Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy in vitro

Shane K. Green, Giulio Francia, Ciro Isidoro, and Robert S. Kerbel

1Molecular and Cellular Biology Research, Sunnybrook and Women’s College Health Sciences Center and Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada and 2Laboratory of Molecular Pathology, Department of Medical Sciences, “Amedeo Avogadro” University, Novara, Italy

Abstract
Multicellular resistance, a subtype of therapeutic resistance manifested in cancer cells grown as three-dimensional multicellular masses, such as spheroids in vitro and solid tumors in vivo, occurs with respect to a variety of anticancer treatment strategies including chemotherapy, ionizing radiation, and even host-mediated antibody-dependent cellular cytotoxicity. Previous studies from our laboratory have shown that multicellular resistance to chemotherapy demonstrated by aggregates of EMT-6 murine mammary carcinoma cells can be overcome by using hyaluronidase to disrupt intercellular adhesive interactions and associated patterns of protein expression. In this proof of principle study, we explored the concept of antiadhesive chemosensitization in the context of human cancer cells by using a monoclonal antibody to disrupt E-cadherin-mediated cell-cell interactions in multicellular spheroids of HT29 human colorectal adenocarcinoma. In so doing, we found that disruption of E-cadherin-mediated adhesion sensitizes multicellular spheroids of HT29 in vitro to treatment with 5-fluorouracil, paclitaxel, vinblastine, and etoposide but not cisplatin. Furthermore, we have found that antibody-mediated blockade of E-cadherin function leads to decreased expression and activity of protein kinase C α and β1, both of which have previously been implicated in chemoresistance exhibited by HT29 cells; however, we have found that the chemosensitization effects of the anti-E-cadherin antibody are independent of its influence on protein kinase C β1. [Mol Cancer Ther. 2004;3(2):149–159]

Introduction
The term “multicellular resistance” (1, 2) was coined to describe a clinically relevant subtype of therapeutic resistance exclusively manifested in cancer cells grown as three-dimensional multicellular masses, such as spheroids in vitro and solid tumors in vivo. This particular form of resistance occurs with respect to a variety of anticancer approaches including chemotherapy (3–5), ionizing radiation (5, 6), and erbB-dependent host defense mechanisms (7). Because multicellular resistance can only be recapitulated experimentally when cells are studied under conditions in which they are allowed to adhere to one another (i.e., in a three-dimensional context), our investigations into this phenomenon predominantly involve the use of cancer cells grown as multicellular spheroids in vitro. Decades of research have firmly established that cancer cells grown in vitro as three-dimensional spheroids more accurately mimic the drug sensitivity/resistance behavior of those found within solid tumors in vivo in a preclinical or clinical setting (8) compared with those cultured under conventional two-dimensional monolayer conditions.

Previous studies from our laboratory have shown that multicellular resistance to chemotherapy manifested within spheroids of EMT-6 murine mammary carcinoma cells can be overcome by using bovine testicular hyaluronidase, an enzyme capable of disrupting intercellular interactions and possibly related signaling pathways (5). Subsequent studies showed that disruption of EMT-6 spheroids via hyaluronidase caused a marked decrease in expression of the cyclin-dependent kinase inhibitor p27kip1, which acted as a de facto mediator of multicellular resistance in the aforementioned system through its capacity to induce G1 arrest in cells in spheroid culture (9). Similarly, G1 arrest mediated by p27kip1 has also been implicated in cell adhesion-mediated drug resistance in leukemia (10). It has also been shown that forced overexpression of p27kip1 via transfection can confer increased resistance to several chemotherapeutic agents on HT29 human colorectal adenocarcinoma cells grown as subconfluent monolayers (11). When normal HT29 cells are grown in three-dimensional spheroid culture, they undergo E-cadherin-dependent growth suppression mediated by up-regulation of endogenous p27kip1 (12).

As such, in the present study, we sought to determine whether an antiadhesive agent (i.e., a monoclonal antibody directed against the potent homophilic cell adhesion molecule E-cadherin called SHE78-7; 13) could be used to disrupt cell-cell interactions and related signaling pathways in multicellular spheroids of HT29 in vitro, thereby sensitizing the cells to chemotherapeutic drug treatment. It should be noted, however, that as E-cadherin is down-regulated or absent in a large percentage of advanced...
Materials and Methods

Cell Lines and Culture Conditions

HT29 cells were obtained from the American Type Culture Collection (Rockville, MD). HT29-PKC cells were generated as described previously (16). HT29-V* cells were generated by transfection of the empty mammalian expression vector pCEP4 (Invitrogen, Carlsbad, CA; kind gift of S. Chakrabarty, University of Texas MD Anderson Cancer Center, Houston, TX) into HT29 via Lipofect-AMINE 2000 (Invitrogen) with subsequent clonal selection in 500 μg/ml Hygromycin B (Roche Diagnostics Co., Indianapolis, IN). All cells were routinely maintained as monolayer cultures in complete media (RPMI 1640; Life Technologies, Inc./Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) and 100 units/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Inc./Invitrogen) in a humidified atmosphere at 37°C with 5% CO2. Spheroids of HT29-V1/HT29-PKC cells were formed in three-dimensional culture using the liquid overlay technique as described previously (3). Briefly, cells were plated in 24-well dishes coated with 1% SeaPlaque agarose (Bio Whittaker Molecular Applications, Rockland, ME) in complete media with (5 × 10^6 cells/well in 300 μl) or without (1 × 10^6 cells/well in 500 μl) 1 μg/ml SHE78-7 for 48 h prior to further manipulation (e.g., drug addition for chemosensitivity assays). (Note: After 48 h in culture, wells with and without SHE78-7 contained equal numbers of HT29 cells.) In all cases, spheroids received an additional 500 μl fresh complete media after 48 h in culture. All cell culture plates were obtained from Nunc Technologies (Burlington, ON, Canada).

Antibodies

SHE78-7 monoclonal (murine IgG2a) antibody against E-cadherin was obtained from Takara Shuzo Biomedicals Co., Ltd. (Shiga, Japan). Anti-PKC α (H-7), anti-PKC β1 (C-16), and anti-PKC ζ (C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PKC α/βII antibodies (which also cross-react with phospho-PKC β1) were purchased from Cell Signaling Technology (Mississauga, ON, Canada). Anti-pan-ERK and anti-p27kip1 antibodies were obtained from Transduction Laboratories (Mississauga, ON, Canada). Nonspecific murine myeloma IgG2a (Zymed, San Francisco, CA) was dialyzed in serum-free RPMI 1640 (Life Technologies, Inc./Invitrogen) using Slide-A-Lyzer dialysis cassettes (Sorvall, Raleigh, NC) to remove sodium azide before use.

Chemotherapeutics

Paclitaxel (Taxol; Bristol Myers Squibb, Montreal, QC, Canada), etoposide (Vepesid; Bristol Myers Squibb), 5-fluorouracil (5-FU; Adrucil; Pharmacia & Upjohn, Mississauga, ON, Canada), and vinblastine sulfate (Faulding Canada, Montreal, QC, Canada) were obtained from the pharmacy at Sunnybrook and Women’s College Health Sciences Center (Toronto, ON, Canada). [3H]-labeled etoposide (MT-786), vinblastine (MT-657), 5-FU (MT-686), and Taxol (MT-582) were obtained from Moravek Biochemicals (Brea, CA).

Cmosensitivity Assays

Cells were plated in three-dimensional culture for 48 h in 500 μl complete media as described above, after which chemotherapeutics (Taxol, vinblastine, etoposide, or 5-FU) were added at twice the desired final concentration in 500 μl complete media for 24 h. Cells were then harvested, rinsed in PBS to remove residual drug, placed in 0.1% trypsin-EDTA (Life Technologies, Inc./Invitrogen) in PBS for 10–15 min at 37°C (to disaggregate intact spheroids), counted in duplicate, and replated as follows: Recovery experiments (Fig. 1): 10,000 cells/well (8 wells/group) were plated in 100 μl complete media in 96-well plates [flat-bottomed (A) or round-bottomed with poly(2-hydroxyethylmethacrylate) coating (Aldrich Chemical Co., Milwaukee, WI; B and C) as described previously; 7] with (C) or without (A and B) 1 μg/ml SHE78-7 and allowed to recover for 72 h prior to assaying. Two-dimensional cultures (A) were assayed via addition of methanethiosulfonate (MTS) assay reagents as per manufacturer’s instructions (Promega, Madison, WI) for 1–2 h at 37°C followed by quantification of the colorimetric change indicative of metabolic activity with a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Three-dimensional cultures (B and C) were pulsed for 5 h at 37°C with 2 μCi/well methyl-[3H]thymidine (Amersham Life Science, Buckinghamshire, United Kingdom), after which cells were harvested (Packard BioSciences Filtermate harvester; PerkinElmer Life Sciences, Woodbridge, ON, Canada) onto multiscreen plates (Millipore, Bedford, MA) and [3H]thymidine incorporation was measured using a Packard TopCount-NXT microplate scintillation counter (PerkinElmer Life Sciences). Colony formation assays (Figs. 2 and 6, C and D): 200, 500, 1000, or 5000 cells/well in 4 ml complete media (six-well plates, 3 wells/group) were incubated in a humidified atmosphere at 37°C with 5% CO2 for 10 days to allow colony formation. Colonies were then fixed at room temperature for 5 min in 3:1 methanol/acetic acid and stained for 5 min with crystal violet solution to allow colony visualization. Colony counts were conducted manually under a mounted illuminated magnifying glass.

Flow Cytometry

Immediately following drug treatment in three-dimensional culture as described above, cells were harvested...
(and kept on ice from this point on), rinsed in PBS, placed in 0.1% trypsin-EDTA in PBS for 10–15 min at 37°C, resuspended in complete media to inactivate trypsin, rinsed twice in PBS, and fixed in cold 70% ethanol for 1 h. Fixed cells were then rinsed twice in PBS and stained with 50 μg/ml propidium iodide (Sigma Chemical Co., Mississauga, ON, Canada) in PBS for 1 h, protected from light. DNA content was then visualized and graphed using a FACS Calibur analyzer (BD Biosciences, San Jose, CA).

**[3H]-Labeled Drug Retention Experiments**

HT29 cells were plated in three-dimensional culture as described above. [3H]-labeled paclitaxel, vinblastine, etoposide, or 5-FU was then added at twice the desired final concentration in 500 μl complete media for 24 h, after which cells were harvested, rinsed in PBS, placed in 0.1% trypsin-EDTA in PBS for 10–15 min at 37°C, resuspended in complete media to inactivate trypsin, and transferred onto ~1 × 2.5-cm strips of blotting paper (VWR, West Chester, PA). Paper strips were dried overnight and placed into scintillation vials with 5 ml scintillation fluid to allow quantification of [3H] accumulation within cells by a LS6000IC [3H] scintillation counter (Beckman Instruments (Canada) Inc., Mississauga, ON, Canada). [3H] counts were normalized to the number of cells (i.e., counts per 2 × 10^4 cells) determined from an identical experiment running concurrently using cold (i.e., not [3H]-labeled) drug.

**Western Blotting**

Cells were collected, rinsed with PBS, and placed in lysis buffer [20 mM Tris (pH 7.5), 137 mM NaCl, 100 mM NaF, 10% glycerol, 1% NP40, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin] for 30 min on ice. Total protein in lysates was quantified using Bradford reagent (Bio-Rad Laboratories), and 50 μg (Fig. 5A) or 20 (Figs. 5B and 6, A and B) of denatured protein were resolved on a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred onto Immobilon-P membranes (Millipore), which were then blocked in 10% skim milk in Tris-buffered saline + Tween 20 (TBS-T) buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20] for 1 h at room temperature and blotted in primary antibody for 1 h at room temperature. Membranes were rinsed in TBS-T, exposed to horseradish peroxidase-conjugated secondary antibody (Promega) for 1 h at room temperature, and rinsed again in TBS-T. Bound antibody was visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ). (Note: Blots shown in Fig. 4A are all from a single membrane that was stripped and reprobed with each indicated primary antibody.)

**Statistics**

Graphing and statistical analyses were performed using the GraphPad Prism software package v3.0 (GraphPad Software Inc., San Diego, CA). Error bars in all figures represent SE. Significance was set at \( P < 0.05 \).

**Results**

**Blocking Antibodies to E-Cadherin Chemosensitize HT29 Cells Grown as Multicellular Spheroids in Vitro**

We have shown previously that exposure to the anti-E-cadherin monoclonal antibody SHE78-7 (13) causes complete disruption of E-cadherin-mediated cell-cell adhesion.
in several human cancer cell lines grown under three-dimensional conditions in vitro, including colon (HT29 and DLD-1), breast (BT20 and MCF-7), and lung (L23) cancer lines (12). Moreover, we have demonstrated that fully preformed spheroids of HT29 can be completely disaggregated (i.e., into a single cell suspension) via 24-h exposure to 1 μg/ml SHE78-7 (7). In the present study, we sought to determine whether this antiadhesive activity of SHE78-7 could be used to overcome, at least in part, multicellular resistance in HT29 spheroids, thereby sensitizing the disaggregated cells to treatment with a variety of chemotherapeutic agents.

We cultured HT29 cells in three-dimensional conditions in vitro (in which they normally form compact, viable spheroids) with or without the addition of 2 μg/ml SHE78-7 to culture media to prevent spheroid formation. Cells were left untreated with drug for 48 h to allow full compaction of intact spheroids, after which fresh complete media was added to all wells, alone or containing Taxol (0.05, 0.5, and 5 μM), etoposide (10 or 50 μM), or cisplatin (5 μM). Cells were then collected and incubated with trypsin (to disrupt intact spheroids), and equal numbers of cells from each group were then replated to assess cell survival post-treatment as two-dimensional monolayers (Fig. 1A) or in more clinically relevant three-dimensional conditions, either as intact spheroids (Fig. 1B) or as single cell suspensions (i.e., in media containing 2 μg/ml SHE78-7; Fig. 1C). Seventy-two hours after replating, cells were tested for relative metabolic activity, via MTS assay (Fig. 1A), or for differences in cell proliferation, as measured by [3H]thymidine uptake (Fig. 1, B and C).

In so doing, we found that blocking E-cadherin-mediated adhesion between HT29 cells grown in three-dimensional culture significantly increased their sensitivity to treatment with a range of dosages of the chemotherapeutic agents Taxol and etoposide but not cisplatin in comparison with cells treated as intact multicellular spheroids (Fig. 1). The addition of 2 μg/ml nonspecific murine IgG2a (an isotype match to the SHE78-7 antibody) to cells when plated as spheroids had no effect on spheroid formation or the therapeutic response of the component cells (data not shown). Thus, we concluded that SHE78-7 specifically chemosensitized HT29 cells in three-dimensional culture to both Taxol and etoposide. Furthermore, with the sole exception of cells replated as spheroids following treatment with 5 μM Taxol (Fig. 1B), the chemosensitization effect conferred by SHE78-7 occurred irrespective of whether the cells were cultured post-treatment in a two- or three-dimensional setting. Additional experiments showed that, as compared with the parental cells growing in two-dimensional culture, spheroids of HT29 did not manifest multicellular resistance to cisplatin even at higher concentrations, ranging from 10 to 50 μM (data not shown).

Subsequently, we performed colony formation assays to compare the reproductive viability of HT29 cells from intact versus disrupted spheroids following treatment with chemotherapeutic drugs (Fig. 2). To gain additional insight into the breadth of the spectrum of drugs to which the observed chemosensitization could apply, we tested not only Taxol (a microtubule targeting drug; Fig. 2A) and etoposide (a topoisomerase II inhibitor; Fig. 2B) as above but also vinblastine (a microtubule destabilizing agent, with a different mode of activity from Taxol; Fig. 2C) and, perhaps most importantly, 5-FU (an inhibitor of thymidylate synthase; Fig. 2D), which is currently used in the clinical management of colorectal carcinoma.

As shown in Fig. 2, HT29 cells in intact spheroids retained substantially greater colony formation ability than SHE78-7-disaggregated cells following treatment with all four drugs. The greatest differences in colony formation
ability were observed with Taxol and etoposide treatment as well as treatment with a relatively low dose (0.1 μM) of vinblastine; however, highly reproducible differences, indicative of bona fide SHE78-7-mediated chemosensitization, were indeed seen when cells were treated with 1 μM vinblastine or with 5-FU at concentrations of at least 100 μM.

**Chemotherapeutic Treatment of HT29 Spheroids in the Presence of SHE78-7 Causes Enhanced Cell Cycle Perturbations**

We have previously demonstrated that E-cadherin-mediated adhesion between HT29 cells cultured for 48 h as three-dimensional multicellular spheroids causes the cells to up-regulate p27kip1 and undergo G1 arrest (12). Furthermore, we demonstrated that the use of SHE78-7 to block these adhesive interactions prevented the up-regulation of p27kip1 over 48 h, thereby stimulating cell proliferation, which theoretically should render the cells more sensitive to treatment with chemotherapeutics that preferentially target dividing cells. Figure 3A shows the levels of p27kip1 in HT29 cells cultured under the experimental conditions described herein; specifically, levels were determined in HT29 cells grown in vitro for up to 4 days (with fresh media added after 48 h) as monolayers, intact spheroids, or disaggregated cell suspensions (i.e., ± SHE78-7). As expected, p27kip1 levels steadily increased in HT29 cells in intact spheroids but not when spheroid formation was prevented with E-cadherin blocking antibodies (Fig. 3A).

To evaluate what effect SHE78-7 treatment and/or chemotherapy had on the overall cell cycle profiles of HT29, we analyzed the profiles of cells left idle or treated for 24 h with the aforementioned chemotherapeutics, at concentrations ranging over several orders of magnitude, as intact or disrupted spheroids (i.e., ± SHE78-7; Fig. 3). We found that with the exception of 1 and 10 μM doses of vinblastine, none of the drugs had any discernable effect on the profiles of cells within intact spheroids, as they remained almost entirely arrested in the G1 phase (Fig. 3, B–E; “−SHE78-7”). As anticipated from its prevention of p27kip1 up-regulation, cell-cell disruption via SHE78-7 in the absence of drug abrogated the growth arrest and caused recruitment of cells from G1 to S phase, as evidenced by the comparatively diminished G1 peaks (Fig. 3, B–E; “+SHE78-7”).

More noteworthy, however, was the fact that treatment with Taxol (Fig. 3B) or etoposide (Fig. 3C) in the presence of SHE78-7 caused a massive efflux from cells in G1 to the S and G2-M phases of the cell cycle; those treated with at least 0.05 μM Taxol were almost entirely arrested in G2-M. Similar effects were seen with vinblastine treatment (Fig. 3E): doses of vinblastine of at least 1 μM did have a notable effect on the profiles of cells in intact spheroids, giving rise to a large sub-G1 peak suggestive of apoptosis, although the viable cells nonetheless remained largely in G1; however, in disaggregated cells, there was a significant recruitment of cells into G2-M in response to the drug.

Although no G2-M arrest was seen in disaggregated cells treated with 5-FU, there were nonetheless significant differences between profiles of these cells and those of cells treated in spheroids. At all dosages, the G1 peak was static in spheroid cells whereas it shortened and broadened in disrupted cells treated with 5-FU, suggestive of progression of these cells into late G1 and/or S phase (Fig. 3D).

**Antiadhesion Mediated by SHE78-7 Causes Greater Intracellular Accumulation of Chemotherapeutics**

Numerous studies (5, 19–22) have demonstrated that intercellular contact-dependent reduction in sensitivity to chemotherapy-induced cell death is not exclusively the result of restricted penetration of therapeutic agents into multicellular cancerous masses. Nonetheless, other studies have shown that some chemotherapeutics, such as those...
with large molecular weights (e.g., 853.92 kDa for Taxol, 888.58 kDa for etoposide, and 909.07 kDa for vinblastine) and/or rapid intracellular interaction, face barriers with respect to penetration into cells in multicellular formations (23, 24) or even confluent monolayer cultures (25). As such, antiadhesion may help to overcome multicellular chemoresistance, at least in part, by eliminating such penetration barriers.

To investigate what effects, if any, SHE78-7-mediated abrogation of E-cadherin-mediated adhesion would have on drug accumulation within HT29 cells in three-dimensional culture, we performed a set of experiments similar to the chemosensitization experiments described above but using [3H]-labeled chemotherapeutics. Immediately following a 24-h exposure to [3H]-labeled Taxol, etoposide, vinblastine, or 5-FU in the presence or absence of SHE78-7, cells were collected and assayed for the quantity of [3H], and therefore the quantity of drug, they had accumulated (Fig. 4); quantities were normalized to cell counts, obtained from a parallel experiment using cold (i.e., [3H]-free) drug at the same concentration(s). In all cases, disruption of E-cadherin-mediated intercellular adhesion led to an increase in intracellular accumulation of drug, although the differences seen with etoposide and 0.5 μM Taxol were not statistically significant. Some results, however, were quite remarkable: for example, SHE78-7 treatment allowed a greater than 100% increase in drug accumulation in two treatment groups (i.e., 0.05 μM Taxol and 0.1 μM vinblastine).

SHE78-7-Mediated Inhibition of E-Cadherin Leads to Decreased Expression and Activation of PKC α and β1 but Not PKC ζ

Previous studies (reviewed in 26) have implicated several isoforms of PKC in chemo/radioresistance in a variety of neoplasms. In several recent studies, interference with the cytoprotective influence of PKC has been shown to lower the threshold for initiation of apoptosis by cytotoxic stimuli (e.g., chemotherapeutics including 5-FU, paclitaxel, vinblastine, mitomycin C, cisplatin, and doxorubicin; 26). With respect to HT29 in particular, recent studies have implicated PKC α (15, 16), β (16, 17), and ζ (18) as possible modulators of sensitivity to a wide range of chemotherapeutic agents, notably including Taxol, vinblastine, and 5-FU. These studies, however, each used HT29 grown under standard two-dimensional monolayer conditions in which it is impossible to study any role that PKC may play in multicellular resistance. As such, we endeavored to determine what effects, if any, SHE78-7-mediated disruption of spheroids of HT29 would have on expression and/or activity of PKC α, β1, and ζ.

As shown in Fig. 5A, the use of blocking antibodies to prevent E-cadherin-mediated intercellular adhesion in HT29 cells grown in three-dimensional culture for up to 4 days causes decreased expression of PKC α and β1 but not PKC ζ. Those isoforms that decreased did so within 24 h, with the cells maintaining a comparatively lower level of expression for more than 96 h (Fig. 5A). Interestingly, while there have been many studies demonstrating the influence of PKC on E-cadherin expression and distribution (27–29), and activation of PKC by E-cadherin (30), this is the first demonstration, to the best of our knowledge, that interference with E-cadherin-mediated adhesion can directly affect expression of PKC isoforms at the protein level. To determine if this down-regulation was specific to interference with E-cadherin-mediated adhesion and not a nonspecific effect of disaggregation, we examined expression of PKC α and β1 in cells from spheroids disrupted via exposure to 0.1% trypsin-EDTA in PBS for 2 h at 37°C. In so doing, we saw no change in expression of either PKC isoform compared with levels of intact spheroids in complete media, although control spheroids in PBS alone showed elevated expression (data not shown). As a result, we cannot yet confidently rule out nonspecific disaggregation as being responsible, at least in part, for the change in PKC α and β1 expression.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** SHE78-7 treatment causes greater intracellular accumulation of chemotherapeutics. Intact or disrupted spheroids of HT29 were exposed to [3H]-labeled paclitaxel (A), etoposide (B), vinblastine (C), or 5-FU (D) for 24 h, after which the intracellular accumulation of [3H]-labeled drug was determined. Columns, average of two (B and C) or three (A and D) independent experiments. *, P < 0.05.
In addition, we found that the total amount of phosphorylated PKC α and β was reduced in disaggregated HT29 cells compared with those in intact spheroids (Fig. 5B), indicating a net loss in PKC α and/or β activity. This loss of phosphorylation was unaffected by 24-h exposure to 500 μM 5-FU or 10 μM etoposide (the highest doses of each drug used in the above chemosensitization experiments) but, surprisingly, was absent in disaggregated cells treated for 24 h with 0.5 μM Taxol. **SHE78-7-Mediated Abrogation of Multicellular Resistance Is Independent of PKC α**

Because, as mentioned above, PKC α and β1 have been shown previously to be modulators of chemosensitivity in monolayers of HT29, we postulated that the decreased expression and activity of one or both of these isoforms caused by blocking E-cadherin may be responsible, at least in part, for the ability of SHE78-7 to abrogate multicellular resistance in three-dimensional cultures of HT29. To investigate this possibility with respect to PKC β1, we performed colony formation assays in the same manner as described above; however, instead of using normal HT29 cells, we used a HT29 line that had previously been stably transfected to overexpress PKC β1 ("HT29-PKC"; 16) and compared their response to drug ± SHE78-7 against the response of HT29 cells transfected with an empty vector ("HT29-V1").

![Image](http://example.com/image)

**Figure 5.** SHE78-7-mediated inhibition of E-cadherin leads to decreased expression and activation of PKC α and β1 but not PKC γ. A, HT29 cells were plated at t = 0 in subconfluent monolayers or in three-dimensional culture ± 1 μM SHE78-7 and subsequently harvested daily for 4 days. Protein from each sample was Western blotted for PKC α/β1/γ isoforms. B, HT29 cells from previous chemosensitization experiments (Fig. 2) were lysed immediately following drug exposure and lysates were Western blotted for phospho-PKC α/β. ERK levels are shown to demonstrate equal loading.

**Discussion**

Multicellular resistance (2, 3) differs from many other resistance mechanisms that are unicellular in nature, such as that conferred by overexpression of P-glycoprotein (31) or the multidrug resistance-associated protein (32), in that it is uniquely manifested *in vitro* when cancer cells are grown as three-dimensional masses (*i.e.*, it is nonemergent in cells in monolayer culture), thus making spheroids ideal tools for its study. Because this form of resistance is dependent on intercellular adhesion, we have been investigating the use of antiadhesive molecules to disturb the integrity of three-dimensional cancerous masses, in hopes of circumventing and/or disrupting the mechanism(s) responsible for multicellular resistance.

In all of the experiments outlined in this study, HT29 cells were grown in three-dimensional culture as large (≈1 mm in diameter) intact or disrupted spheroids, with disruption conferred via addition of 2 μg/ml SHE78-7 to the culture media, for 48 h prior to drug addition. Within 48 h, intact spheroids achieved full intercellular adhesion and compaction, both of which are necessary for the emergence of multicellular resistance, and, with the exception of cells in the outermost layers, underwent growth arrest. Disrupted cells, however, remained as proliferating single cell suspension within 2 days of culture. As a result, to ensure that any observed differences in responsiveness to chemotherapy could not be the result of...
differences in the amount of treatment given per cell, 50% fewer HT29 cells were plated (per well) in the presence of SHE78-7 than were plated to form intact spheroids, which gave rise to equal numbers of cells after 48 h in culture.

In the chemosensitization experiments, the failure of SHE78-7 to enhance the activity of cisplatin was not entirely unexpected, as previous studies have shown cisplatin to be equally effective against cells in spheroids as against those in monolayer (33, 34). However, the potential usefulness and versatility of this approach to sensitization is demonstrated by the fact that antiadhesive treatment did sensitize HT29 to four other drugs tested, each of which has a different mechanism of action (although it should be acknowledged that they are all preferentially active against dividing cells). Furthermore, because 5-FU, unlike the others, is commonly used clinically to treat colorectal cancers (albeit in combination with other drugs such as leucovorin, irinotecan, or oxaliplatin; 35), the enhancement of its cytotoxic activity via antiadhesive treatment can be seen as providing some additional validation of the potential of this therapeutic strategy.

As the resistance studied strictly manifests in three-dimensional cultures, three-dimensional recovery conditions were employed in addition to traditional two-dimensional conditions in initial experiments to ensure that chemosensitization as measured with monolayer cultures was not artifactual. Furthermore, because antiadhesives, if used in vivo, unlike the others, is commonly used clinically to treat colorectal cancers (albeit in combination with other drugs such as leucovorin, irinotecan, or oxaliplatin; 35), the enhancement of its cytotoxic activity via antiadhesive treatment can be seen as providing some additional validation of the potential of this therapeutic strategy.

As the resistance studied strictly manifests in three-dimensional cultures, three-dimensional recovery conditions were employed in addition to traditional two-dimensional conditions in initial experiments to ensure that chemosensitization as measured with monolayer cultures was not artifactual. Furthermore, because antiadhesives, if used in vivo as chemosensitizers, may or may not persist even after chemotherapeutics have been metabolized, we chose to examine post-therapeutic recovery in three-dimensional cultures with and without the presence of antiadhesives. Interestingly, these experiments (i.e., Fig. 1) seemingly indicated that chemosensitization was more obvious and/or thorough when cells were observed post-treatment as

three-dimensional cultures. However, this may simply be indicative of inherent differences between the two assay techniques, as MTS (which we have found to be unreliable when used on spheroids, likely due to limited penetration of the substrate and/or metabolite) is used to quantify metabolic activity, whereas [3H]thymidine incorporation measures cell proliferation. As a result, direct comparisons between two-dimensional and three-dimensional recovery conditions may be inappropriate. Nonetheless, the data were sufficiently in agreement to validate the use of two-dimensional colony formation assays for subsequent sensitization experiments.

Because the proliferative state (i.e., relative growth fraction) of any cancerous mass can greatly affect its response to treatment, we continued to monitor differences in cell proliferation and cycling behavior by examining cell cycle profiles of HT29 treated with the aforementioned chemotherapeutics as intact or disrupted spheroids. As stated above, we found that most cells in intact spheroids remained arrested in G1 following drug treatment, seemingly indicating that they were relatively unaffected. In contrast, antiadhesive treatment released cells from G1 arrest, allowing subsequent exposure to a range of concentrations of Taxol, etoposide, and vinblastine to cause a substantial percentage of cells to arrest in G2-M. Such G2-M arrest is likely indicative of the impending cell death, as cells that lack functional p53, such as HT29, often arrest in G2-M in response to treatment with a variety of chemotherapeutic drugs, including those employed herein, prior to undergoing apoptosis or mitotic catastrophe.

5-FU does not cause cells deficient in p53 activity to undergo G2-M arrest (36–38); therefore, it was not surprising that no such arrest was seen in our experiments. Studies have suggested that thymidylate synthase
inhibition (e.g., via 5-FU) may cause cells with compromised p53 activity to arrest in a single peak spanning the G1-S boundary (36) or fully in S phase (37, 38). Fittingly, our data show that the G1 peaks from disaggregated HT29 cells did markedly shift (i.e., indicating progression from G1 into S) in response to 5-FU, suggesting an enhanced cytotoxic response of cells in the presence of SHE78-7 compared with those treated in its absence. Collectively, the flow cytometry data suggest that the ability of the SHE78-7 antibody to free cells from an arrested state of growth via interruption and/or prevention of the upregulation of cell cycle-related mediators of drug resistance, including p27kip1, is likely paramount to its activity as a chemosensitizer.

Another possible contributing factor is the fact that blocking E-cadherin-mediated adhesion prevents the creation of architectural barriers to penetration for therapeutic agents. Indeed, when we used radio-[3H]-labeled drugs to ascertain the amount of drug found within cells following treatment as intact or disrupted spheroids, we found apparent increases in drug accumulation in disrupted cells in all cases, although some increases were not statistically significant. Interestingly, the large increase (i.e., >100%) in the quantity of intracellular Taxol achieved with the lower of the two concentrations tested (i.e., 0.05 μM) was somewhat surprising, in that Taxol itself has some antiadhesive activity when used against spheroids of HT29 (S. K. Green, unpublished data), which should theoretically reduce architectural impediments to penetration, at least to some extent. This disruptive effect of Taxol may explain, at least in part, why the intracellular amount of Taxol was not significantly different between cells in the presence or absence of SHE78-7 when treated with 0.5 μM of drug. Nonetheless, as shown in Figs. 1 and 2, at that concentration, Taxol was indeed significantly more effective in the presence of SHE78-7 treatment (i.e., under conditions in which cells were more accessible to drugs). On this basis, coupled with the fact that etoposide was also more effective in the presence of SHE78-7 in spite of insignificant increases in intracellular accumulation of the drug, we propose that SHE78-7 acts as a chemosensitizer, at least in part, independently of its effects on drug delivery to cells in multicellular spheroids, consistent with previously published observations (5, 39).

An effort was made in the aforementioned drug accumulation studies to use dosages of drug similar to those used in the chemosensitization experiments to maximize the relevance of the results. Unfortunately, due to the concentration of the reagent as provided by the manufacturer, this was not technically feasible with [3H]-5-FU, which instead had to be administered at concentrations ~2 orders of magnitude lower than those used for the rest of the studies. Nonetheless, in spite of these low concentrations and the relatively small size of the molecule (130.08 kDa), significant differences in accumulation of 5-FU were observed at both dosages employed. One possible explanation for this may be that the intracellular interactions of 5-FU with nucleic acids are sufficiently rapid to limit its penetration into cells in the heart of a large multicellular mass, although further experiments would be required to confirm or refute this hypothesis.

Although the data do suggest that antiadhesion improves delivery of some chemotherapeutics into multicellular masses, they do not yet compellingly implicate the circumvention of potential penetration difficulties as a predominant factor in the chemosensitization activity of SHE78-7. Furthermore, the effect(s) of any such increased accumulation of drug on therapeutic efficacy under these conditions is ultimately uncertain; this is especially true in light of previous work that has shown that cells in spheroid may display increased resistance to therapy even when they (unexpectedly) accumulate higher intracellular levels of drug (21). As such, we chose to investigate possible molecular mechanisms that may play a more substantial role in SHE78-7-mediated chemosensitization.

Together with the findings from previous studies (9–12), our results suggest a substantial role for p27kip1 in the multicellular resistance demonstrated by HT29 spheroids. However, further experiments involving specific inhibition of p27kip1, perhaps via an antisense (9) or dominant-negative genetic approach, will be necessary to firmly establish the relative role played by p27kip1. Nonetheless, it is probable that other molecules contribute significant effects, as evidenced by the fact that although overexpression of p27kip1 in monolayer cultures of HT29 confers enhanced resistance to cisplatin (11), contact-mediated increases in p27kip1 levels in HT29 spheroids were seemingly insufficient to generate multicellular resistance against this drug. Indeed, previous studies have implicated altered expression of several additional molecules in the emergence of contact-mediated resistance, including the cyclin-dependent kinase inhibitors p18 and p21Cip1/Waf1 (40–41), the β containing integrins (42–43), and the DNA mismatch repair protein PM2S.3

In addition, due to their cytoprotective influence, several PKC isoforms have the ability to significantly affect the therapeutic responses of cancer cells (26), including HT29. For example, Filomenko et al. (18) found that transfection of HT29 with a kinase-defective PKC ζ sensitizes those cells to cisplatin. Other studies have shown that manipulation of expression levels of PKC α and/or β can significantly alter the chemosensitivity of HT29 in vitro (15–17).

Because our data show that HT29 cells do not demonstrate multicellular resistance to cisplatin (at least under the experimental conditions employed herein), it was not unexpected to find that SHE78-7 treatment of HT29 cells in three-dimensional culture caused no change in the expression of PKC ζ. Nevertheless, there remains the possibility that SHE78-7 may abrogate multicellular resistance in HT29, at least in part, by decreasing the extent to

which PKC α and/or β1 can exert their cytoprotective activity, as blocking E-cadherin-mediated cell-cell adhesion between HT29 cells in three-dimensional culture caused significant down-regulation and diminished activity of both isoforms. However, our finding that the chemosensitization effect of the SHE78-7 antibody was as substantial with HT29-PKC cells expressing high levels of PKC β1 as it was with vector-transfected control cells led us to conclude that the antibody’s ability to decrease the expression and activity of PKC β1 was unrelated to its ability to act as an antiadhesive chemosensitizing agent.

Although a role for PKC β1 in multicellular resistance in HT29 has been largely, if not entirely, ruled out, there remains the distinct possibility of other de facto mediators, including PKC α, the activity of which could theoretically be compromised via antiadhesive treatment. Indeed, in a recent study, in which two-dimensional electrophoresis and mass spectrometry were used to compare the protein expression profiles of monolayers and spheroids of HT29, significant changes in expression levels of several proteins that could influence therapeutic response were reported (44). For example, increased expression of several cytokeratins (that may play roles in apoptosis induced by DNA damaging agents) and decreased expression of α- and β-tubulin, the molecular target of Taxol, were found in spheroids. Further experiments will be necessary to elucidate the roles of these and/or other putative mediators of multicellular resistance not only in HT29 but also in several other cell systems.

In summary, our data support the conclusion that intercellular adhesion may represent a potentially useful target for therapeutic agents designed to act as chemosensitizers for the treatment of human tumors. This is, however, an in vitro study, and as such, caution must be observed in any translation of these results into an in vivo or clinical context. Although we have intentionally used a three-dimensional in vitro system that recapitulates many of the characteristics of solid tumors (45), it remains to be seen whether such an anti-adhesive-mediated chemosensitization strategy could be effectively used on human cancers in vivo. In this regard, previous in vivo studies from our laboratory (7) showed that treatment with the SHE78-7 antibody alone conferred a significant prolongation of survival to nude mice carrying i.p. xenografts of HT29 [a model system that emulates peritoneally disseminated cancers, as in advanced colon (46, 47) or ovarian carcinoma (48)]. The survival advantage provided by SHE78-7 was due to its ability to disrupt free-floating i.p. multicellular aggregates of HT29 while simultaneously recruiting an enhanced Fc-dependent host immune response (i.e., antibody-dependent cellular cytotoxicity; 7). However, mice in these experiments eventually succumbed to outgrowth of the xenograft, leading us to postulate that the immunosensitization effects of the antibody may be enhanced via concurrent administration of chemotherapy; such experiments, however, have not yet been undertaken. The successful completion of these and further in vivo studies would be required to validate this therapeutic strategy.

Finally, it should be noted that in a bona fide clinical situation, targeting E-cadherin would likely prove ineffective, as not only is this molecule down-regulated or absent in advanced primary carcinomas (with the notable exception of ovarian carcinoma, in which E-cadherin expression is increased; 49) but also because it is so widely expressed (i.e., on all epithelia) that host toxicity would presumably be unacceptably high. However, we propose that this study provides proof of principle that cell adhesion, whether mediated by cadherins, integrins, or other adhesion molecules, represents a potentially useful therapeutic target for the reversal of multicellular drug resistance in solid tumors and/or avascular multicellular clusters in peritoneally disseminated cancers. Moreover, there are some mediators of intercellular adhesion that are selectively or preferentially up-regulated in tumor cells compared with normal cells (e.g., CEACAM-1 in non-small cell lung cancer (50), ICAM-1 and MUC-18 in melanoma (51), and CD44 or N/P-cadherin in other solid tumors (52–54)). Thus, it is conceivable that such up-regulation could be sufficient, at least in theory, to provide a therapeutic window for the use of such a strategy. Somewhat counterintuitively, such an approach may prove useful even in hematological malignancies, as β1-containing integrins appear to mediate a form of resistance operationally similar to multicellular resistance in leukemia and myeloma, termed cell adhesion-mediated drug resistance (10, 42, 43, 55).

Acknowledgments

We thank Kazuki Yamamoto (Takara Shuzo Biomedicals) for generously providing the SHE78-7 antibody, Gary Chui for technical assistance, and Cassandra Cheng for excellent administrative assistance.

References

10. Hazlehurst LA, Damiano JS, Buyukikul P, Pledger WJ, Dalton WS. Adhesion to fibronectin via β1 integrins regulates p27Kip1 levels and


Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy *in vitro*

Shane K. Green, Giulio Francia, Ciro Isidoro, et al.


Updated version  Access the most recent version of this article at: [http://mct.aacrjournals.org/content/3/2/149](http://mct.aacrjournals.org/content/3/2/149)

Cited articles  This article cites 51 articles, 21 of which you can access for free at: [http://mct.aacrjournals.org/content/3/2/149.full#ref-list-1](http://mct.aacrjournals.org/content/3/2/149.full#ref-list-1)

Citing articles  This article has been cited by 9 HighWire-hosted articles. Access the articles at: [http://mct.aacrjournals.org/content/3/2/149.full#related-urls](http://mct.aacrjournals.org/content/3/2/149.full#related-urls)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.