Impact of Chemotherapeutic Drugs on Cancer Cell Proliferation, Morphology and Metabolic Activity

Razmik Mirzayans*, Bonnie Andrais, and David Murray
Department of Oncology, University of Alberta, Canada

Abstract
Solid tumors and solid tumor-derived cell lines typically contain a small proportion of giant cells with a highly enlarged nucleus or multiple nuclei. Although giant cells enter a state of dormancy and cease to proliferate, they exhibit resistance to anticancer agents, acquire growth-promoting factors, and can give rise to progeny with stem cell-like properties that can repopulate the tumor. In the present study we determined the response of the MDA-MB-231 breast carcinoma cell line to chemotherapeutic drugs in terms of proliferation, morphology and metabolic activity. We report that treatment with moderate (non-toxic) concentrations of oxaliplatin (≤10 µM) or paclitaxel (≤20 nM) triggers proliferation arrest which largely reflects the development of giant cells that remain adherent to the culture dish, retain viability, and exhibit the ability to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to its water insoluble formazan metabolite. The single-cell MTT assay demonstrated that the metabolic activity per cell is much higher (>3 times) in drug-treated cultures, which are enriched with giant cells, than in sham-treated controls. These results give credence to a growing body of evidence suggesting that targeting dormant cancer cells could be a critical strategy for minimizing the chance of relapse following conventional cancer treatment.

ABBREVIATIONS
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DAPI: 4’,6-diamidino-2-phenylindole

INTRODUCTION
Tumours are typically heterogeneous and contain cancer cells with differing sizes and genomic contents (reviewed in [1]). The bulk of cancer cells within the majority (~90%) of solid tumors and solid tumor-derived cell lines are aneuploid. Such cells have an alteration (often gain) of chromosome number which is not a multiple of the diploid (2n) complement. A small proportion of cancer cells, however, are considered “giants” by virtue of their markedly increased cytoplasmic content. The majority of giant cancer cells contain either a highly enlarged nucleus (reflecting polyploidy) or multiple nuclei. Such giant cancer cells are observed not only in tumor-derived cell lines in vitro [2-4], but also in animal models [5] as well as specimens from cancer patients [6-11].

If cancer cells encounter certain types of stress, they can become highly enlarged (e.g., reflecting polyploidy/multinucleation) and enter a state of dormancy (reviewed in [12]). Given that giant (dormant) cancer cells cease to proliferate or proliferate at a very slow rate, they are often scored as “dead” in the conventional preclinical assays that are performed with cultured cells or animal models. However, giant cancer cells remain viable and can give rise to progeny with stem cell-like properties.

We recently reported studies with human solid tumor-derived cell lines with differing p53 status and demonstrated that exposure to moderate doses of ionizing radiation (≤8 Gy) or the chemotherapeutic drug cisplatin (≤10 µM) predominantly triggers proliferation arrest rather than cell death, and that proliferation arrested cells exhibit enlarged morphology and remain adherent to the culture dish for long times (weeks) post-treatment. Furthermore, single-cell observations demonstrated that virtually all enlarged cells that remain adherent for the duration of the experiment retain viability and exhibit the ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) [13-15]. In the current study we report that treatment of the MDA-MB-231 breast carcinoma cell line with the chemotherapeutic drugs oxaliplatin and paclitaxel also results in the development of highly enlarged (giant) cells that retain viability and metabolic activity.

Collectively, these results reinforce the notion that targeting dormant cancer cells is a promising strategy for preventing disease recurrence following conventional cancer treatment.

MATERIALS AND METHODS
Cells and Culture Conditions
The MDA-MB-231 breast carcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). This cell line expresses mutant p53 which is auto-phosphorylated (e.g., on Ser15) and exhibits gain-of-function properties [16,17].
Cells were cultured as monolayers as described [13]. All cultures were free of *Mycoplasma* contamination.

**Reagents**

A mouse monoclonal antibody to β-actin (C4) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An Alexa Fluor 488 secondary antibody (goat anti-mouse IgG) was purchased from Invitrogen (Eugene, OR, USA). The fluorescent tracer 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), the vital dye trypan blue (Millipore Sigma, Oakville, Ontario, Canada), the tetrazolium dye MTT (Roche Diagnostics, Penzberg, Germany), and the chemotherapeutic drugs (Millipore Sigma) were used as recommended by the manufacturers.

**Treatment with Genotoxic Agents**

Treatment with chemotherapeutic drugs was performed by incubating cells in growth medium containing the indicated concentrations of a drug for 3 days. The amount of solvent (dimethyl sulfoxide) in the treatment medium was always <0.1% (vol/vol).

**Proliferation Inhibition Assay**

Cells were plated in 60-mm dishes (10⁵ cells/5 mL medium/dish) and incubated overnight. After treatment with a chemotherapeutic drug (or sham-treatment) for 3 days, adherent cells were harvested by the use of trypsin and counted by a cell counter (Coulter, Hialeah, FL, USA).

**Immunofluorescence Assay**

Cells on coverslips were washed with PBS, fixed in methanol (see below) at -20°C, incubated with 0.5% Triton X-100 for 5 min, and washed again with PBS. Cells were incubated with PBS containing 5% nonfat dry Carnation milk for 30 min, stained with anti-β-actin antibody (C4) and then with AlexaFluor 488 goat anti-mouse IgG. Antibodies were used at dilutions suggested by the suppliers in PBS containing 5% nonfat dry milk. Coverslips were incubated on 30-µl drops of each antibody on Parafilm® for 1 h at room temperature in a humidified chamber. After each antibody incubation, cells were washed with PBS-TWEEN 20 (3 min) and PBS (three times, 3 min each). Cells were examined with an Axiophot microscope (Carl Zeiss, Jena, Germany) using a band filter of 450–490 nm. Images were captured using the Metamorph program and processed using Adobe Photoshop.

Pilot experiments indicated that fixing cells in methanol for short times (~20 min) or long time (months) did not influence the intensity of immunostaining. For long time fixing and storage, methanol was added to dishes as needed to avoid drying of cells.

**Single-Cell MTT Assay**

Metabolic activity of individual cells was determined using the single-cell MTT assay as described [13,18]. To this end, cells were plated in 35-mm dishes (~20,000 cells/2 mL medium/dish) and incubated overnight. The cells were then incubated for 3 days with either fresh medium (controls) or with medium containing different concentrations of a chemotherapeutic drug. The treatment (or sham-treatment) medium was replaced with fresh medium containing MTT (final concentration, 0.5 mg/mL) and the cells were returned to the incubator. After incubation with MTT for ~1 h, the medium was removed and the cells were air dried. A small drop (~20 µl) of glycerol/PBS (9:1 vol/vol) mounting medium was placed in the middle of each dish, and a coverslip was carefully lowered onto the drop of mounting medium in such a way as to prevent the formation of bubbles. Bright-field microscopy with a 20x objective was performed either immediately or after storing the dishes in the dark at room temperature.

**RESULTS AND DISCUSSION**

We have developed an experimental approach to determine the effect of genotoxic stress on different parameters in the same culture of a solid tumour-derived cell line under identical experimental conditions [13-15]. These include proliferation arrest by direct cell counting coupled with single-cell analysis of cell morphology, viability, and metabolic activity. The advantages of such optimized single-cell assays over the conventional cell population averaged assays (e.g., colorimetric/fluorimetric assays performed in a multiwell plate format) are discussed elsewhere [18]. Employing this approach, we have reported the responses of a large number of cancer cell lines to ionizing radiation [13,14] and of two cell lines (MDA-MB-231 breast carcinoma and A549 lung carcinoma) to cisplatin [15]. Below we present studies with MDA-MB-231, one of the most commonly used breast cancer cell lines in medical research laboratories, following treatment with moderate (non-toxic) concentrations of oxaliplatin and paclitaxel; for comparison, some of our published results obtained following treatment with cisplatin are also reproduced. Our intent is to illustrate that the fate of MDA-MB-231 cultures is similar irrespective of the type of genotoxic agent used (ionizing radiation, cisplatin, oxaliplatin and paclitaxel). This fate manifests as a proliferation arrest (rather than cell death) which is largely associated with the development of giant cells that remain adherent to the culture dish, retain cell membrane integrity, and metabolize MTT.

The results of proliferation arrest and viability (trypan blue exclusion) assays following incubation with the chemotherapeutic drugs for 3 days are presented in Figure 1. A 50% proliferation arrest (IC₅₀) was observed after treatment with ~2 μM cisplatin, ~2 μM oxaliplatin, and ~8 nM paclitaxel. The highest concentrations of these drugs that we used resulted in an inhibition of cell proliferation of >90%; virtually all adherent cells maintained cell membrane integrity as judged by the trypan blue-exclusion assay. Under these conditions we observed cell detachment in only <5% of cells (data not shown).

The results obtained by the actin/DAPI immunostaining and the single-cell MTT assays are presented in Figures 2-4. A 3-day incubation with a chemotherapeutic drug resulted in the development of giant cells (Figures 2), all of which metabolized MTT (Figures 3 and 4). MTT metabolites are water insoluble; they develop into giant cells (Figures 2), all of which metabolized MTT (Figures 3 and 4). MTT metabolites are water insoluble; they remain adherent to the culture dish, retain cell membrane integrity, and metabolize MTT. The results of proliferation arrest and viability (trypan blue exclusion) assays following incubation with the chemotherapeutic drugs for 3 days are presented in Figure 1. A 50% proliferation arrest (IC₅₀) was observed after treatment with ~2 μM cisplatin, ~2 μM oxaliplatin, and ~8 nM paclitaxel. The highest concentrations of these drugs that we used resulted in an inhibition of cell proliferation of >90%; virtually all adherent cells maintained cell membrane integrity as judged by the trypan blue-exclusion assay. Under these conditions we observed cell detachment in only <5% of cells (data not shown).

The results obtained by the actin/DAPI immunostaining and the single-cell MTT assays are presented in Figures 2-4. A 3-day incubation with a chemotherapeutic drug resulted in the development of giant cells (Figures 2), all of which metabolized MTT (Figures 3 and 4). MTT metabolites are water insoluble; they remain adherent to the culture dish, retain cell membrane integrity, and metabolize MTT. The results of proliferation arrest and viability (trypan blue exclusion) assays following incubation with the chemotherapeutic drugs for 3 days are presented in Figure 1. A 50% proliferation arrest (IC₅₀) was observed after treatment with ~2 μM cisplatin, ~2 μM oxaliplatin, and ~8 nM paclitaxel. The highest concentrations of these drugs that we used resulted in an inhibition of cell proliferation of >90%; virtually all adherent cells maintained cell membrane integrity as judged by the trypan blue-exclusion assay. Under these conditions we observed cell detachment in only <5% of cells (data not shown).

The results obtained by the actin/DAPI immunostaining and the single-cell MTT assays are presented in Figures 2-4. A 3-day incubation with a chemotherapeutic drug resulted in the development of giant cells (Figures 2), all of which metabolized MTT (Figures 3 and 4). MTT metabolites are water insoluble; they remain adherent to the culture dish, retain cell membrane integrity, and metabolize MTT. The results of proliferation arrest and viability (trypan blue exclusion) assays following incubation with the chemotherapeutic drugs for 3 days are presented in Figure 1. A 50% proliferation arrest (IC₅₀) was observed after treatment with ~2 μM cisplatin, ~2 μM oxaliplatin, and ~8 nM paclitaxel. The highest concentrations of these drugs that we used resulted in an inhibition of cell proliferation of >90%; virtually all adherent cells maintained cell membrane integrity as judged by the trypan blue-exclusion assay. Under these conditions we observed cell detachment in only <5% of cells (data not shown).

The results obtained by the actin/DAPI immunostaining and the single-cell MTT assays are presented in Figures 2-4. A 3-day incubation with a chemotherapeutic drug resulted in the development of giant cells (Figures 2), all of which metabolized MTT (Figures 3 and 4). MTT metabolites are water insoluble; they remain adherent to the culture dish, retain cell membrane integrity, and metabolize MTT.
Figure 1 Effects of the indicated chemotherapeutic drugs on the proliferation and viability of MDA-MB-231 cells, determined by direct cell counting and the trypan blue-exclusion assays, respectively. Cells were incubated with a drug for 3 days and then evaluated. Arrows indicate drug concentrations resulting in 50% proliferation arrest. Bars, standard error (SE) of at least 3 experiments, each run in duplicates. TB, trypan blue. The results obtained following cisplatin treatment are reproduced from Mirzayans et al. [15].

Figure 2 Fluorescence images showing the morphology of MDA-MB-231 cells after treatment with the indicated drugs or sham treatment (control). For treatments, cells were incubated with cisplatin (10 µM), oxaliplatin (10 µM), or paclitaxel (20 nM) for 3 days.

Figure 3 Bright-field microscopy images showing the metabolic activity of MDA-MB-231 cells before (control) and after incubation with cisplatin (10 µM), oxaliplatin (10 µM), or paclitaxel (20 nM) for 3 days. Metabolic activity was measured by the ability of the cells to convert the MTT reagent to its formazan metabolite (dark granules and crystals). Images were acquired after incubation of cells with MTT for ~1 h.

image) are converted to gray scale and then inverted (e.g., Figure 5A, right image). This manipulation of images results in dark (black) backgrounds and bright (white) signals (reflecting cellular MTT formazan granules and crystals) that are ideal for image analysis. The level of metabolic activity per cell (estimated from signal intensity in the “region of interest”) was much higher (>3 fold) for chemotherapeutic drug-treated cultures (which were highly enriched with giant cells) than for sham-treated controls (Figure 5B).

CONCLUSION

Our studies reported here and elsewhere [13-15] demonstrate
Figure 4 Percentages of giant cells and MTT-positive cells in cultures of the MDA-MB-231 cell line before (control) and after treatment with cisplatin (10 µM), oxaliplatin (10 µM), or paclitaxel (20 nM) for 3 days. Only adherent cells were evaluated. Bars, SE of at least 3 experiments, each run in duplicate. More than 200 cells were evaluated for each treatment condition. The difference between control and drug-treated cultures for the frequency of giant cells was significant (p<0.05) as determined by the student’s t-test. Cells with a diameter and nuclear content of at least four times greater than those of bulk control (small) cells were scored as giants. The results obtained following cisplatin treatment are reproduced from Mirzayans et al. [15].

Figure 5 (A) Representative images of MDA-MB-231 cells used for evaluating metabolic activity (see text for details). Blue and red ovals mark typical regions of interest (reflecting MTT metabolites) and corresponding background regions used for image analysis, respectively. (B) Densitometric evaluation of MTT metabolite level (metabolic activity) in control (sham-treated) cultures and in cultures that were incubated for 3 days with cisplatin (10 µM), oxaliplatin (10 µM) or paclitaxel (20 nM). Densitometry was performed using the Andor IQ analysis software package of Kinetic Imaging (Primer Biotech, Weston, Ontario, Canada). Signal intensity for regions of interest was subtracted from that of the corresponding background regions. Mean values of at least 30 cells are presented for each sample. The difference between control and drug-treated cultures for signal intensity in regions of interest was highly significant (p<0.001) as determined by the student’s t-test. Bars, SE. ROI, region of interest; BG, background. The results obtained following cisplatin treatment are reproduced from Mirzayans et al. [15].

that cancer therapeutic agents administered at moderate, clinically relevant doses can promote the development of giant cells at a high frequency in solid tumor-derived cell lines and that virtually all giant cells that remain adherent to the culture dish post-treatment retain cell membrane integrity and exhibit the ability to metabolize MTT. Considering that similar findings have been reported in in-vivo systems (reviewed in [12]), including the observation of tumorigenic/chemotherapy-resistant giant cells in specimens from cancer patients (e.g., [8]), these findings raise the major concern that giant cancer cells might contribute to metastasis, therapy resistance and disease relapse in patients. The single-cell assays employed here should thus be valuable for identifying pharmacological agents capable of killing giant cancer cells before they will have the opportunity to promote tumor repopulation.

ACKNOWLEDGEMENTS

This work was supported by the Canadian Breast Cancer Foundation–Prairies/North West Territories region.

REFERENCES

2. Illidge TM, Cragg MS, Fringes B, Olive P, Erenpresia JA. Polyploid giant


