A sensitive assay for the evaluation of cytotoxicity and its pharmacologic modulation in human solid tumor-derived cell lines exposed to cancer-therapeutic agents

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Dedicated to Professor Antoine (Tony) A. Noujaim in recognition of his outstanding contributions to radio-pharmacy, diagnostic oncology and the immunotherapy of cancer.

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Abbreviations: HDS, high density survival; CFA, colony-forming ability; CaMKII, calcium/calmodulin-dependent protein kinase II; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; EDTA, ethylene-diaminetetraacetic acid; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Key words: Cytotoxicity assays; human cancer cell lines; ionizing radiation; cisplatin.

ABSTRACT - Purpose: Reliable *in vitro* cytotoxicity assays are essential for determining the responses of human normal and cancer-derived cells to therapeutic agents and also for the identification and pre-clinical evaluation of new drugs capable of selectively augmenting the susceptibility of cancer cells to conventional therapies. The clonogenic survival assay is considered as the "gold standard" in this regard because it measures the sum of all modes of cell death, encompassing both early and late events such as delayed growth arrest. In this assay, however, the impact of cell-to-cell communication is disregarded because the cells are plated out at very low densities. In addition, here we provide evidence that human breast cancer cell lines cannot be reliably evaluated by clonogenic assays. We developed a novel long-term, High Density Survival (HDS), assay that circumvents the various intrinsic shortcomings of the conventional cytotoxicity assays. **Methods:** In the HDS assay, the cells are maintained at a high density for 24 h prior to, and for 24 h after, exposure to a DNA-damaging agent to facilitate intercellular communication. After a carefully scheduled subculturing for ~7 days, cultures are assessed for the extent of growth. **Results:** The degree of radiosensitivity and cisplatin sensitivity evaluated by the HDS assay in human cancer cells was comparable to that measured by the clonogenic assay. Pharmacological inhibitors of CaMKII and/or PI3K signaling elicited a greater degree of radiosensitization when determined by the HDS assay than the clonogenic assay. In all these experiments, there was no relationship between the degree of cytotoxicity measured by the clonogenic survival and viability assays. In the HDS assay, all seven human breast cancer cell lines that we tested exhibited a high degree of radioresistance. **Conclusions:** The novel HDS assay appears to be a powerful tool for evaluating cancer cell responses to therapeutic agents under conditions which incorporate some aspects of intercellular communication.

INTRODUCTION

It is a pleasure to contribute to this special issue in honor of Dr. Antoine (Tony) Noujaim. Over the past two decades, Dr. Noujaim devoted extensive efforts in developing cancer therapeutics, and his remarkable accomplishments have placed him high on the list of Canada's true champions in this area. In line with Dr. Noujaim's endeavors, we have been exploring the molecular mechanisms underlying the cytotoxic effects of DNA-damaging agents in human normal and cancer-derived cell lines in an attempt to design pharmacological approaches for selectively potentiating the susceptibility of cancer cells to such agents.

It is now well understood that cancer therapeutic agents not only induce early apoptotic and necrotic cell death, but also trigger sustained growth-arresting events through, for example, accelerated senescence (1,2) and mitotic catastrophe (3,4), responses which are manifested at late times (several days) after the introduction of DNA damage. The clonogenic survival assay provides an integrated readout of all of these early and late responses and has therefore been considered as the "gold standard" for the assessment of cytotoxicity

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(5,6). Several shortcomings of clonogenic assays in the context of cancer therapy have long been recognized (7). Importantly, the impact of intercellular communication (both direct cell-to-cell interaction through gap junctions and communication through diffusible factors) is overlooked because in such assays the cells must be plated out at very low densities in a large volume of medium. In addition, many solid tumor-derived cell lines (e.g., most breast cancer cell lines) cannot be reliably evaluated by clonogenic assays because of their poor cloning efficiencies and because some cell lines do not yield a good single-cell suspension by conventional approaches (e.g., after exposure to trypsin). Numerous short-term techniques have been developed that circumvent some of the problems associated with clonogenic assays (8-10), but unfortunately they primarily detect early apoptosis and necrosis.

We therefore developed a "High Density Survival" (HDS) assay which offers three advantages in determining the responses of human solid tumor-derived cell lines to therapeutic agents: (i) there is no need for the preparation of a single-cell suspension; (ii) cell-to-cell contact (and thus some aspects of intercellular signaling) (11,12) is maintained during the genotoxic treatment and for an extended time (24 h) thereafter; and (iii) cytotoxicity is evaluated at relatively long times (e.g., 7 days and beyond) post-treatment. We compared the HDS, colony-forming ability (CFA), and viability assays in evaluating the cytotoxic effects of ionizing radiation and cisplatin in human cancer cells. In addition we compared these three assays for determining the effects of pharmacological inhibitors of calcium/calmodulin-dependent protein kinase II (CaMKII) and phosphatidylinositol 3-kinase (PI3K) on the radiosensitivity of cancer cells. Employing the novel HDS assay, we also determined the degree of radiosensitivity of a panel of seven human breast cancer cell lines that could not be evaluated by the clonogenic assay. The outcome of these and related studies forms the subject of this communication.

MATERIALS AND METHODS

Cells, cell culture and treatment

Pertinent characteristics of the human cell lines employed in this study are given in **Table 1**. Cells were routinely cultured as monolayers in DMEM/F12 nutrient medium supplemented with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin sulfate in a 37 °C chamber incubator providing a humidified atmosphere of 5% CO_2 in air. All cultures were free of *Mycoplasma* contamination. Exposure to ^{60}Co γ radiation was performed in a Gammacell 220 unit as described (16). Treatment with cisplatin (Mayne Pharma, Kirkland, PQ, Canada) was performed by incubating cells in growth medium containing the indicated concentration of the drug for 2 h at 37 °C. Following incubation, the medium was replaced with fresh medium lacking cisplatin. The PI3K inhibitor wortmannin and the CaMKII inhibitor 1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-*L*-tyrosyl]-4-phenylpiperazine (KN62) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Stock solutions of wortmannin and KN62 (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at –70 °C. To determine the effects of these protein kinase inhibitors on the radiosensitivity of HCT116 cells, cultures were treated with each inhibitor for 1 h prior to irradiation and for 24 h post-irradiation. Control cultures were incubated in medium containing 0.1% (v/v) DMSO.

Clonogenic survival assay

Cells of an exponentially-growing monolayer culture were harvested by the use of 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.53 mM ethylenediaminetetraacetic acid (EDTA) (3 min incubation at 37 °C) and suspended in ~5 mL of PBS. Using a 5-mL pipette, the cells were pipetted up and down several times, forcing them through the tip of the pipette to break up the clumps. One mL of this suspension was diluted in ~20 mL of medium and immediately the cells were counted using a Coulter counter (Coulter, Hialeah, FL, USA). After microscopic examination to ensure a reasonable quality of single-cell suspension, the cells were diluted in a volume of medium to yield ~60 cells/mL. Five-mL samples of the resultant single-cell suspension were then pipetted in 60-mm dishes. Using the same protocol, normal human fibroblasts (strain GM38) were plated out at 300 cells per dish in 100-mm dishes (10 mL medium/dish). After plating, the cells were incubated for ~4 h and then exposed to different doses of γ rays (between 0 and 8 Gy) or treated for 2 h in growth medium with different concentrations

Table 1. Characteristics of human cell lines studied.

Cell line [a]	Description	Cloning efficiency (%) [b]	Single cells (%) [c]	
			4 h	16 h
GM38	Normal human fibroblasts	37 ± 3	95 ± 4	92 ± 6
SK-N-SH	Neuroblastoma	21 ± 4	91 ± 3	83 ± 6
HCT116	Colon carcinoma	54 ± 1	78 ± 6	39 ± 8
A172	Malignant glioma	28 ± 2	87 ± 3	51 ± 4
MDA-MB-435s	Melanoma? [d]	43 ± 3	80 ± 6	42 ± 4
SKBR3	Breast carcinoma	ND [e]	40 ± 6	20 ± 8
MCF7	Breast carcinoma	ND	44 ± 3	18 ± 6
UACC893	Breast carcinoma	<1%	50 ± 2	39 ± 7
BT-483	Breast carcinoma	ND	51 ± 3	42 ± 3
MDA-MB-175-VII	Breast carcinoma	ND	48 ± 2	11 ± 2
CRL2230	Breast carcinoma	ND	51 ± 6	26 ± 4
SUM52PE	Breast carcinoma	<1%	42 ± 5	28 ± 4
SUM185PE	Breast carcinoma	<1%	39 ± 5	29 ± 5

[a] The normal fibroblast strain GM38 was purchased from the Institute for Medical Research (Camden, NJ, USA). The "SUM" cell lines were generously provided by Dr. Stephen P. Ethier from the University of Michigan Human Breast Cell/Tissue Bank and Data Base (The University of Michigan Medical School, Ann Arbor, MI, USA). The remaining tumor cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA).

[b] The cloning efficiency of each cell line was determined by plating out 300 cells/60-mm dish and incubating them for ~10 days to permit the formation of macroscopic colonies. The percent cloning efficiency values were calculated by dividing the number of colonies recovered by the number of cells plated, and multiplying the outcome by one hundred. The means (± SE) of at least three determinations are presented.

[c] The quality of single-cell preparation was determined by suspending cells in culture medium and seeding them at very low densities. The cells were fixed in methanol at the indicated times after seeding and evaluated for the percentages of single cells. The means (± SE) of at least three determinations are presented.

[d] Although the MDA-MB-435s cell line has been widely used as a model in breast cancer research, there is convincing evidence that it is of melanoma origin (13-15).

[e] Not determined.

of cisplatin (between 0 and 4 μg/mL). Cultures were incubated for 18 days with one medium renewal at day 7 (fibroblasts) or for 10 days without medium renewal (tumor cells). The cells were then fixed and stained with crystal violet, and the number of survivors (i.e., colonies containing >50 cells) scored. Survival curves were constructed by plotting CFA (expressed as a percentage of the sham-irradiated control cultures) on a logarithmic scale as a function of the radiation dose or cisplatin concentration administered on a linear scale.

High density survival assay

Cells of an exponentially-growing monolayer culture were harvested by the use of trypsin/EDTA, counted, plated in 35-mm dishes at $5x10^5$/dish, and incubated for 24 h. The cells were then exposed to various doses of γ rays (or sham-irradiated), or were treated with various concentrations of cisplatin for 2 h (or sham-treated), and incubated for another 24 h. The cells of each dish were then detached by the use of trypsin/EDTA, and 1/10 of the content of each dish was seeded into a 100-mm dish (containing 10 mL fresh medium) and incubated for 5 days. The cells of each dish were again detached and 1/10 of the content of each dish was seeded into 60-mm dishes (3 dishes for each time point). The cells were incubated for 24 h in fresh medium, and for a further 24 h in medium containing either 0.01 μCi/mL of [*methyl*-^{14}C]-thymidine (specific activity, 55 mCi/mmol) or 0.01 μCi/mL of [*methyl*-^{3}H]-thymidine (stock specific activity, 5 Ci/mmol). The radiolabeled nucleosides were purchased from Amersham Pharmacia Biotech (Baie D'Urfe, PQ, Canada). The amount of radioactivity incorporated into the cells of each dish was then determined as described (17). The degree of cell killing by a particular radiation/cisplatin treatment was determined from the amount of radioactivity incorporated in cells of treated dishes compared to sham-treated control dishes. Survival curves were constructed by plotting the numbers of [^{14}C]-

thymidine or [^{3}H]-thymidine counts (expressed as a percentage of control cultures) on a logarithmic scale as a function of the radiation dose or cisplatin concentration administered on a linear scale.

Microscopic evaluation of cells stained with Hoechst 33342/propidium iodide

Exponentially-growing monolayer cultures were plated in 60-mm dishes at 0.5 x 10^5 cells/dish, exposed to γ radiation or treated with cisplatin as described above, and incubated for three days. Cells in each dish were stained with Hoechst 33342 (for evaluation of the total number of cells) and propidium iodide (for evaluation of non-viable cells) as follows. Hoechst 33342 (Sigma, St. Louis, MO, USA) was added to the culture medium to give a final concentration of 1 μg/mL. After ~20 min of incubation at 37 °C, floating cells were collected by centrifugation and suspended in PBS (0.5 mL) containing 10 μg/mL propidium iodide (Sigma). This solution was then added to the original dish containing adherent cells. Cells were viewed under an inverted fluorescence microscope (Nikon Diaphot 300; Melville, NY, USA) and evaluated for loss of viability (percentage of propidium iodide-stained cells).

Annexin V/flow cytometry analysis of apoptosis

Cells were plated out in 60-mm culture dishes at 5 x 10^5/dish, incubated overnight, exposed to 0 or 4 Gy of γ radiation, and incubated for 48 or 72 h. As a positive control, cells in one dish were treated with a high concentration of cisplatin (20 μg/mL) for 2 h, and incubated for 48 h to induce apoptosis in a high proportion of cells. After completion of the incubation periods, cells were detached by exposure to trypsin/EDTA, rinsed in PBS, and treated with propidium iodide and Annexin V-FITC as outlined in the Annexin V-FITC kit protocol of the manufacturer (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry was performed using a standard protocol (18).

RESULTS

Evaluation of the quality of single-cell suspensions following exposure to trypsin/EDTA in human cancer cell lines

For the CFA assay, it is essential to plate out single cells at cloning densities, otherwise the outcome of the experiment will be biased. Thus, before comparing different assays for the assessment of radiosensitivity, we examined the cancer cell lines shown in Table 1 for the quality of single-cell preparation after exposure to trypsin/EDTA. For this purpose, a monolayer culture of a cell line was detached by exposure to trypsin/EDTA and suspended into single cells as detailed in Materials and Methods for the clonogenic survival assay. The cells were diluted in culture medium and were immediately plated out at very low densities; the cell number was not determined in these experiments in order to minimize the time between cell detachment and subsequent seeding and thus prevent cell clumping. After seeding, cells were incubated for 4 h or 16 h, fixed in methanol, and assessed for the percentages of single (isolated) cells. The results are presented in Figure 1 and Table 1. At 4 h post-seeding, the quality of single-cell preparation was reasonably good for only four cancer cell lines (A172, SK-N-SH, HCT116, and MDA-MB-435s), with ~10-20% of cells being in clusters (i.e., aggregates of two or more cells). With breast cancer cell lines (e.g., MCF7, SKBR3), however, 49-71% of cells were in clusters, the majority consisting of more than two cells. At 16 h after seeding, a high percentage of cells were in clusters for all but one (SK-N-SH) of the cancer cell lines examined.

As in every CFA assay, for the experiments presented in Table 1 we determined the quality of the single cell suspension prior to seeding in tissue culture dishes. This was done by spreading a small volume (~50 μL) of the cell suspension on a microscope slide and examining the cells under a microscope. The outcome of this measurement (data not shown) was comparable to that performed at ~4 h after seeding (Table 1).

The method of cell detachment and re-seeding used in these experiments is sufficient to ensure a good quality of single cell preparation without compromising the clonogenic potential of cancer cell lines used in our previous studies (e.g., HCT116, A172) (2,12,19).

Given that MCF7 cells have been evaluated by the clonogenic assay in several laboratories, we considered the possibility that our approach might not be optimal for this cell line. We therefore tested different conditions, such as exposure to different concentrations of trypsin (between 0.05 and 25%) for various times (between 3 and 15 min),

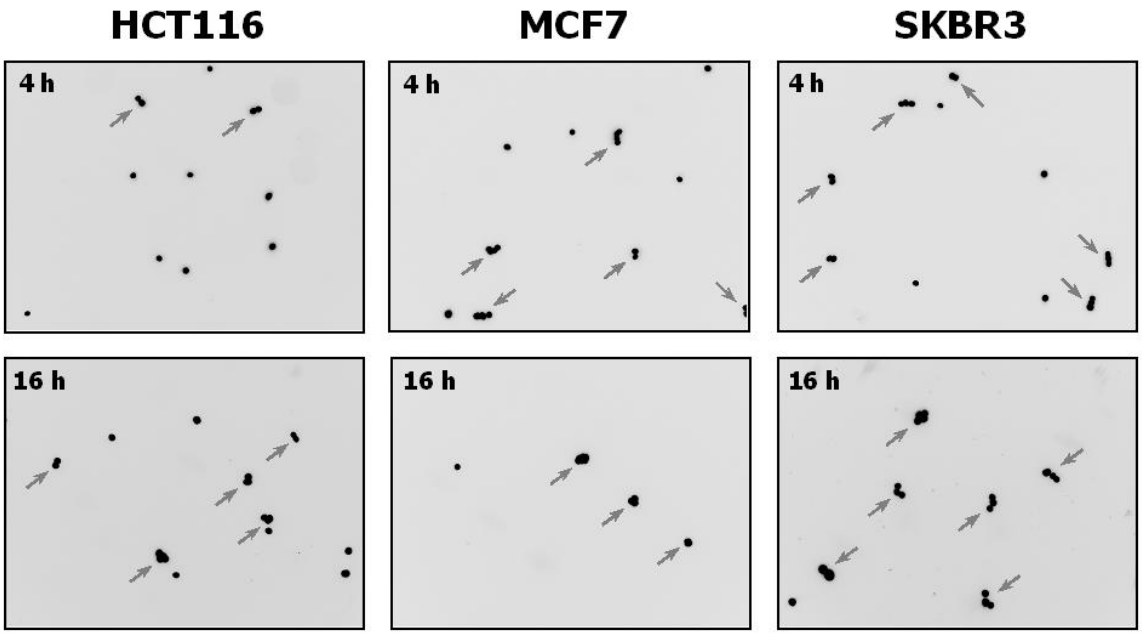

Figure 1. Quality of the single-cell preparation for the indicated cell lines. Cells were detached from the dishes by exposure to trypsin/EDTA, suspended in culture medium, and immediately plated out at low densities. After incubation for 4 h or 16 h, the cells were fixed in methanol, stained with 4',6'-diamidino-2-phenylindole (DAPI), and examined under a fluorescence microscope. Arrows indicate aggregates of two or more cells.

combined with forcing the cells through the tip of a pipette for different times. Although some of the "harsher" treatments of MCF7 cells led to a moderate improvement in the quality of single cell preparation, they also resulted in very poor cloning efficiency (data not shown).

Comparison of different assays for the evaluation of radiosensitivity and cisplatin sensitivity

We used the four cancer cell lines that yielded a reasonable single-cell suspension within 4 h post seeding to compare the degree of radiosensitivity as determined by the following three assays: CFA, HDS, and viability. Normal human fibroblasts (strain GM38) were included in some experiments as a control. Loss of viability in all cell lines was determined by staining the cells with Hoechst 33342 (to label all cells) and propidium iodide (to selectively label non-viable cells) at 72 h after radiation or cisplatin exposure. In addition, HCT116 cells were evaluated by flow cytometry for apoptosis (Annexin V staining) and loss of viability (propidium iodide staining) at 48 h and 72 h after irradiation.

The outcomes of the CFA and HDS experiments are presented in Figure 2. In the CFA assay (solid circles), the cancer cell lines A172, SK-N-SH and HCT116 showed a degree of radiosensitivity similar to normal fibroblasts, which is consistent with our previously published studies (2). On the other hand, the cancer cell line MDA-MB-435s was significantly more radioresistant than normal fibroblasts. The HDS assay performed with these same cancer cell lines (Figure 2, open circles) yielded results that were quite consistent with the CFA assay. It should be noted that the population doubling times of these cell lines differed substantially, ranging from ~16 h in HCT116 to ~30 h in SK-N-SH. The finding that these two cell lines exhibited comparable degrees of radiosensitivity in both the HDS and CFA assays indicates that neither of these assays is significantly influenced by the growth rates of cancer cells.

In contrast, a very different pattern of radiosensitivity was apparent when the cells were evaluated by the viability assay (Figure 3) versus the CFA or HDS assays (Figure 2). In the former assay, which was performed at 72 h post-irradiation, MDA-MB-435s cells showed the highest degree of radiosensitivity when compared to A172, SK-N-SH and HCT116 cells (i.e., the reverse of the observations made with the CFA and HDS

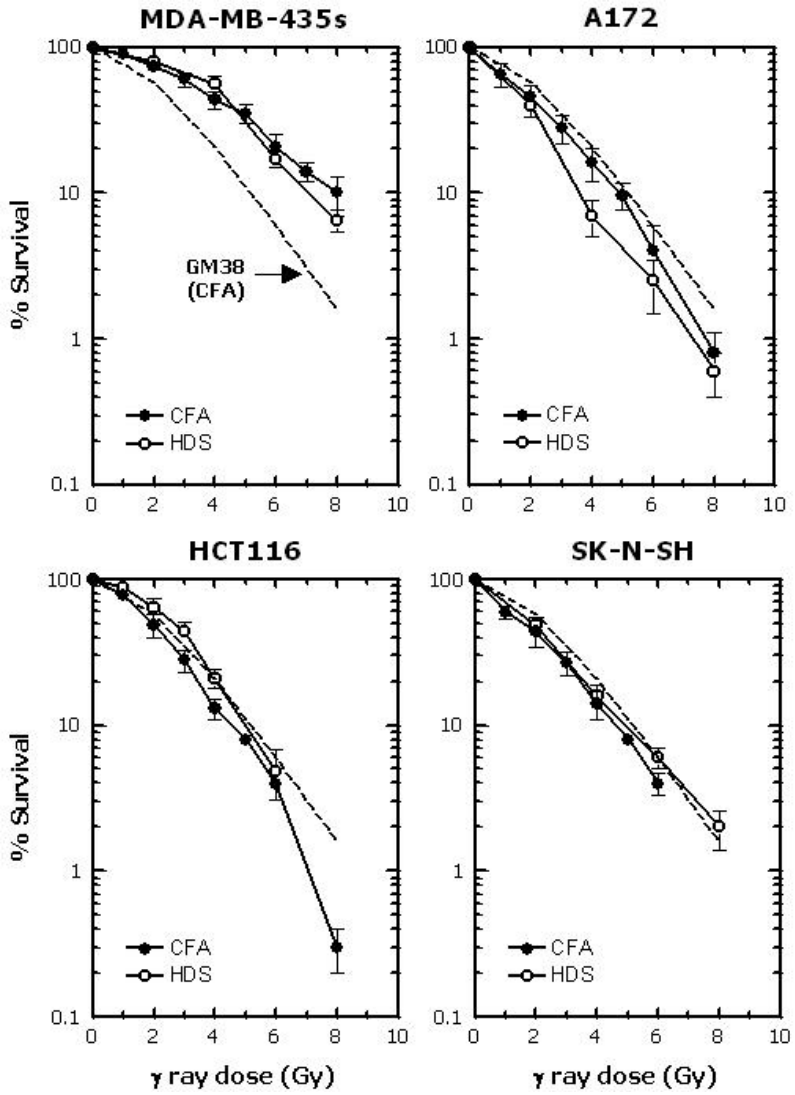

Figure 2. Comparison of the CFA and HDS assays for evaluation of radiosensitivity in the indicated cancer cell lines. The mean (±SD) values of at least two experiments are presented for each cell line. The dashed curve marks the response of the normal human fibroblast strain GM38 (average of three experiments), which was evaluated by the CFA assay in parallel with the A172, SK-N-SH and HCT116 cell lines.

assays). In addition, exposure of HCT116 cells to a moderate dose of γ radiation (4 Gy) resulted in ~80% cytotoxicity when measured by CFA or HDS assays (Figure 2), but in only marginal (~10%) cytotoxicity in the viability assay (Figure 3).

It is possible that, during the 72-h post-irradiation period, a significant proportion of cells might have been at relatively early stages of apoptosis such that they might not yet have lost their membrane integrity and would thus be scored as "viable" in the study presented in Figure 3. The results of the Annexin V/flow cytometry experiment presented in Figure 4 make this explanation unlikely, however.

Exposure of HCT116 cells to radiation (4 Gy) followed by incubation for 48 or 72 h resulted in early apoptosis (i.e., Annexin V positive cells) and late apoptosis (Annexin V plus propidium iodide positive cells) in only <9% of the cells. On the other hand, exposure of HCT116 cultures to cisplatin (20 μg/ml), which was used as a positive control,

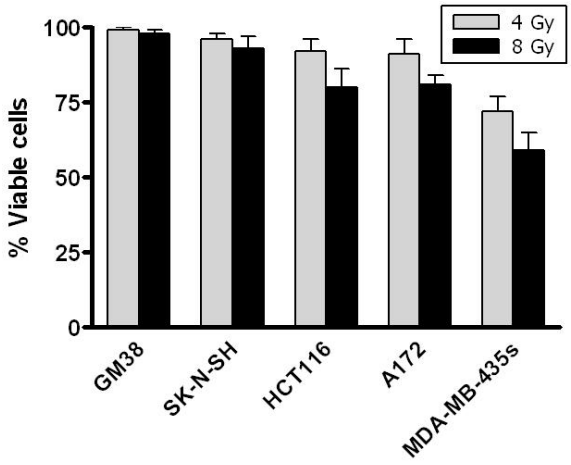

Figure 3. Effect of γ radiation on viability of the indicated cell lines. Exponentially-growing cultures were exposed to radiation and incubated for 3 days. Adherent and floating cells were combined, stained with Hoechst 33342/propidium iodide, and evaluated under a fluorescence microscope. Cells that were not stained with propidium iodide under these conditions were considered viable. The mean (±SE) values of at least two experiments (each run in triplicate) are presented for each cell line.

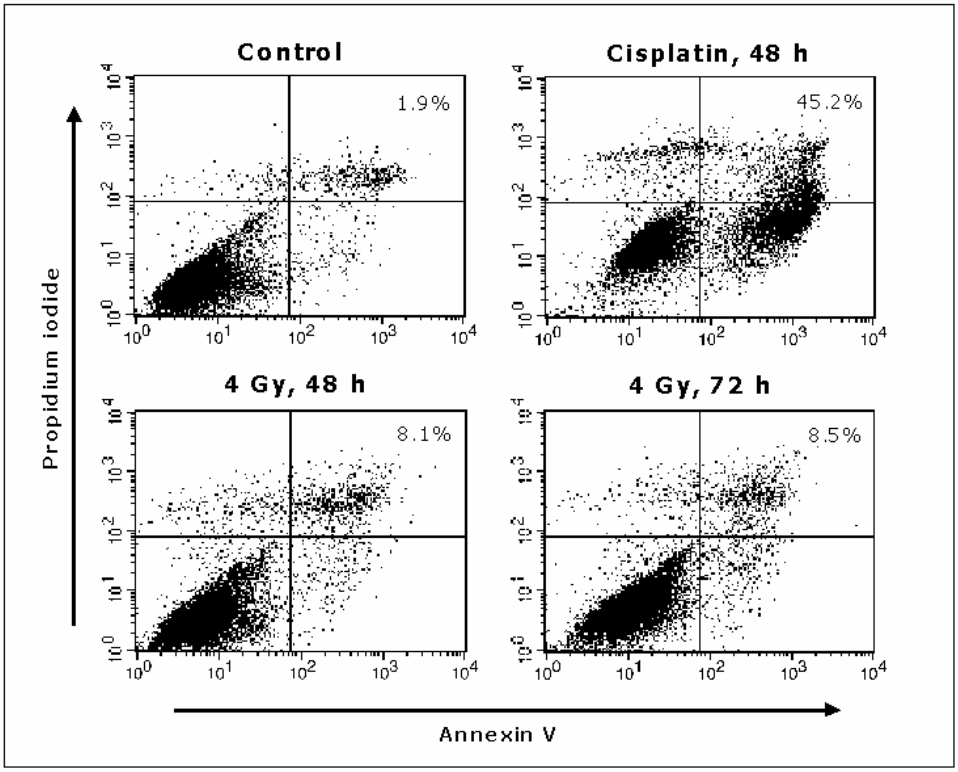

Figure 4. Representative flow cytometry plots of HCT116 cells stained with Propidium iodine/Annexin V-FITC at the indicated times after exposure to γ radiation (4 Gy) or cisplatin (20 μg/mL). The percentages of Annexin V-positive cells (upper right plus lower right panels) are shown in each panel. This experiment was repeated with similar outcome.

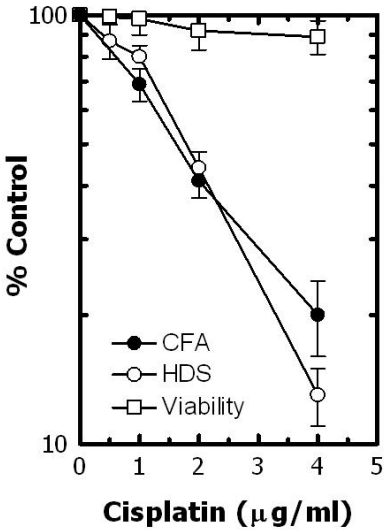

Figure 5. Evaluation of cisplatin-induced cytotoxicity by the CFA, HDS and viability assays in MDA-MB-435s cells. The mean (±SE) values of two independent experiments (each run in triplicate) are presented.

resulted in apoptosis in ~45% of the cells.

Figure 5 compares the cytotoxic effect of cisplatin in MDA-MB-435s cells when determined by the CFA, HDS and viability assays. The outcome was similar to that of the experiments with radiation-exposed cells. Treatment with 4 μg/mL cisplatin, for example, resulted in a high and comparable degree of cytotoxicity in the CFA and HDS assays, but in only marginal cytotoxicity in the viability assay.

Comparison of the CFA, HDS and viability assays for determining the influence of pharmacological inhibitors of CaMKII and PI3K on radiosensitivity

Our previous studies with normal human fibroblasts showed that radiation exposure results in rapid activation of the CaMKII signaling pathway and that this response can be inhibited by incubation of cells with either KN62 (an inhibitor of CaMKII) (20, 21) or wortmannin (an inhibitor of PI3K and PI3K-like protein kinases, as well as of the CaMKII-dependent signaling pathway) (21). Treatment with these kinase inhibitors also increased the degree of radiosensitivity of normal fibroblasts when measured by the CFA assay (21). Here we determined the effects of KN62 and wortmannin on the radiosensitivity of cancer cells when evaluated by the HDS and CFA assays, i.e., under high versus extremely low cell density culture conditions, respectively. Loss of viability was also determined by the Hoechst 33342/propidium iodide staining approach at 72 h after irradiation. We used HCT116 cells exclusively in these experiments. The cells were treated with each protein kinase inhibitor for 1 h before irradiation and for 24 h post-irradiation. The results are presented in Figure 6. In the HDS assay, treatment with KN62 (10 μM) or wortmannin (5 μM) markedly increased the radiosensitivity of HCT116 cells. In the CFA assay, incubation with 10 μM KN62 did not influence the radiosensitivity of HCT116 cells, whereas wortmannin at 5 μM moderately increased their radiosensitivity. In the viability assay, neither KN62 nor wortmannin influenced the radiosensitivity of HCT116 cells.

We considered the possibility that the differential responses indicated by the use of these assays might be associated with KN62 or wortmannin treatment resulting in a reduced rate of proliferation in irradiated cultures, which would influence the outcome of the HDS assay (in which the extent of cell growth is used as the end point), and probably of the viability assay, but not of the CFA assay. To test this possibility, we carried out a combination of the HDS and CFA assays (denoted as HD/CFA in Figure 6C) in which the cells were maintained at a high density before and 24 h after radiation exposure either in the presence or absence of an inhibitor (as in the HDS assay shown in Figure 6B).

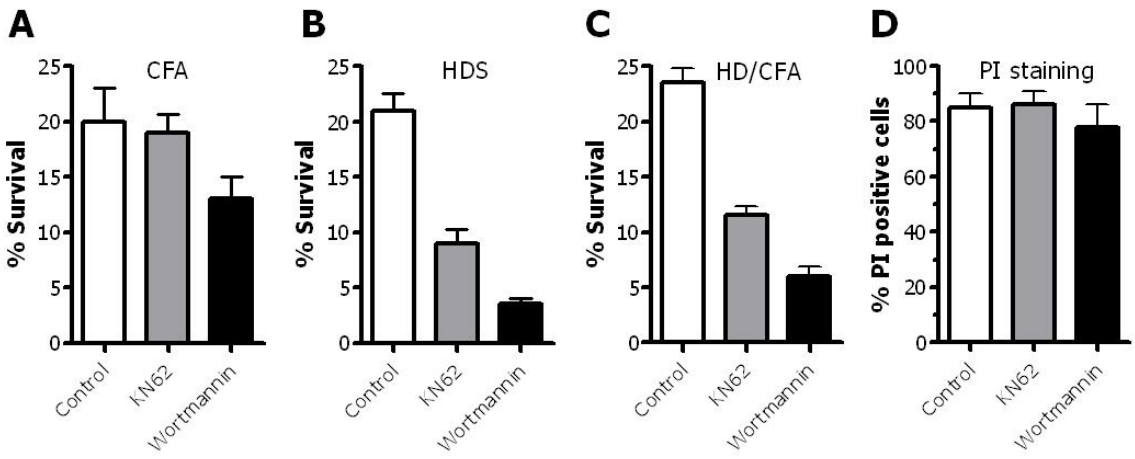

Figure 6. Effects of KN62 (10 μM) and wortmannin (5 μM) on the cytotoxicity of γ radiation (4 Gy) in HCT116 cells as evaluated by the indicated assays. For each assay, the cells were incubated with a protein kinase inhibitor for 1 h before irradiation and for 24 h after irradiation. Viability was evaluated by propidium iodide (PI) staining 72 h after irradiation. The data presented in panel **C** were generated by a combination of the HDS and CFA assays, indicated by HD/CFA, as described in Results. The mean (±SE) values of three determinations of a single experiment are presented in each panel.

After this incubation period the cells were plated out at cloning densities and cytotoxicity was assessed from the loss of clonogenic potential.

The outcome of the HD/CFA assay (Figure 6C), which is expected to be virtually unaffected by post-treatment growth rate, was similar to that of the HDS assay (Figure 6B), which can be influenced by post-treatment growth rate. These results suggest that the differential responses seen in the HDS assay versus the CFA or viability assays (Figure 6) is unlikely to be associated with the kinase inhibitors merely decreasing the growth rate of the cells subsequent to radiation exposure.

The HD/CFA assay was carried out in two sets. In the first set, the cells were plated out at cloning densities in tissue culture dishes without feeder cells (Figure 6C), whereas in the second set the cells were plated out in dishes containing irradiated (10 Gy) HCT116 feeder cells (data not shown). The outcomes of the two sets of experiments were similar, except that the presence of irradiated feeders (100,000 cells per 60-mm dish) resulted in an ~30% decrease in the clonogenic potential of HCT116 cells. The latter observation is perhaps not surprising because HCT116 cells are p53 proficient and radiation exposure is known to trigger the p53-mediated release of numerous cytostatic/cytotoxic factors into the culture medium (22).

Evaluation of the radiosensitivity of human breast cancer cell lines by the HDS assay

Using the HDS assay, we next determined the response of a panel of seven breast cancer cell lines to ionizing radiation. These cell lines were not evaluated by the CFA assay for two reasons. First, as noted above, these cell lines (which included the widely used MCF7 and SKBR3 lines) did not yield a good single-cell suspension following exposure to trypsin/EDTA. Second, the cloning efficiency of some breast cancer cell lines was too low (<1%; Table 1) to obtain reproducible data in the CFA assay.

The results of multiple HDS experiments performed with these breast cancer cell lines following exposure to ionizing radiation are averaged in Figure 7. The A172 glioma, SK-N-SH neuroblastoma and HCT116 colon carcinoma cell lines, which showed a degree of radiosensitivity comparable to normal fibroblasts, were included in these experiments as a reference. Each of the breast cancer cell lines examined showed a high degree of radioresistance when compared to HCT116, SK-N-SH and A172 cells.

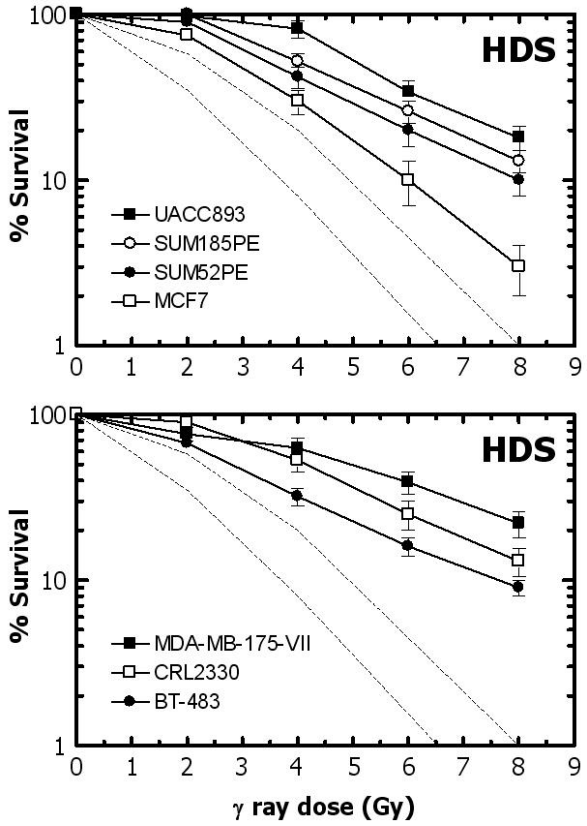

Figure 7. Evaluation of radiation-induced cytotoxicity by the HDS assay in the indicated cell lines. The dotted curves mark the range of responses of A172, SK-N-SH and HCT116 cell lines that are known to show a degree of radiosensitivity comparable to normal human fibroblasts. The mean (±SE) values of at least two experiments (each run in triplicate) are presented for each cell line.

DISCUSSION

The CFA assay has been invaluable for determining the cytotoxic effects of environmental and man-made genotoxic agents in certain mammalian cell types. Primary human fibroblast cultures, for example, are particularly suitable for this assay because they yield a good single-cell suspension following exposure to trypsin/EDTA and require relatively long times (>12 h) to resume cycling after seeding in tissue culture dishes at cloning densities. Some tumor cell lines (e.g., the panel of breast cancer cell lines used in the present study), however, do not yield a reasonable quality of single-cell suspension following exposure to trypsin/EDTA (see below). Thus, the degree of cell killing determined by the CFA assay with such cell lines would be skewed towards resistance, unless the extent of multiplicity (aggregates of two or more cells) can be accurately determined prior to genotoxic treatment, and the data are corrected by the use of appropriate mathematical models.

In addition, it has long been recognized that the experimental conditions for clonogenic assays are far different from the native tumor microenvironment, e.g., because in such assays the impact of intercellular communication on the cytotoxic effects of genotoxic agents is not recapitulated (7,23). Several colorimetric viability-based assays have been developed over the past two decades which can be performed with high-density cultures and thus incorporate some features of intercellular communication (24 and refs. therein). Although such colorimetric assays are indispensable for high throughput drug screening studies (10,25,26), their use in exploring cancer cell responses to genotoxic agents is limited because they are performed at relatively short times (typically 48 h) after genotoxic treatment and thus primarily score loss of viability associated with

early apoptosis and necrosis. It is now well established that exposure of many human cancer cell lines to ionizing radiation and chemotherapeutic agents triggers a permanent growth arrest through the process of accelerated senescence in a proportion of the cell population (1,2,4,27,28). Senescent cancer cells retain cell membrane integrity and remain metabolically active for prolonged times (over 7 days) post-treatment (4,32), and would therefore be scored as "survivors" in short-term assays of loss of cellular viability, including the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay which has emerged as a favored method for assessment of cytotoxicity (28-30).

The HDS assay reported here circumvents some of the shortcomings associated with the conventional cytotoxicity assays. Importantly, the HDS assay is not influenced by the quality of the single-cell preparation and can be performed with cell lines (e.g., SUM185PE) exhibiting extremely low cloning efficiencies. In addition, in the HDS assay the cells are plated out at high densities to facilitate cell-to-cell interactions before and for 24 h after genotoxic treatment. Like the CFA assay, the HDS assay involves incubation of cells for prolonged times (7 days and beyond) after genotoxic treatment, and therefore provides an integrated readout of all cytotoxic responses, encompassing early apoptosis and delayed permanent growth arrest (e.g., accelerated senescence).

Whereas the clonogenic cytotoxicity assay solely measures the failure of individual cells to form macroscopic colonies (i.e., aggregates of >50 cells), the HDS assay takes into account not only the loss of clonogenic potential of the cells, but also their rate of proliferation consequent to genotoxic treatment. Despite this difference, the degree of radiosensitivity measured by the HDS assay was found to be similar to that measured by the CFA assay. This observation was made with each of the four human solid tumor-derived cell lines that we examined, which included SK-N-SH and HCT116 that exhibit marked differences in their growth rates (population doubling times, ~30 h and ~16 h, respectively). In addition, we observed similar degrees of cisplatin sensitivity in a representative cancer cell line (MDA-MB-435s) when evaluated by these two assays. On the other hand, as expected, there was no relationship between the degree of cytotoxicity measured by a short-term (viability) assay and long-term (CFA and HDS) assays in response to radiation or cisplatin exposure. These results suggest that: (i) the HDS and CFA assays generate comparable outcomes in terms of the measurement of cytotoxicity; (ii) the HDS assay is not influenced by the growth rate of cancer cells; and (iii) early apoptotic/necrotic cell death does not contribute significantly to the overall cytotoxic effects of ionizing radiation and cisplatin in these examples.

Our studies involving the use of pharmacological inhibitors of CaMKII and PI3K to modulate the cytotoxic effect of ionizing radiation suggested that the HDS assay can give quite different results from the CFA and the viability assays (Figure 6). The basis for this conclusion is that treatment of HCT116 cells with KN62 (a CaMKII inhibitor) did not produce any significant influence on their radiosensitivity when evaluated by the CFA or viability assays, whereas treatment with this drug markedly potentiated their radiosensitivity when measured by the HDS assay. In addition, wortmannin, a potent inhibitor of PI3K and PI3K-like protein kinases (e.g., ATM) (31, 32), which also abrogates the radiation-triggered activation of CaMKII (21), produced a greater influence on the radiosensitivity of HCT116 cells when evaluated by the HDS assay than the CFA or viability assays. Among the three assays, therefore, the HDS assay appears to be the most suitable for identifying agents that might potentiate the cytotoxic effects of ionizing radiation *in vivo* and probably other cancer therapeutic agents.

In a previous study from our laboratory, exposure of HCT116 cultures to ionizing radiation was shown to result in growth arrest through accelerated senescence in a high proportion of the cells (2). In a preliminary experiment, we observed that treatment of HCT116 cultures with wortmannin or KN62 markedly increased their propensity to undergo accelerated senescence in response to radiation exposure (unpublished observations). As a working model, therefore, we hypothesize that the potentiating effects of these inhibitors on radiation-triggered senescence might be manifested to a greater extent in high-density cultures than in isolated cells, which could account for the

differential outcomes of the HDS and CFA assays shown in Figure 6.

Although the clonogenic cytotoxicity assay has been universally used with solid tumor-derived cell lines, either with or without the addition of soft agar, the quality of the single-cell suspension (which is critical for this assay) has rarely been reported. Because cancer cells generally do not cease to proliferate immediately after detachment from culture dishes, we (2,19,33) and others (34-36) have kept the time interval between cell seeding and genotoxic treatment as short as possible (typically ~4 h in our experiments). In some other studies, however, the cells were incubated for longer times (e.g., overnight) before genotoxic exposure (37-39). We determined the quality of the single-cell suspension under these conditions for twelve human cancer cell lines. At ~4 h after seeding, cultures of four cell lines (A172, SK-N-SH, HCT116, and MDA-MB-435s) yielded a reasonable distribution of single cells, with ~80-90% of the cells existing in isolation. Thus, the clonogenic survival data obtained with these cell lines under similar conditions (genotoxic treatment before or shortly after seeding) should be considered relatively unbiased. On the other hand, for all of the breast carcinoma cell lines that we examined the majority (>50%) of cells existed in clusters of two or more cells when determined shortly after seeding. At 16 h after seeding, cultures of all cancer cell lines shown in Table 1 contained >45% of cells in clusters. Undoubtedly, a given cancer cell line grown in different laboratories can yield a different quality of single cell suspension as a result of, for example, differences in culture conditions, method of cell detachment and reseeding, as well as differences in the pattern of gene expression, as has been documented for the MCF7 cell line (40). Our results, nonetheless, underscore the importance of carefully assessing the quality of single cell preparation prior to genotoxic treatment in each clonogenic experiment involving cancer cell lines.

We reported previously that the human cancer cell lines A172, SK-N-SH and HCT116 show a degree of radiosensitivity in the CFA assay that is comparable to that of normal human fibroblasts (2, 19). In the present study we have confirmed these results using the CFA assay. Employing the HDS assay, we have further demonstrated that all seven human breast cancer cell lines examined here exhibit a high degree of radioresistance when compared to A172, SK-N-SH and HCT116 cells. These results warrant further studies to establish the generality of this intriguing observation, and also to determine the basis for the radioresistant phenotype in an attempt to design pharmacological approaches for selectively potentiating the sensitivity of breast cancer cells to conventional therapies. The HDS assay reported here should be instrumental in this regard.

CONCLUSION

Although cancer therapy has witnessed many exciting developments over the years, cure of cancer still remains as elusive as understanding the disease itself. New strategies to combat cancer are being developed, one of the most exciting of which is the use of pharmacological modulators of different signal transduction pathways either alone or in combination with conventional cancer therapeutic agents. Based on evidence provided in this communication, in concert with the emerging evidence implicating a key role for intercellular communication in determining the cellular response to genotoxic stress, model systems that incorporate aspects of intercellular communication, such as the HDS assay developed by us, should be instrumental in these and related studies. While the HDS assay measures the sum of all modes of cytotoxic events, it is not influenced by artifacts such as poor quality of the single-cell preparations and poor cloning efficiencies often associated with the conventional CFA assay. In short, the novel HDS assay facilitates the evaluation of cancer-cell responses to therapeutic agents under conditions that are more relevant to the native tumor microenvironment than those used in conventional *in vitro* cytotoxicity assays.

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