to original volume with equal parts conditioned and fresh medium. Thereafter, half the medium of dishes or spinners was removed and replaced with fresh growth medium daily.

For cisplatin treatment of cells after release from CultiSphers, cells were dissociated from CultiSphers as described below, then plated at  $4 \times 10^6$  or  $2 \times 10^6$  cells per gelatin-coated 15-cm dish for 4 or 24 h, respectively, ensuring a density at treatment of approximately  $4 \times 10^6$  cells per dish. After release from CultiSphers and replating, the cells in monolayers at 4 and 24 h displayed normal monolayer morphology and could not be removed by agitation alone. Cells in monolayers were then treated with 10  $\mu$ g/ml cisplatin.

For doxorubicin accumulation and clonogenic studies, A549 cells were grown on gelatin-coated dishes ( $2 \times 10^5$  cells/dish followed by 48 h growth) or in CultiSphers (562 cells/bead) and were treated with various doses (clonogenic assay) or 5  $\mu$ g/ml (110  $\mu$ M for uptake studies) doxorubicin for 1 h at 37°C in growth medium. After 1 h, cells in CultiSphers were released from beads with collagenase as described below. Cell suspensions were then washed in ice-cold PBS, collected by centrifugation, and kept on ice until analyzed by flow cytometry. To provide the same washing for cells in monolayers and

CultiSpheres, monolayers were washed two times with PBS, incubated in drug-free growth medium for 1 h, then released from dishes with a 2-min trypsin treatment. Cell suspensions were then washed in ice-cold PBS, collected by centrifugation, and kept on ice until analyzed by flow cytometry. Under these conditions, efflux of drug is minimal in cells, such as A549, that do not express P-glycoprotein (Willingham et al., 1986).

**Clonogenic Survival Assay.** To determine cell survival after drug treatment, single cell suspensions from CultiSpheres and monolayers were prepared by collagenase. After drug treatment, cells in monolayers or CultiSpheres were washed three times with whole medium and once with Hanks' balanced salt solution. Gelatin-containing CultiSpheres were digested to release the cells by adding 1 ml of 800 U/ml collagenase in Hanks' balanced salt solution per 1 mg of CultiSpheres at 37°C for 1 h, with agitation every 10 min. Known numbers of cells were plated for standard colony-forming assay (3–6 plates per cell dilution, 3–5 dilutions per drug concentration) in 6-cm or 6-well plates. Dilutions with greater than  $5 \times 10^4$  cells/dish were plated in 10-cm dishes. Surviving fraction was calculated in reference to the plating efficiency of untreated controls (two separate triplicate controls per experiment). Plates were incubated undisturbed for 10 to 12 days. Surviving colonies were stained with 0.5% methylene blue in 50% ethanol, visualized on a Leica StereoZoom 4 dissecting microscope (Leica Inc., Deerfield, IL), and colonies of at least 50 cells on plates containing 50 to 150 colonies were scored as positive.

**Cisplatin-DNA Adduct Measurements.** CultiSpheres were seeded in 250-ml spinner flasks, allowed to grow to ~200 to 500 cells/bead, and monolayers plated as above. Cells were treated with either 10  $\mu$ g/ml cisplatin for 1 h, 10  $\mu$ g/ml cisplatin for 4 h, or 40  $\mu$ g/ml cisplatin for 4 h, washed with PBS, and single cell suspensions prepared. DNA was prepared using the Puregene DNA isolation kit. Purity and yield were confirmed by gel electrophoresis and spectroscopy. DNA was analyzed quantitatively for Pt-DNA adducts by atomic absorption spectroscopy as previously described (Johnson et al., 1994).

**Flow Cytometry.** For cell cycle analysis, samples of cell suspensions were fixed in 50% ethanol, then prepared for flow cytometric analysis by incubation in PBS containing 0.1% Triton-X100, 25  $\mu$ g/ml propidium iodide, and 100  $\mu$ g/ml RNase A for 30 min, 37°C. Cells were analyzed on a Becton Dickinson FACScan with CELLQuest (Becton Dickinson, San Jose, CA) and MultiCycle AV (Phoenix Flow Systems, San Diego, CA) analysis software. For doxorubicin accumulation analysis, cell volume was monitored by forward scatter and granularity by side scatter, respectively, and a single gate applied to all samples (under conditions in which over 95% of all cells were contained within the gate). The doxorubicin fluorescence distribution was measured in the gated population of cells by excitation at 488 nm and emission at 585 nm (Luk and Tannock, 1989). With this methodology the fluorescence of each cell and the distribution of fluorescence of the population of cells was measured as previously described (McGown et al., 1983). For each sample, a statistical marker spanning the single peak of fluorescence was used to calculate the mean fluorescence (CELLQuest cytometry analysis software). Greater than 96% of the total number of gated cells were included in the statistical analysis for each sample. To estimate the relative uptake of doxorubicin after 1 h of treatment (and 1 h of efflux), the mean fluorescence in the presence of doxorubicin was normalized to the mean fluorescence in the absence of doxorubicin.

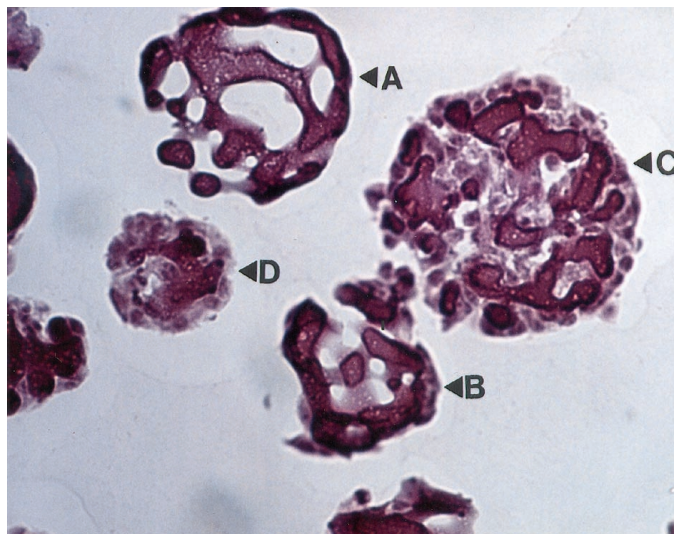
**Immunoblot Analysis.** Cellular protein was prepared from collagenase-released cells that were washed once with cold PBS, then lysed on ice for 30 min in 1% Nonidet P-40 in PBS with 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 2 mM 4-(aminoethyl)benzene-sulfonyl fluoride, 10 mM sodium fluoride, and 5 mM sodium pyrophosphate. Samples were collected by centrifugation at 8200g for 30 min at 4°C, and the solubilized protein quantitated by the Bio-Rad protein assay. Samples were stored at -70°C. For immunoblotting, 40  $\mu$ g protein/

lane was electrophoresed in precast Bio-Rad Mini-Protein II 12% SDS-polyacrylamide gels and electrotransferred to 0.2- $\mu$ m nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Quantitation was confirmed visually with Coomassie blue staining after polyacrylamide gel electrophoresis. Membranes were blocked overnight in 10 mM Tris-HCl, pH 8; 150 mM NaCl; 0.5% Tween 20 (TBST) and 5% nonfat dry milk at 4°C. Membranes were then incubated in polyclonal antibody to p27/kip1 at 1:2000 in TBST with 5% nonfat dry milk for 1 h at room temperature, followed by  $3 \times 15$  min washes in TBST with 5% nonfat dry milk at room temperature. Membranes were incubated with goat anti-rabbit antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) at 1:15,000 for 1 h at room temperature in TBST with 5% nonfat dry milk, followed by two 15-min washes in TBST with 0.5% dry milk and three 15-min washes in TBST. The membrane was then incubated with the chemiluminescence reagent (Amersham) for 1 min, drained, and exposed to autoradiograph film.

**Statistical Evaluation of Data.** Averages, S.D.s, and unpaired two-tailed student's *t*-tests at the 95% confidence interval were performed using Prism 2.0 software (GraphPad Software, San Diego, CA).

## Results

**CultiSphere Culture System.** We characterized a multicellular growth system, macroporous gelatin microcarrier beads called CultiSphere-G (Nikolai and Hu, 1992) to study the role that cell contact and matrix interactions play in determining the response of cells to chemotherapeutic agents (Rasey et al., 1996). Figure 1 shows a typical histological section through a population of CultiSphere beads seeded with A549 cells. Using the manufacturer's suggested seeding conditions, a range of cell densities was observed. Some beads were not populated, some beads were sparsely populated (Fig. 1, A and B; ~100 cells/bead), whereas others were densely populated (Fig. 1, C and D; ~1000 cells/bead). In the beads that were populated, cells grew well on both the external and internal surfaces of the bead. There were areas of single cells or monolayer-like growth as well as areas of extensive cell-cell contact. Even at high cell/bead ratios, some beads were not occupied. Therefore, the number of cells/bead is an average measure of overall occupancy. Longer growth



**Fig. 1.** Photomicrograph of histological section of A549 CultiSpheres after 5 days of growth. Cells were seeded in CultiSpheres and prepared for histology. A and B, sparsely occupied CultiSpheres; C and D, densely occupied CultiSpheres.



periods in CultiSphers resulted in an increase in areas of multicellular contact and decreased single cells (data not shown). Extensive cell-cell contact within beads occurred while channels were maintained for drug (Table 1 and see *Results* below) and nutrient access (Rasey et al., 1996).

Cell cycle distribution was evaluated relative to bead occupancy to assess the effect of extensive cell contact in CultiSphers. For both A549 and KB-3-1 cells grown as CultiSphers, flow cytometric cell cycle analysis indicated that occupancies of over 800 cells per bead resulted in an increased G<sub>1</sub> population compared with exponentially growing monolayers, suggesting that at greater than 800 cells/bead, some cells were exiting the cell cycle (data not shown). Occupancies of less than 200 to 400 cells per bead produced no significant change in G<sub>1</sub> status (data not shown). Our previous studies suggested that release of cells from beads in culture contributed to an apparent difference in doubling time between monolayers and CultiSphers (Rasey et al., 1996). To examine this question, cell cycle studies using a 1-h pulse of 2-bromo-5-deoxyuridine indicated by flow cytometry that the cell cycle transit time for untreated monolayers and CultiSphers at 255 cells/bead were similar (data not shown). Thus, seeding and growth conditions that produced cell/bead ratios of 200 to 400 cells per bead, typically 3 to 5 days postseeding, were used for subsequent experiments because they yielded cultures with extensive cell contact, similar cell cycle transit, and log phase growth.

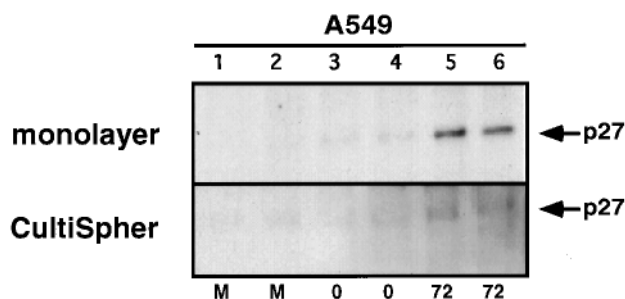
Cell contact has been shown to induce the expression of the cell cycle inhibitor p27/kip1, resulting in cell cycle arrest (Polyak et al., 1994). Expression of p27 has also been correlated with decreased drug sensitivity of a wide variety of human tumor cell lines grown as multicellular spheroids (St. Croix et al., 1996a). In contrast to these data, A549 monolayer and CultiSpher cultures expressed little p27 protein (Fig. 2, lanes 1 and 2, top and bottom panels) at the time of drug treatment (3–5 days postseeding), conditions in which cells in CultiSphers formed extensive cell-cell contacts. Following a 1-h treatment with 10  $\mu$ g/ml cisplatin and 72 h of recovery (Fig. 2, top panel, lanes 5 and 6; data not shown), cells in monolayers expressed increased levels of p27 relative to mock-treated samples. In contrast, 72 h following the same cisplatin treatment, cells in CultiSphers (Fig. 2, bottom panel, lanes 5 and 6) expressed very little p27. These data indicate that under similar growth conditions used to pre-

pare CultiSpher cultures for drug treatment, p27/kip1 is not extensively expressed.

**Analysis of Chemotherapeutic Drug Sensitivity of Cells Grown in CultiSpher Culture.** Relative sensitivities to clinically relevant concentrations of drugs were then determined by clonogenic survival assay (Fig. 3). A549 cells grown as CultiSphers (Fig. 3A) were much less sensitive to cisplatin compared with A549 cells grown as monolayers, as indicated by an increased survival fraction. To determine whether the observed decrease in cellular drug sensitivity was drug-specific, A549 cells grown as monolayers and in CultiSphers were exposed to clinically relevant concentrations of 4-HC or doxorubicin for 1 h or to paclitaxel for 24 h and clonogenic cell survival determined. A549 cells in CultiSphers were also less sensitive to 4-HC (Fig. 3B), paclitaxel (Fig. 3C), and doxorubicin (Fig. 3D) compared with A549 monolayers. To assess whether this phenomenon was cell type-specific, the cisplatin sensitivities of KB-3-1 cervical cancer cells grown as monolayers and in CultiSphers were determined. The results demonstrate that KB-3-1 (Fig. 3E) cells were also less sensitive to cisplatin when grown in CultiSphers than when grown in monolayer culture. Statistical analysis using Student's *t* test at 15  $\mu$ g/ml cisplatin showed significantly different survival fractions for cells grown as monolayers versus in CultiSphers ( $P = .002$  for A549 cells in Fig. 3A and  $P < .001$  for KB-3-1 cells in Fig. 3E). Further analysis by *t* test showed significant differences in survival fractions of A549 monolayers versus CultiSphers at 200  $\mu$ g/ml 4-HC ( $P < .0001$ ; Fig. 3B), 100 nM paclitaxel ( $P < .0001$ ; Fig. 3C), and at 1  $\mu$ M doxorubicin ( $P = .0009$ ; Fig. 3D).

If the decreased sensitivity to cisplatin of cells grown in CultiSphers is a consequence of multicellular growth, disruption of multicellular interactions before drug treatment might alter the sensitivity of the cells. To examine this possibility, cells from CultiSphers were released and replated onto gelatin-coated dishes as monolayers for 4 or 24 h and then treated for 1 h with cisplatin. As shown in Fig. 3F, A549 cells grown in CultiSphers and replated for 4 h before treatment with cisplatin exhibited the same dose-dependent sensitivity as cells treated in CultiSphers. However, cells grown in CultiSphers and then replated for 24 h before treatment exhibited the same reduction in dose-dependent sensitivity as cells grown as monolayers. These data are similar to results observed for alkylating agent treatment of cells released from spheroids (Kobayashi et al., 1993) and suggest that the multicellular contact effect observed when cells are grown in CultiSphers persists for at least 4 h but is reversible by 24 h after multicellular growth is disrupted.

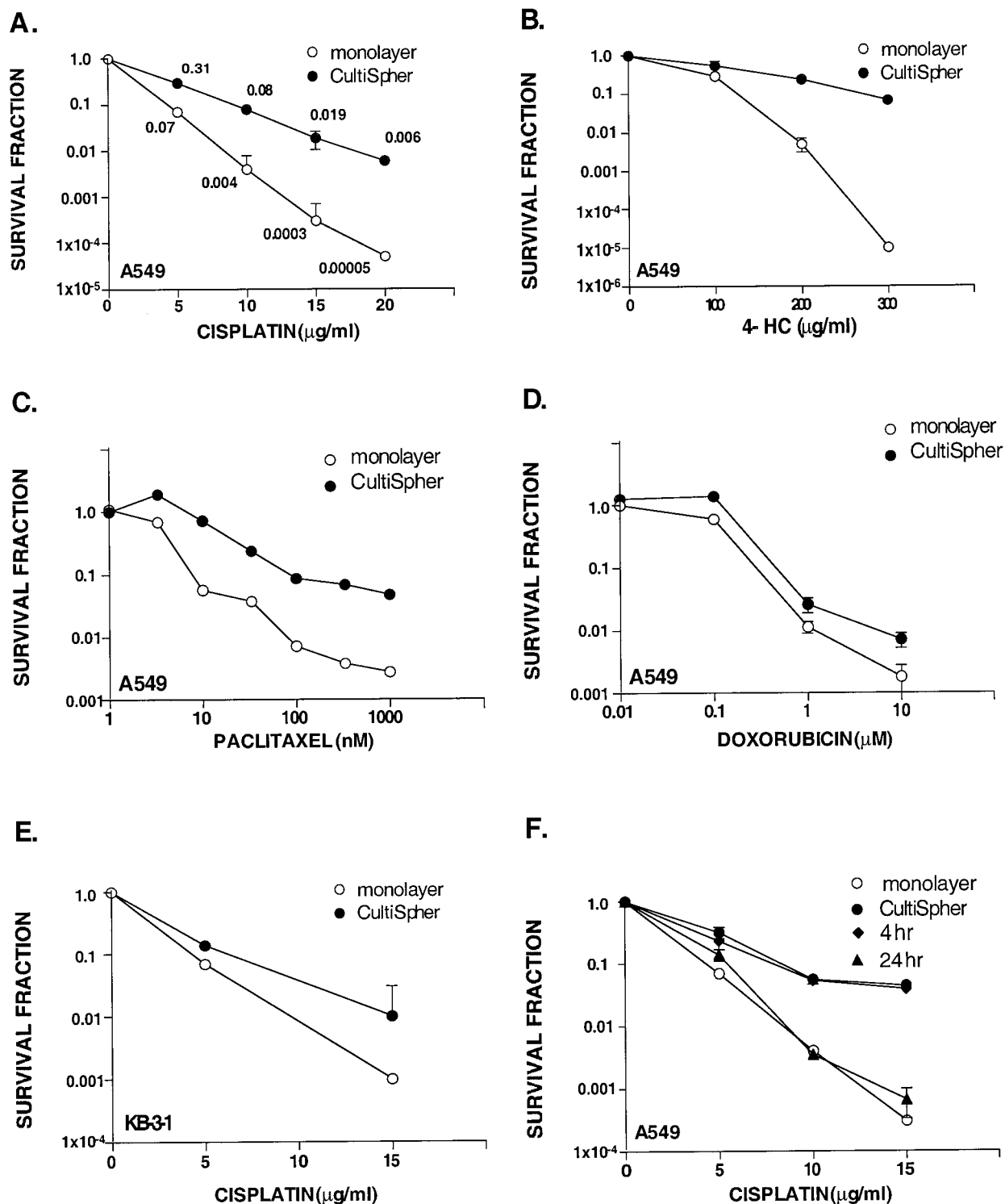
**Analysis of Doxorubicin Accumulation and Cisplatin DNA Adduct Formation in A549 Cells Grown as Monolayers and in CultiSphers.** The decreased sensitivity of cells in CultiSphers might be explained by decreased drug accumulation or by lower drug concentration in a subset of cells. This possibility was examined using two methods. First, flow cytometry was used to determine doxorubicin accumulation and distribution within the cell population of monolayer and CultiSpher A549 cultures. Doxorubicin was chosen because it has been shown that treatment of cells grown as multicellular spheroids results in large doxorubicin concentration gradients, and thus it represents a stringent test of drug accumulation and distribution (McGown et al.,



**Fig. 2.** Immunoblots of p27/kip1 in A549 monolayers and CultiSphers. A549 cells were grown as monolayers or in CultiSphers and treated with 10  $\mu$ g/ml cisplatin. Whole cell lysates were prepared from cells before treatment (M, mock) at 1 h and at 72 h after treatment. Duplicate aliquots of each lysate were probed with p27 antibody. Times of exposure of immunoblots to film were 5 min and 30 min for monolayers and CultiSphers, respectively. Clonogenic survival of cells used to make cell lysates is shown in Fig. 3A.

1983; Sakata et al., 1994). Cells were incubated with 5  $\mu\text{g}/\text{ml}$  doxorubicin for 1 h at 37°C, released by trypsin or collagenase treatment, and washed free of drug using ice-cold growth medium to prevent doxorubicin diffusion. Doxorubi-

cin epifluorescence was measured in duplicate samples by flow cytometry (McGown et al., 1983; Luk and Tannock, 1989). The flow cytometry data (Fig. 4) shows that when normalized to autofluorescence the shift in mean fluo-



**Fig. 3.** Clonogenic survival assay results for A549 and KB-3-1 cells grown as monolayers or in Cultispheres. Cells were treated for 1 h with cisplatin, 4-HC, and doxorubicin or for 24 h with paclitaxel. Data are represented as mean  $\pm$  S.D. of three to six replicates from at least two separate experiments. Values for A549 monolayers at 5 and 20  $\mu\text{g}/\text{ml}$  and A549 Cultispheres at 20  $\mu\text{g}/\text{ml}$  cisplatin represent triplicates from a single trial. Error bars are concealed within data point size for some values. A, A549 cells treated with cisplatin. B, A549 cells treated with 4-HC. C, A549 cells treated with paclitaxel. D, A549 cells treated with doxorubicin. E, KB-3-1 cells treated with cisplatin. F, Effect of plating cells from Cultispheres onto gelatin-coated monolayers for 4 or 24 h before cisplatin treatment. Cells were treated as Cultispheres or plated for 4 or 24 h as monolayers before treatment with the indicated doses of cisplatin. Data are representative of two separate experiments.

cence following doxorubicin treatment was about 2-fold greater for cells in CultiSpheres compared with cells in monolayer. These data suggest that cells in CultiSpheres accumulated more, not less, doxorubicin than cells in monolayers in this experiment. In repeated experiments, cells in CultiSpheres never had lower mean fluorescence than cells in monolayers, indicating that cells in CultiSpheres and monolayers had at least equal doxorubicin uptake. Furthermore, the shapes of the histograms for the accumulation of doxorubicin were very similar (Fig. 4), suggesting that the distribution of doxorubicin, on a per cell basis, was similar in cells treated in monolayers and in CultiSpheres.

Second, to confirm that Pt-DNA adduct formation was comparable in monolayers and CultiSpheres, atomic absorption spectroscopy was performed using isolated DNA. Pt-DNA adduct formation following 1 h of treatment with 10  $\mu\text{g}/\text{ml}$  cisplatin in A549 cells grown as monolayers and in CultiSpheres is shown in Table 1. Essentially equivalent amounts of Pt-DNA adduct formation were observed. However, the ability of atomic absorption spectroscopy to accurately measure low levels of Pt-DNA adducts is limited and true equivalency at this adduct level could not be concluded. Therefore, A549 cells grown as monolayers and in CultiSpheres were treated with 10 or 40  $\mu\text{g}/\text{ml}$  cisplatin for 4 h to increase total Pt-DNA adduct formation and assure measurement accuracy (Table 1). In this second experiment, as in the

first, A549 cells in monolayers and CultiSpheres accumulated essentially equivalent amounts of Pt-DNA adducts per microgram DNA.

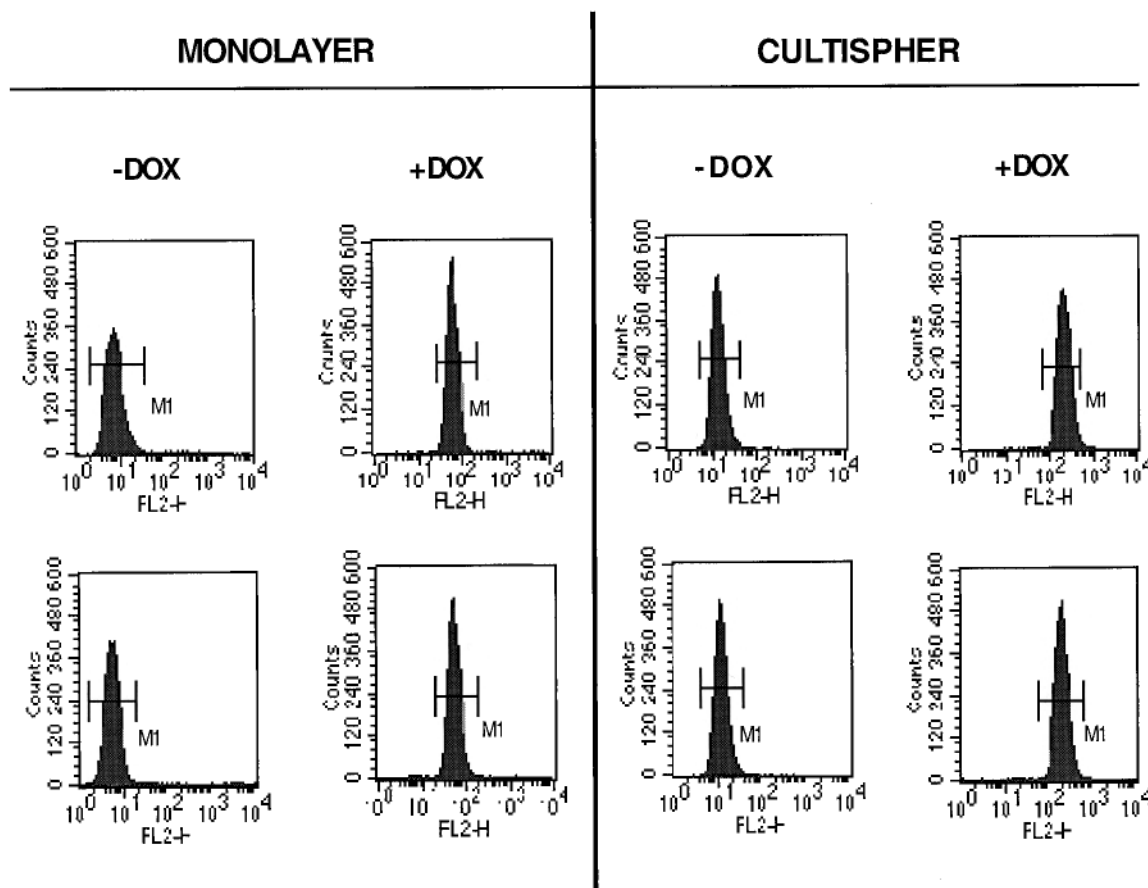
**Role of p53 in Response to Cisplatin Treatment in Cells Grown in CultiSpheres and Monolayers.** The decreased sensitivity of response to DNA damage by cisplatin of cells grown in CultiSpheres (Fig. 3) suggested that levels of DNA damage recognition and response proteins, such as p53, might be different in cells in the two growth conditions. To examine this possibility, cell lines that differed in p53 expression were used. Derivatives of the parental A549 human lung tumor cell line, A549/LXSN, and A549/16E6, as well as the p53 null line NCI-H1299, were grown as monolayers and

TABLE 1

Cisplatin adduct levels in A549 monolayers and CultiSpheres

Pt-DNA adduct formation and platinum accumulation in A549 cells monolayers and CultiSpheres using atomic absorption spectroscopy (AAS). Mean value and standard error calculated from two separate trials.

Sample	Cisplatin $\mu\text{g}/\text{ml}$	Time <i>hr</i>	pg Pt/100 $\mu\text{g}$ DNA
Monolayer	10	1	$5.4 \pm 2.9$
CultiSphere	10	1	$5.3 \pm 1.5$
Monolayer	10	4	$24.0 \pm 2.1$
CultiSphere	10	4	$27.2 \pm 0.3$
Monolayer	40	4	$120.3 \pm 4.2$
CultiSphere	40	4	$144.4 \pm 26.2$



**Fig. 4.** Doxorubicin epifluorescence assayed by flow cytometry. A549 cells grown as monolayers or in CultiSpheres were treated without (–Dox) or with 5  $\mu\text{g}/\text{ml}$  doxorubicin (+Dox) for 1 h at 37°C. Following release from monolayers and CultiSpheres, cellular doxorubicin accumulation was assayed. Cell volume (forward and side scatter) was used to determine population of cells in which fluorescence was measured with a single gate applied to all samples. Autofluorescence (–Dox) or doxorubicin fluorescence (+Dox) was determined in duplicate samples (rows 1 and 2) from a single experiment. M1 indicates statistical marker for determination of mean fluorescence.

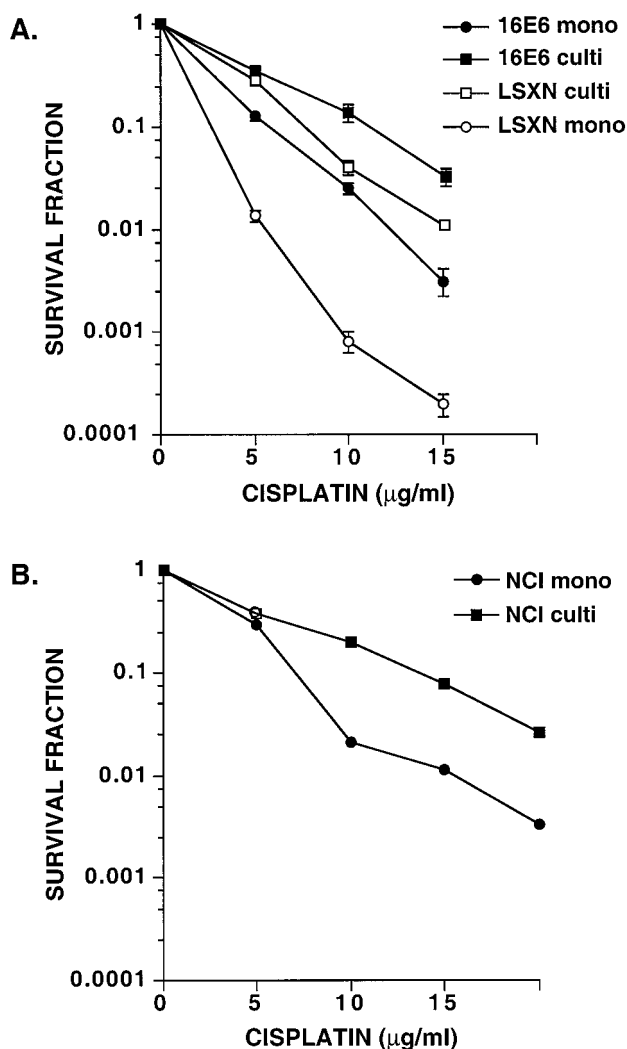
CultiSphers and then treated with cisplatin. A549/LXSN cells, containing an empty retroviral vector, have similar, low basal levels of p53 expression when grown both as monolayers and in CultiSphers (data not shown). A549/16E6 cells contain a retroviral vector expressing the human papilloma virus strain 16 E6 protein, which abrogates wild-type p53 function by facilitating its degradation via the ubiquitin pathway (Scheffner et al., 1990; Butz et al., 1995; Rolfe et al., 1995). The absence of p53 protein in the A549/16E6 cell line was confirmed by Western blot analysis (data not shown) before the clonogenic experiments described below.

The cisplatin sensitivity of A549/LXSN and A549/16E6 cells was examined following growth in monolayers and CultiSphers. A549/LXSN cells had identical sensitivity to cisplatin as the A549 parental line (data not shown). The results shown in Fig. 5A indicate that A549/16E6 cells were less sensitive to cisplatin, both as monolayers and in CultiSphers, than A549/LXSN or A549 parental cells (Fig. 3A) grown in identical fashions. To further verify that decreased

sensitivity to cisplatin of cells in CultiSphers was independent of p53 status, the sensitivity of p53 null human lung tumor cell line NCI-H1299 was examined. As shown in Fig. 5B, NCI-H1299 cells grown in CultiSphers were less sensitive to cisplatin than the same cells grown as monolayers. Statistical analysis using Student's *t* test at 15  $\mu\text{g/ml}$  cisplatin showed significantly different survival fractions for cells grown as monolayers versus in CultiSphers ( $P < .0001$  for A549/LXSN and A549/16E6 cells in Fig. 5A and for NCI-H1299 cells in Fig. 5B).

**Cell Cycle Response to Drug-Induced Damage in Cells Grown in CultiSphers and Monolayers.** Cisplatin treatment induces  $G_2/M$  cell cycle arrest (Sorenson and Eastman, 1988; Rolfe et al., 1995) in many, but not all, cell types containing wild-type p53 (Sorenson et al., 1990; Fan et al., 1994, 1995; Fujiwara et al., 1994; Hawkins et al., 1996; Kawasaki et al., 1996). To determine whether growth conditions altered this response, asynchronous cultures of A549/LXSN and KB-3-1 cells grown as monolayers and in CultiSphers were treated with 10  $\mu\text{g/ml}$  cisplatin for 1 h, washed, allowed to recover in situ, and then serially harvested for flow cytometry analysis for up to 120 h after treatment. Aggregate data from 10 separate cell cycle experiments indicate that S phase fractions for monolayers ( $31.77 \pm 18.11\%$ ) and CultiSphers ( $22.89 \pm 15.27\%$ ) are not statistically significant from each other using Student's *t* test ( $P = .71$ ). This suggests that cell cycle profiles at the time of drug treatment for monolayers and CultiSphers are not significantly different. As shown in Fig. 6, by 48 h after treatment both A549/LXSN monolayers (Fig. 6A) and KB-3-1 monolayers (Fig. 6B) exhibit a  $G_2$  arrest that continues until at least 96 to 120 h post-treatment. However, a prolonged  $G_2/M$  arrest of A549 cells grown as monolayers following cisplatin treatment was not observed in every experiment, and did not correlate well with cell survival (data not shown). When either A549/LXSN or KB-3-1 cells were grown in CultiSphers and treated with cisplatin, a portion of the cells appear to remain in  $G_1$ . Between 72 to 120 h after cisplatin treatment the proportion of cells in  $G_1$  increased relative to 48 h, suggesting that some cells progress past  $G_2/M$  and arrest in  $G_1$ . In addition, no sub- $G_1$  DNA content, an indicator of apoptosis, was observed following treatment of A549 or KB-3-1 cells with cisplatin. It should be further noted that neither A549 nor KB-3-1 cells respond to cisplatin, at the doses used in these studies, by undergoing classical apoptosis (no sub- $G_1$  DNA content, minimal poly(ADP)ribose polymerase cleavage, minimal DNA laddering, minimal annexin V binding (M. Ihnat and M.M. Cornwell unpublished observations)).

The cell cycle response of A549 cells to paclitaxel treatment is shown in Fig. 6C. Cells were treated with 33 nM paclitaxel for 24 h, conditions that resulted in cell survival similar to 10  $\mu\text{g/ml}$  cisplatin. Following 24 h of paclitaxel treatment, A549 monolayers exhibited a pronounced  $G_2/M$  arrest. By 48 h following treatment, cells in monolayers displayed a considerable fraction of cells with sub- $G_1$  DNA, an indicator of apoptosis. The sub- $G_1$  content of cells in monolayers increased at 72 and 96 h postpaclitaxel treatment. Similar to cells in monolayer, A549 cells grown in CultiSphers accumulated in  $G_2/M$  following paclitaxel treatment for 24 h. In contrast to cells in monolayers, the sub- $G_1$  content of cells in CultiSphers did not increase substantially until 72 and 96 h postpaclitaxel treatment, and the fraction of cells with



**Fig. 5.** Clonogenic survival assay results for A549/LXSN, A549/16E6, and NCI-H1299 cells. Cells grown as monolayers or in CultiSphers were treated with cisplatin. A, A549/LXSN and A549/16E6 cells were treated for 1 h with cisplatin. Data represent at least two independent experiments. Error bars omitted for clarity. B, NCI-H1299 cells were treated for 1 h with cisplatin. Data represent mean  $\pm$  S.D. of three to six individual samples from a single experiment.



sub-G<sub>1</sub> DNA content was lower than in paclitaxel-treated monolayers.

## Discussion

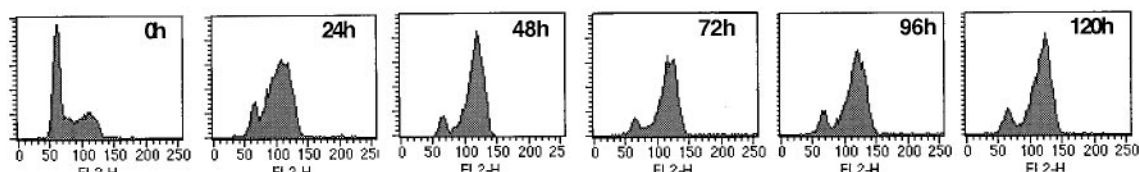
These studies suggest that the growth of human cancer cell lines in macroporous, microcarrier beads can alter their response to cancer chemotherapeutic agents compared with growth as dispersed monolayer culture. We have previously demonstrated that cells in CultiSpheres exhibit substantial three-dimensional contact without central necrosis and lack an edge-to-center gradient of proliferation or evidence of a hypoxic fraction (Rasey et al., 1996).

In the present work, we demonstrate that cells in CultiSpheres can be grown with similar cell cycle profiles as monolayers under conditions in which similarly low levels of the cell cycle inhibitor p27/kip1, which causes cell cycle arrest,

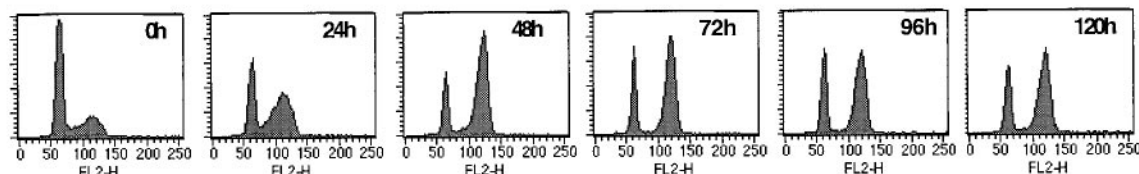
are expressed. In our studies, cells grown as monolayers lacking p53 (A549/16E6, NCI-H1299) are less sensitive to cisplatin treatment when compared with similar cells with an intact p53 (A549, A549/LXSN). Cells lacking p53 function have been shown to have increased (Hawkins et al., 1996) as well as decreased (Piovesan et al., 1998) sensitivity to cisplatin, thus the role of p53 in drug resistance has yet to be fully understood. Furthermore, three human epithelial-derived cancer cell lines with differing p53 status (wild-type, E6-expressing, and null) all demonstrate significantly altered drug responses in CultiSphere compared with monolayer culture. These data suggest that the decreased cisplatin sensitivity of cells in CultiSpheres is p53-independent. In addition, altered responses to drug treatment are observed under culture conditions that produce similar growth and drug distribution.

### A. Cisplatin

A549/LXSN  
monolayers

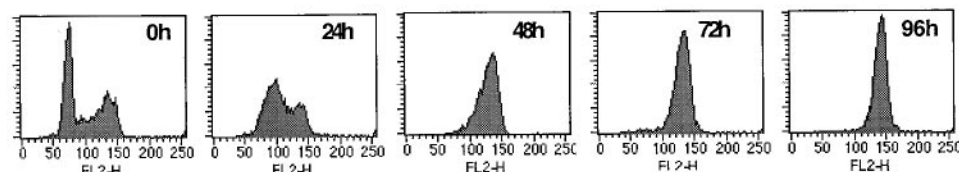


A549/LXSN  
CultiSpheres

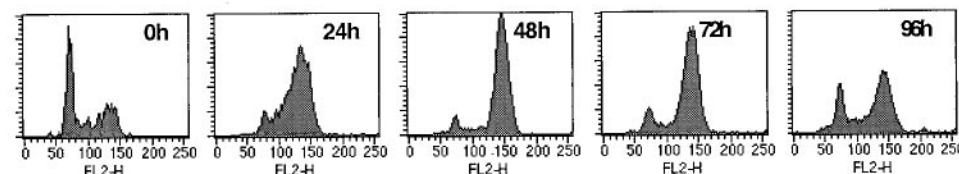


### B. Cisplatin

KB-3-1  
monolayers

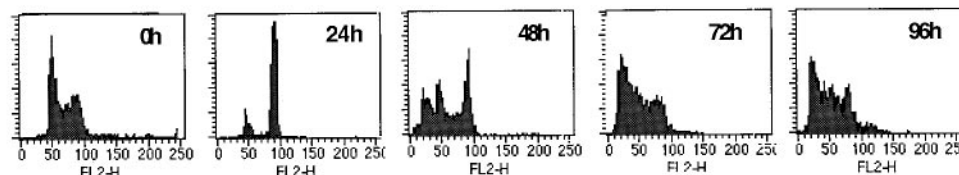


KB-3-1  
CultiSpheres

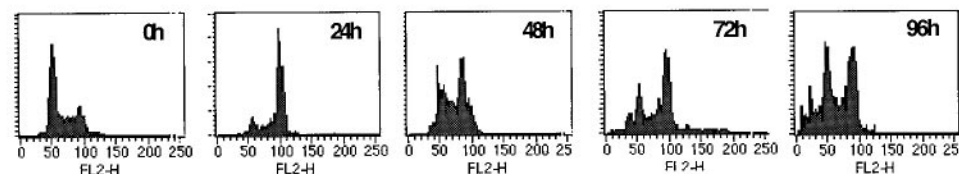


### C. Taxol

A549  
monolayers



A549  
CultiSpheres



**Fig. 6.** Fluorescence histograms for A549/LXSN cells (A), KB-3-1 cells (B), and A549 cells (C) grown as monolayers or in CultiSpheres, treated for 1 h with 10  $\mu$ g/ml cisplatin (A and B) or paclitaxel (C). At indicated times following treatment cells were ethanol fixed, incubated with propidium iodide, and analyzed by flow cytometry. Cell cycle profiles for A549 parental cells after treatment with cisplatin were identical with A549/LXSN (data not shown).



Determining equivalent drug uptake in cells grown in monolayers and CultiSphers is crucial, because it is known that multicellular growth can alter the total accumulation of drug per cell by causing drug gradients (Durand, 1990; Erlichman and Wu, 1992; Johnson et al., 1994; Ramachandran et al., 1995). We use two approaches to document that drug distribution is comparable in cells grown in monolayers and CultiSphers. First, we demonstrate using doxorubicin epifluorescence that cells in monolayers and CultiSphers have similar drug distribution histograms. This indicates that, unlike cells grown in spheroids (Sutherland et al., 1979; Sakata et al., 1994), all the cells in the CultiSphers accumulate doxorubicin to a similar degree and to at least an amount comparable with cells grown as monolayers. Second, we demonstrate that cells in monolayers and CultiSphers form similar amounts of Pt-DNA adducts as monolayer cultures. Thus, differences in Pt-adduct formation are not likely to account for the observed differences in drug sensitivity. These data also suggest that cellular mechanisms that alter postuptake cisplatin metabolism (such as altered glutathione levels and multidrug resistance protein pump activity) do not account for the decreased drug sensitivity of cells in CultiSphers.

We report that cells grown in CultiSphers at the time of treatment with chemotherapeutic agents express very little p27/kip1, a mediator of cell cycle arrest (Polyak et al., 1994). This contrasts with studies using multicellular spheroids as a model, in which increased p27 expression has been correlated with decreased drug sensitivity (St. Croix et al., 1996a). Thus we believe that the decreased drug sensitivity observed with cells grown in CultiSphers is not due to an arrested population of cells as a result of growth in three dimensions, as is the case with cells grown as multicellular spheroids.

We also did not find an obvious correlation between cell cycle response and clonogenic survival in monolayer versus CultiSpher cultures after cisplatin treatment. Although the majority of A549 cells and all KB-3-1 monolayer cells enter a prolonged  $G_{2/M}$  arrest by 48 h after 10  $\mu\text{g/ml}$  cisplatin treatment, A549 cells in monolayers do not always remain in  $G_{2/M}$  at longer time points following treatment. However, when A549 or KB-3-1 cells are grown in CultiSphers before cisplatin treatment, in every experiment a portion of both cells fail to exit  $G_1$ , while some cells progress past  $G_{2/M}$ . Thus, with asynchronous A549 cultures, a clear correlation cannot be made between decreased sensitivity of cells in CultiSphers and cell cycle response following cisplatin treatment. Experiments using synchronous cell populations to further investigate the altered cell cycle response of cells in CultiSphers are in progress.

Through immunoblot analysis of proteins associated with apoptosis and markers of apoptotic cell death, we found that 10  $\mu\text{g/ml}$  cisplatin treatment for 1 h does not kill A549 cells through classical apoptotic pathways (data not shown). It was further observed that many A549 cells remain attached to cell culture dishes following 10  $\mu\text{g/ml}$  cisplatin treatment but do not proliferate to form colonies (data not shown). To confirm that cisplatin-treated cells from both growth modes were viable post-treatment, equal numbers of cells from A549 monolayers and CultiSphers were plated after a 1 h cisplatin treatment and then assayed for viability using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell dehydrogenase assay

or by trypan blue staining. At 48 and 72 h post-treatment, no difference in MTS dehydrogenase activity or trypan blue dye exclusion was observed following treatment of cells in monolayers and CultiSphers (data not shown). Thus, we speculate that growth of cells in CultiSphers increases the ability of cisplatin-treated cells to proliferate rather than induces the cells to die. If correct, the effect of cisplatin on proliferation rather than viability may account for the observations made by Frankel et al. (1997) in which no difference in cisplatin sensitivity between cells grown as monolayers and multicellular spheroids was observed. In that study the sensitivity of cisplatin-treated cells was measured by MTS and [ $^3\text{H}$ ]thymidine incorporation, not by clonogenic cell survival.

The sensitivities to chemotherapeutic drugs that form DNA adducts (cisplatin and 4-HC), intercalate into DNA, and alter cellular membranes (doxorubicin), as well as to an agent that does not directly adduct DNA (paclitaxel), are all decreased by multicellular growth in CultiSphers. Thus, it is possible that the decreased drug sensitivities of cells grown in CultiSphers relative to monolayers are independent of drug mechanism of action. The decreased sensitivity to paclitaxel of cells grown in CultiSphers compared with monolayers suggests that this phenotype is not solely related to augmented DNA repair of drug-induced lesions. In addition, the observation that paclitaxel induces sub- $G_1$  content (an indicator of apoptosis) in A549 monolayers and CultiSphers, whereas cisplatin does not, also suggests that decreased drug sensitivity of cells grown in CultiSphers is not due to a defect in the apoptotic pathway, but rather to a qualitative change in apoptotic response.

Although it is probable that there are distinct drug-specific pathways within the cellular damage response signaling network, we speculate that a set of common events occurring before drug treatment, dependent on multicellular growth, alters the cellular damage response through specific signaling pathways. Data suggesting that the CultiSpher resistance phenotype is reversed or lost after replating cells for 24 h, but not for 4 h, before drug treatment supports our speculation that growth conditions before drug exposure determine the clonogenic survival of cells. Our current working hypothesis is that multicellular growth of cells in CultiSphers activates survival factor signaling networks (Evan et al., 1995), which in turn inhibit drug-induced cell death signals and promote cell proliferation.

Our aim was to develop a model in vitro culture system that is similar to three-dimensional cellular contact in vivo while retaining the advantages of homogeneous, comparable, easily manipulated cell populations for molecular analysis. We demonstrated that extensive cell-cell contact exists in CultiSphers in a cell population that is similar to dispersed monolayers in several important parameters, especially comparable cell cycle transit time and drug uptake. We are currently investigating whether growth in CultiSphers alters drug response by facilitating cell-cell interactions, and thereby the formation of gap junctions, extracellular matrix deposition, and ligand-receptor interaction, etc., or whether changes in drug response are independent of cell-cell contact. In summary, this work demonstrates that the growth of cells in CultiSphers provides a relatively simple and useful model system with advantages over monolayer culture to advance the molecular characterization of cell contact-induced drug resistance.

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