Published in final edited form as:

Int J Radiat Biol. 2012 December ; 88(12): 1028–1038. doi:10.3109/09553002.2012.683511.

Induction of lethal bystander effects in human breast cancer cell cultures by DNA-incorporated Iodine-125 depends on phenotype

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Abstract

Purpose—This study uses a three-dimensional cell culture model to investigate lethal bystander effects in human breast cancer cell cultures (MCF-7, MDA-MB-231) treated with ¹²⁵I-labeled 5-iodo-2'-deoxyuridine (¹²⁵IdU). These breast cancer cell lines respectively form metastatic xenografts in nude mice in an estrogen-dependent and independent manner.

Materials and methods—In the present study, these cells were cultured in loosely-packed three-dimensional architecture in a Cytomatrix™ carbon scaffold. Cultures were pulse-labeled for 3 h with ¹²⁵IdU to selectively irradiate a minor fraction of cells, and simultaneously co-pulse-labeled with 0.04 mM 5-ethynyl-2'-deoxyuridine (EdU) to identify the radiolabeled cells using Click-iT® EdU and flow cytometry. The cultures were then washed and incubated for 48 h. The cells were then harvested, serially diluted, and seeded for colony formation. Aliquots of cells were subjected to flow cytometry to determine the percentage of cells labeled with ¹²⁵IdU/EdU. Additional aliquots were used to determine the mean ¹²⁵I activity per labeled cell. The percentage of labeled cells was about 15% and 10% for MCF-7 and MDA-MB-231 cells, respectively. This created irradiation conditions wherein the cross-dose to unlabeled cells was small relative to the self-dose to labeled cells. The surviving fraction relative to EdU-treated controls was measured.

Results—Survival curves indicated significant lethal bystander effect in MCF-7 cells, however, no significant lethal bystander effect was observed in MDA-MB-231 cells.

Conclusions—These studies demonstrate the capacity of ¹²⁵IdU to induce lethal bystander effects in human breast cancer cells and suggest that the response depends on phenotype.

Keywords

Auger electron; I-125; iododeoxyuridine; bystander effects; breast cancer; human tumour cells

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Prediction of biological responses to radiopharmaceuticals used in medicine, and to radioactive materials internalized by members of the general population through accidental or malevolent means, is challenging (Howell et al. 2006). This is partly due to several factors, such as non-uniformities in radionuclide distribution at the macroscopic, cellular, and subcellular levels (Humm 1986, Jönsson et al. 1992, Neti and Howell 2003, 2004, 2006, Akudugu et al. 2011). The different mechanisms by which radionuclides decay and the different radiation types that are correspondingly emitted (Auger electrons, beta particles, alpha particles, etc) introduce additional levels of complexity in understanding biological responses to internal radioactivity. The patterns of radiation exposure that arise as a consequence of these properties can be highly non-uniform, and lead to conditions that are favorable for eliciting radiation-induced bystander effects. As summarized in a recent review (Blyth and Sykes 2011), many studies have provided strong evidence to support the existence of a variety of radiation-induced bystander effects (Brooks et al. 1974, Nagasawa and Little 1992, Mothersill and Seymour 1997, Azzam et al. 1998, Wright 1998, Zhou et al. 2000, Autavaporn et al. 2011, Buonanno et al. 2011). These effects may be toxic or protective in cells that are not directly irradiated; they can result from a variety of signals from neighboring irradiated cells (Wright and Coates 2006). Although bystander effects have been mainly demonstrated for exposure to high linear energy transfer (LET) radiations, they have also been observed after low-LET radiation exposures (Mothersill and Seymour 1997).

Although there is now a plethora of bystander studies in the literature (Blyth and Sykes 2011), there have been relatively few investigations that have involved incorporated radioactivity (Brooks et al. 1974, Bishayee et al. 1999, 2001, Howell and Bishayee 2002, Xue et al. 2002, Gerashchenko and Howell 2004, 2005, Boyd et al. 2006, 2008, Kishikawa et al. 2006). In 3-dimensional tightly-packed multicellular cluster models, deoxyribonucleic acid (DNA)-incorporated ^{125}I -labeled 5-iodo-2'-deoxyuridine (^{125}IdU) and tritiated thymidine ($^3\text{HTdR}$) resulted in increased cytotoxicity in Chinese hamster cells (Bishayee et al. 1999, 2001, Howell and Bishayee 2002, Persaud et al. 2005, 2007). The radiochemicals ^{125}IdU and $^3\text{HTdR}$ are well-known to impart radiotoxicity akin to high- and intermediate-LET radiations, respectively. Using human colon LS174T adenocarcinoma cells labeled with ^{125}IdU or ^{123}IdU , it was demonstrated that while DNA-incorporation of ^{125}I inhibits growth in bystander cells (Xue et al. 2002), incorporation of ^{123}I stimulates growth in bystander cells in vivo (Kishikawa et al. 2006). A similar proliferative bystander response had been observed in $^3\text{HTdR}$ labeled liver epithelial cells (Gerashchenko and Howell 2004), a response that depended on the number of adjacent labeled cells (Gerashchenko and Howell 2005). However, bystander effects, as measured by the magnitude of G_1 arrest, micronucleus formation, or changes in mitochondrial membrane potential, were not observed in normal AG1522 human fibroblasts grown in 3-dimensional architecture in a CytomatrixTM carbon scaffold and treated with ^3H -deoxycytidine (Pinto et al. 2006, 2010). In summary, bystander responses elicited as a consequence of irradiation by internal radionuclides have been mixed and depend on the bystander signal producer and recipient cell types, experimental model, radionuclide, radiopharmaceutical, and fraction of

cells labeled with the radiopharmaceutical. Therefore, further elucidation of the conditions under which incorporated radioactivity can cause bystander effects is warranted.

In the present work, studies were conducted to determine if ^{125}IdU can cause bystander effects in human breast cancer cells. To achieve this, MDA-MB-231 or MCF-7 were pulse-labeled concomitantly with ^{125}IdU and 5-ethynyl-2'-deoxyuridine (EdU), and radiation-induced cell death was measured using the colony forming assay. Flow cytometry techniques were used to detect EdU as a surrogate for selectively identifying cells that had incorporated ^{125}IdU . The dependence of bystander effects on the phenotype of human breast cancer, fraction of ^{125}IdU -labeled cells, and the radiation absorbed dose to the labeled and unlabeled cells was evaluated. The implications of ^{125}IdU -induced bystander effects in risk estimation for individuals accidentally exposed internal radioactivity, and for patients undergoing radionuclide based therapy and/or routine nuclear medicine diagnostic procedures are also discussed.

Materials and methods

Cell lines and routine cell culture

The MCF-7-luc-F5 (MCF-7) and MDA-MB-231-luc-D3H1 (MDA-MB-231) human breast tumor cells, obtained from Caliper Life Sciences (Hopkington, MA, USA), were derived from pleural effusions taken from women with invasive ductal carcinoma and adenocarcinoma, respectively. They respectively form metastatic xenografts in nude mice in an estrogen-dependent and independent manner. Growth media and supplements were purchased from Mediatech (Manassas, VA, USA), unless otherwise stated, and the MCF-7 and MDA-MB-231 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640, cat no. 10-040-CV, lot no. 10040486) and minimum essential medium (MEM, cat no. 10-010-CV, lot no. 10010244), respectively. The media contained 10% fetal calf serum (cat no. 10437, lot no. 539574) and 2 mM L-glutamate (cat no. 25030, lot no. 624236) from Gibco[®] Life Technologies (Grand Island, NY, USA), and with 100 units/ml penicillin, 100 µg/ml streptomycin (cat no. 30-002, lot no. 30002188), 10 mg/ml non-essential amino acids (cat no. 25-025, lot no. 25025084). Cells were grown as monolayers in Becton Dickinson (BD, Franklin Lakes, NJ, USA) Falcon 175-cm² flasks (cat no. 353028) and were used for experiments (passages 10–18) upon reaching 80–90% confluence.

Radiochemicals and quantification of radioactivity

The radiochemical ^{125}IdU was either obtained from Perkin Elmer Life Sciences (Waltham, MA, USA) in a solution of water:ethanol:methanol (53:46:1) at a specific activity of 81.4 TBq/mmol (cat no. NEX072) or was synthesized using a kit (kindly provided by K. Wang and A.I. Kassis) according to previously reported procedures (Foulon et al. 1995). Radiochemical purity was checked with reverse phase hydrocarbon impregnated thin layer chromatography plates (cat no. 52521) from Analtech (Newark, DE, USA) and high performance liquid chromatography grade water as the solvent. The activity in ^{125}I samples was determined with a calibrated Packard Cobra Quantum Model E5003 automatic gamma counter (Meriden, CT, USA). The ^{125}I overall counting efficiency was 61%.

Three-dimensional cultures with radiolabeled cells

The 3-dimensional in vitro Cytomatrix™ cell culture model using Cytomatrix™ carbon scaffolds (cat no. 23279) from Ultramet (Pacoima, CA, USA) as described previously for normal AG1522 human fibroblasts (Pinto et al. 2006, 2010), was not suitable for growing the breast cancer cells used in the current study. In the attempts to culture MDA-MB-231 and MCF-7 cells in the Cytomatrix™ scaffold, the cells simply fell off the carbon scaffold upon their division, possibly due to poor anchorage even when the Cytomatrix™ units were precoated with fibronectin. Therefore, this cell culture model was modified in two different ways (Protocol A and Protocol B) as described below.

Protocol A—The cells were trypsinized, resuspended in complete growth media, and were centrifuged for 5 min at 200 g. Cell pellets were resuspended in Matrigel™ (BD, cat no. 354234) on ice (30×10^6 cells per ml). Aliquots of 200 μ l containing 6×10^6 cells were pipeted onto Cytomatrix™ carbon scaffolds in CELLSTAR® sterile ultralow attachment 48-well plates (cat no. 677 - 102) from Greiner Bio-One (Monroe, NC, USA) on ice. Observation with inverted and dissecting microscopes confirmed that cells distributed throughout the Cytomatrix™. The plates were incubated for 1 h at 37°C with 5% CO₂ and 95% air to allow the Matrigel™ to set. The Matrigel™/Cytomatrix™/cell units were then transferred into new 48-well plates, and the cultures were pulse-labeled for 3 h with ¹²⁵IIdU (0–1.85 MBq/ml) to selectively irradiate a minor fraction of cells, and simultaneously co-pulse-labeled with 0.04 mM 5-ethynyl-2'-deoxyuridine (EdU) to identify the radiolabeled cells using the Molecular Probes® Click-iT® EdU Alexa Fluor® 488 cell proliferation kit (Life Technologies) and flow cytometry. EdU and ¹²⁵IIdU are similarly and non-competitively incorporated into DNA during S-phase. Control tubes contained 0 MBq/ml ¹²⁵IIdU and 0.04 mM EdU. The total media volume for labeling was 400 μ l. After pulse-labeling, aliquots (5 μ l) of the culture media were transferred in triplicate into 12 $\times$ 75-mm tubes for determination of the radioactivity concentrations. The cultures were then thoroughly washed and split into two sets for the assessment of ¹²⁵IIdU/EdU incorporation and cytotoxicity, respectively.

To determine the mean radioactivity per cell and the proportions of cells that incorporated ¹²⁵IIdU/EdU, cells were immediately retrieved from the Matrigel™/Cytomatrix™ units using MatriSpense™ cell recovery solution (BD, cat no. 354253) as per supplier's guide. Cells were washed, resuspended in 3.5 ml of medium, and were passed four times through a 20 G needle. Aliquots (50 μ l) of cell suspensions were transferred in triplicate into 12 $\times$ 75-mm tubes for subsequent determination of the mean radioactivity per cell (see below). The remaining cells were used to determine the cell concentration with a Beckman Coulter Model Z2 (Brea, CA, USA), and for the assessment of ¹²⁵IIdU/EdU incorporation. For toxicity measurements, cultures were transferred into sterile Corning® Costar® ultralow attachment 24-well plates (cat no. 3473, Lowell, MA, USA) containing 2 ml of pre-warmed growth media, immediately after pulse-labeling with ¹²⁵IIdU and EdU, and were incubated for 48 h. After this irradiation period, the cells were harvested from the Matrigel™/Cytomatrix™ units using MatriSpense™ and trypsin as needed, syringed, counted, serially diluted, and plated in Falcon 60 $\times$ 15 mm tissue culture dishes for colony formation.

Protocol B—Trypsinized cells were resuspended in complete suspension growth media, passed four times through a 20 G needle, and were counted. Aliquots of 6×10^6 cells were transferred into BD Falcon 17×120 -mm polypropylene conical tubes (cat no. 352097). Two sets of tubes were prepared for assessing $^{125}\text{IdU}/\text{EdU}$ incorporation and cell survival, respectively. The cells were centrifuged for 5 min at 200 g, and the pellets were resuspended in media (400 μl) containing ^{125}IdU and EdU as described above. The tubes were then placed on a Bellco Biotechnology orbital shaker (Vineland, NJ, USA) for 3 h at 37°C , 5% CO_2 , 95% air. Following the labeling, the suspension cell cultures were centrifuged and 5- μl aliquots of the supernatant were transferred in triplicate into 12×75 -mm tubes to determine radioactivity concentrations. The cells were then washed five times by centrifugation (each with 10 ml of medium).

After the last wash, cell pellets of one set of tubes were resuspended in 4 ml of complete media, and the cells were syringed into a single-cell suspension. Aliquots (50 μl) of cell suspensions were transferred in triplicate into 12×75 -mm tubes to determine mean radioactivity per cell. The remaining cells were counted, and were processed for subsequent assessment of $^{125}\text{IdU}/\text{EdU}$ incorporation. Each cell pellet of the second set of tubes was resuspended in 200 μl of MatrigelTM on ice, and was transferred onto CytomatrixTM carbon scaffolds in sterile ultralow attachment 48-well plates on ice. The plates were incubated for 1 h at 37°C with 5% CO_2 and 95% air to allow the MatrigelTM to set, and 1 ml of culture media was carefully added onto the semi-solid MatrigelTM. The plates were returned to the incubator for 48 h, with the growth media replaced twice daily. The cells were then harvested from the MatrigelTM/CytomatrixTM units using MatriSpenseTM and trypsin as needed, passed four times through a 20 G needle, counted, serially diluted, and plated for colony formation.

Assay of $^{125}\text{IdU}/\text{EdU}$ incorporation

Cellular incorporation of EdU was assayed using the Click-iT[®] EdU Alexa Fluor[®] 488 cell proliferation kit, as described by the supplier, and was used as a surrogate for quantifying the fraction of ^{125}IdU labeled cells. EdU and ^{125}IdU are preferentially and non-competitively incorporated into newly synthesized DNA (including mitochondrial DNA [Lentz et al. 2010]), making the kit suitable for identifying S-phase cells. Therefore, concomitant pulse-labeling of cell cultures with 0.04 mM of EdU and varying concentrations of ^{125}IdU enables identification of ^{125}IdU -labeled cells by the Click-iT[®] EdU kit and flow cytometry techniques using a BD LSR II flow cytometer.

Colony forming assay

Aliquots of cell suspensions containing about 200 cells were seeded in triplicate into 60 mm dishes containing 4 ml of complete medium and incubated for 10 days and 14 days for MDA-MB-231 and MCF-7 colony formation, respectively. The colonies were washed three times with phosphate buffered saline (PBS), fixed in 95% ethanol, stained with 0.01% amido black, washed in tap-water, air-dried, counted, and the surviving fraction of cells relative to controls SF^{colony} was determined. To assess the significance of unlabeled cell killing, the anticipated surviving fraction based on the fraction of cells that had incorporated EdU, f^{labeled} , was also calculated as: $SF^{\text{anticipated}} = 1 - f^{\text{labeled}}$. This, from the perspective of

attempting to observe a bystander response, conservatively represents the minimum surviving fraction assuming that all labeled cells die.

Cellular dosimetry

Dose contributions to labeled and unlabeled cells—The over-whelming preponderance of cellular uptake of EdU and ^{125}IdU was anticipated in cells that were in S phase during the short pulse-labeling period. These are the *labeled* cells which incorporate EdU and ^{125}IdU principally into their nuclear DNA. The radiotoxicity in these labeled cells is expected to be of a high-LET nature with their response being exponentially dependent upon the mean self-dose to the nucleus (N) of the labeled cells $D_{\text{self}}(\text{N} \leftarrow \text{N})$ (Goddu et al. 1997). The nuclei of the labeled and unlabeled cells receive a small self-dose $D_{\text{self}}(\text{N} \leftarrow \text{Cy})$ from decays that occur in their cytoplasm (Cy). These cytoplasmic decays arise as a consequence of uptake of ^{125}IdU during mitochondrial DNA synthesis (Lentz et al. 2010) that is known to be continuous and independent of cell cycle stage (Magnusson et al. 2003). Finally, the nuclei of the labeled and unlabeled cells also receive a small cross-dose $D_{\text{cross}}(\text{N} \leftarrow \text{MC})$ from electrons and photons emitted by decays occurring in other labeled cells in the MatrigelTM/CytomatrixTM unit (MC) (Howell et al. 1989). These are summarized by the following equations:

$$D^{\text{labeled}}(\text{N}) = D_{\text{self}}(\text{N} \leftarrow \text{N}) + D_{\text{self}}(\text{N} \leftarrow \text{Cy}) + D_{\text{cross}}(\text{N} \leftarrow \text{MC}) \quad (1)$$

$$D^{\text{unlabeled}}(\text{N}) = D_{\text{self}}(\text{N} \leftarrow \text{Cy}) + D_{\text{cross}}(\text{N} \leftarrow \text{MC}) \quad (2)$$

The absorbed dose to the labeled and unlabeled cells is estimated using the cellular S value approach (Goddu et al. 1997).

$$D(\text{target}) = \tilde{A}(\text{source}) S(\text{target} \leftarrow \text{source}) \quad (3)$$

where $\tilde{A}(\text{source})$ is the cumulated activity in the source region and $S(\text{target} \leftarrow \text{source})$ is the mean absorbed dose to the target region per unit cumulated activity in the source region.

Mean activity per cell—The ^{125}I activity in aliquots from *Protocol A* and *Protocol B* that contained a known number of cells was determined. The measured mean activity per cell A can be written in terms of the activity in the labeled and unlabeled cells:

$$A = f^{\text{labeled}} A^{\text{labeled}} + (1 - f^{\text{labeled}}) A^{\text{unlabeled}} \quad (4)$$

Representative Click-iT EdU Alexa Fluor 488 fluorescence spectra for MDA-MB-231 and MCF-7 cells are shown in Figure 1. The percentages of cells that have incorporated EdU, and therefore ^{125}IdU , are illustrated by the gates. The average percentages of MDA-MB-231 cells that incorporated EdU in *Protocol A* and *Protocol B* were 10 ± 2 and $22 \pm 2\%$, respectively. For the MCF-7 cell line, an average of 15 ± 3 and $35 \pm 15\%$ of cells were EdU⁺ in *Protocol A* and *Protocol B*, respectively. These labeling percentages were then

expressed as fractions of cell populations that were labeled f^{labeled} . In each case, the errors represent the standard error of the mean (SEM) for $n = 10$ (*Protocol A*) and $n = 12$ (*Protocol B*), respectively.

In addition, to quantify any trace of ^{125}I uptake in unlabeled cells, the highest activity samples of *Protocol B* (i.e., treated with 0.04 mM EdU and 1.85 MBq/ml) were sorted into EdU positive (EdU^+) and EdU negative (EdU^-) fractions using a BD FACS Vantage flow cytometer. Figures 2A and 2B depict Click-iT EdU Alexa Fluor 488 fluorescence spectra for MDA-MB-231 and MCF-7 cells co-pulse-labeled with or without EdU/ ^{125}I IdU. The shift in spectral peaks for the EdU^- populations relative to the unstained control, which did not affect the percentage of EdU^+ cells (Figure 1), suggests that these cells incorporate a small quantity of EdU, and perhaps ^{125}I IdU. To confirm this, the cells were sorted based on fluorescence intensity for subsequent determination of incorporated activity. The fluorescence spectra for the EdU^+ and EdU^- sorted subpopulations are presented in Figures 2C and 2D, and show no spectral overlap between the two subpopulations. This indicates that no cross contamination occurred between the samples during the cell sort. The resulting mean cellular activities in the EdU^+ cells and EdU^- cells for MDA-MB-231 cells were 2.09 and 0.04 mBq/cell, respectively. The corresponding mean cellular activities for the MCF-7 cells were found to be 2.59 and 0.15 mBq/cell, respectively. The ratio of the mean activity per labeled cell A^{labeled} to the mean activity per unlabeled cell $A^{\text{unlabeled}}$ can be defined as:

$$R = \frac{A^{\text{labeled}}}{A^{\text{unlabeled}}} \quad (5)$$

Therefore, $R = 52.3$ and 17.3 for MDA-MB-231 and MCF-7 cells, respectively. Assuming that the mitochondrial uptake of ^{125}I IdU is the same in labeled and unlabeled cells, then the fraction of labeled cell activity in the cytoplasm $f(\text{Cy}) = 1/R$ and the fraction of activity in the nucleus $f(\text{N}) = (1 - 1/R)$. Accordingly, using the cellular activities mentioned immediately above, $f(\text{N}) = 0.981$ and $f(\text{Cy}) = 0.019$ for the MDA-MB-231 cells. Similarly, $f(\text{N}) = 0.945$ and $f(\text{Cy}) = 0.055$ for the MCF-7 cells.

Cumulated activity—To calculate the aforementioned absorbed dose contributions, it is necessary to determine the mean cumulated activity per cell $\tilde{A}(\text{C})$, where C denotes cell. The general procedure for calculating the cumulated activity of radiolabeled iododeoxyuridine in cells was detailed in the Appendix of Neti and Howell (Neti and Howell 2004) and used with modifications to account for cell division during the maintenance period in the present work (i.e., physical half-life T_p in equations of Appendix of Neti and Howell 2004 was replaced with effective clearance half-time T_e). Following this approach, there are three major time periods of interest: The pulse-labeling incubation period ($t_1 = 3$ h), the maintenance period in the three-dimensional Cytomatrix culture ($t_M = 48$ h), and the colony-forming period ($t_{\text{CF}} = 10$ or 14 d). The cumulated activities during these periods are: $\tilde{A}_1 = 0.5 t_1 A_0$, $\tilde{A}_M = 1.44 T_e A_0 (1 - e^{-0.693 t_M/T_e})$, and $\tilde{A}_{\text{CF}} \approx 1.44 A_0 T_e e^{-0.693 t_M/T_e}$, respectively, where A_0 is the mean cellular uptake at the end of the labeling period and T_e is the effective clearance half-time during the colony forming period, and $T_p = 60$ d for ^{125}I . In the present experiments, the cell doubling time for both cell lines is about 24 h and, therefore, $T_e = 23.6$

h. Substitution of the various values yields $\tilde{A}_I = 5400 A_0 \text{ Bq-s}$, $\tilde{A}_M = 9.25 \times 10^4 A_0 \text{ Bq-s}$, and $\tilde{A}_{CF} = 2.99 \times 10^4 A_0 \text{ Bq-s}$, where A_0 is in Bq. The total mean cellular cumulated activity is:

$$\tilde{A}(C) = \tilde{A}_I + \tilde{A}_M + \tilde{A}_{CF} = 1.28 \times 10^5 A_0 \text{ Bq-s} \quad (6)$$

where A_0 is in units of Bq. This cumulated activity was apportioned to the various compartments in the labeled and unlabeled cells as per the absorbed dose calculations below.

Diameter of cell and cell nucleus—Using a Beckman Coulter Model Z2, the cell and nuclear diameters of MDA-MB-231 and MCF-7 cells were measured for use in defining cellular source and target volumes. Briefly, aliquots were taken from single-cell suspensions, analyzed 10 times with the Coulter Counter, and mean cell diameters were calculated. The remaining cells were washed with phosphate buffered saline (PBS) by centrifugation. The resulting cell pellets were resuspended in 1 ml of lysing buffer consisting of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol, and 0.05% NP-40, and were left on ice for 10 min. After two washes with PBS, the nuclei were resuspended and nuclear diameters were determined using the Coulter Counter as described above. The cell and nuclear diameters of the MDA-MB-231 cells were 12.4 ± 0.1 and $7.1 \pm 0.1 \mu\text{m}$, respectively. The corresponding values for the MCF-7 cells were 14.2 ± 0.1 and $8.9 \pm 0.5 \mu\text{m}$, respectively. In each case, the errors represent the SEM for $n = 10$.

Self-dose to the cell nucleus of labeled cells and unlabeled cells—Cellular S values for ^{125}I were interpolated from published tables (Goddu et al. 1997) based on the radius of the cell and cell nucleus. For MDA-MB-231 cells, $S_{\text{self}}(N \leftarrow N) = 9.89 \times 10^{-3} \text{ Gy (Bq-s)}^{-1}$ and $S_{\text{self}}(N \leftarrow Cy) = 3.84 \times 10^{-4} \text{ Gy (Bq-s)}^{-1}$. The values for MCF-7 cells are $S_{\text{self}}(N \leftarrow N) = 5.19 \times 10^{-3} \text{ Gy (Bq-s)}^{-1}$ and $S_{\text{self}}(N \leftarrow Cy) = 2.65 \times 10^{-4} \text{ Gy (Bq-s)}^{-1}$.

By apportioning the activity into the nuclear and cytoplasmic compartments, one can derive the mean self-absorbed doses to the labeled and unlabeled cells:

$$D_{\text{self}}^{\text{labeled}}(N) = \frac{R \tilde{A}(C)}{R f^{\text{labeled}} + f^{\text{unlabeled}}} \times (f(N) S_{\text{self}}(N \leftarrow N) + f(Cy) S_{\text{self}}(N \leftarrow Cy)) \quad (7)$$

$$D_{\text{self}}^{\text{unlabeled}}(N) = \frac{\tilde{A}(C)}{R f^{\text{labeled}} + f^{\text{unlabeled}}} S_{\text{self}}(N \leftarrow Cy) \quad (8)$$

Cross dose to labeled and unlabeled cells—The photons, and some of the Auger and conversion electrons emitted by ^{125}I result in cross-irradiation of neighboring cells in the MatrigelTM/CytomatrixTM (MC) cell culture unit. This cross-dose, D_{MC} , can be estimated by considering the entire unit both as the source and the target. The S-value for the MatrigelTM/CytomatrixTM cell culture unit with a volume of 200 μl , $S(N \leftarrow MC)$, was estimated using a well-established FORTRAN code (Howell et al. 1989). An upper estimate was obtained by assuming that the 200 μl cell culture unit is spherical with a diameter of 7260 μm . The

corresponding upper estimate for the S-value for ^{125}I photons for this geometry is $S(N \leftarrow MC) \sim 2 \times 10^{-11} \text{ Gy (Bq}\cdot\text{s)}^{-1}$. The cumulated activity for this contribution to the total absorbed dose arises only during the maintenance period of 48 h. With each unit containing 6×10^6 cells, the mean cross-dose to the labeled and unlabeled cells is:

$$D_{\text{cross}}^{\text{labeled+unlabeled}}(N \leftarrow MC) 6 \times 10^6 \tilde{A}_M(C) S(N \leftarrow MC) \quad (9)$$

The cells are very sparsely distributed in the MatrigelTM/CytomatrixTM cell culture unit. However, there is a small probability that two cells can be in direct contact. In that event, the cross-dose would be substantially higher than the mean value calculated above. The cellular S values for this particular cross-dose scenario were obtained from an online medical internal radiation dose cellular dosimetry tool (MIRDcell v1.0) published by our laboratory (Wu et al. 2010). The cross-dose S values for MDA-MB-231 and MCF-7 cells are $S_{\text{cross}}(N \leftarrow N) = 5.27 \times 10^{-5} \text{ Gy (Bq}\cdot\text{s)}^{-1}$ and $3.39 \times 10^{-5} \text{ Gy (Bq}\cdot\text{s)}^{-1}$, respectively. The cumulated activity for this contribution to the absorbed dose also arises only during the maintenance period of 48 h. Therefore, this cross-dose contribution which can be received by both labeled and unlabeled cells is:

$$D_{\text{cross}}^{\text{labeled+unlabeled}}(N \leftarrow N) = \tilde{A}_M(C) S_{\text{cross}}(N \leftarrow N) \quad (10)$$

Statistical analysis

To evaluate the significance of unlabeled cell killing, the anticipated surviving fractions (percentage of cells that were unlabeled) were compared with the experimentally determined surviving fractions using the Mann-Whitney rank sum test (SigmaPlotTM, Systat Software, Pomona, CA, USA). This test compares two data sets, and determines whether their medians are statistically different. A *P* value of less than 0.05 indicates a statistically significant difference between the data sets.

Results

Cellular dosimetry

The cellular absorbed doses for a mean unit ^{125}I cell uptake of $A_0 = 1 \text{ mBq}$ are given in Table I. The data presented in Table I summarizes the contributions to the mean absorbed doses to the nuclei of labeled cells $D^{\text{labeled}}(N)$ and unlabeled cells $D^{\text{unlabeled}}(N)$ for each cell line. In this cell culture system, the dose calculations show that the mean absorbed dose to the nuclei of the labeled cells is about 150-fold and 100-fold higher than the unlabeled cells for MDA-MB-231 and MCF-7 cells, respectively. This difference is due primarily to the higher uptake by unlabeled cells in the case of MCF-7 cells.

Cell survival

Cell survival data for MDA-MB-231 cells following ^{125}IdU incorporation are presented in Figure 3. In *Protocol A*, the measured surviving fraction SF^{colony} appeared to be less than anticipated (open circles) based on a conservative assumption that all labeled cells are killed by virtue of the high-LET type radiotoxicity imparted by ^{125}IdU and all unlabeled cells

survive ($SF^{\text{anticipated}} \sim 0.90$, Figure 3A). The data corresponding to *Protocol B* show a similar response (Figure 3B). Similar data is provided for MCF-7 cells in Figure 4. An apparently more robust dose-response is observed for this breast cancer cell line, particularly for *Protocol B* (Figure 4B).

To evaluate the significance of unlabeled cell killing, the anticipated surviving fractions (percentage of cells that were unlabeled) were compared with the experimentally determined surviving fractions using the Mann-Whitney rank sum test, as described in the *Materials and methods* and illustrated in Figure 5. For both cell lines, the data from *Protocol A* show a weak trend toward a higher cell killing than anticipated on the basis of the fraction of ^{125}IdU labeled cells. The P values for MDA-MB-231 and MCF-7 were 0.259 and 0.105, respectively. In *Protocol B*, however, the experimental cell survival was significantly lower than the anticipated cell survival with $P < 0.001$ for both cell lines.

Using the data from *Protocol B* (Figure 3B and Figure 4B), the surviving fraction of unlabeled cells ($SF^{\text{unlabeled}}$) specifically was obtained from the ratio $SF^{\text{colony}}/SF^{\text{anticipated}}$ (Figure 6). In light of the apparent mitochondrial uptake of ^{125}I that delivers self-dose, and the small cross-dose received by the unlabeled cells (Table I), $SF^{\text{unlabeled}}$ is plotted as a function of mean absorbed dose to the unlabeled cell nucleus in Figure 6. The mean fraction of ^{125}IdU labeled (EdU^+) cells for each protocol was used to derive $SF^{\text{anticipated}}$. These data show that unlabeled cell killing in the low-dose region is higher than expected based on dose response curves for low-LET ionizing radiation, particularly given the chronic irradiation conditions in the present experiments. The strength of this observation depends on phenotype.

Discussion

There is now overwhelming evidence to support the phenomenon of radiation-induced bystander effects following external exposure of select subpopulations of cells to low doses of ionizing radiation of varying quality (Blyth and Sykes 2011). However, similar studies involving the use of tissue incorporated radioactivity are relatively few, due largely to the complex and labor-intensive nature of conducting these studies. Extending the knowledge base on possible bystander phenomena in different tissue systems and for the wide spectrum of radionuclides that may be encountered in therapies using radiopharmaceuticals, routine nuclear medicine procedures, or radiation accidents such as Fukushima, is important for improving our capacity to accurately predict their biological effects.

As noted in the *Materials and methods*, the present cell culture model is a modification of that of Pinto et al. (Pinto and Howell 2007, Pinto et al. 2010). Here, human breast cancer cells (MDA-MB-231 and MCF-7), embedded in a matrix of MatrigelTM within a CytomatrixTM carbon scaffold, were pulse-labeled with $^{125}\text{IdU}/\text{EdU}$ (*Protocol A*). The cell survival data in Figure 3A (for MDA-MB-231) and Figure 4A (for MCF-7) show a small increase in cell killing beyond what would be anticipated if all of the labeled cells were killed. However, the statistical analysis in Figure 5 indicates that the difference is not significant when the experiments were conducted under *Protocol A*. The absence of a clear bystander effect in *Protocol A* may be attributable to variable cell losses from the fragile

Matrigel™ within the Cytomatrix™ units that occurred during the transfer steps. The resulting variability in the dose-response data (Figures 3A and 4A) prompted changes in the protocol to minimize these disturbances. When the cells were processed and treated as described in *Protocol B*, ^{125}I IdU incorporation resulted in significant increases in cell killing beyond what would be expected if all of the labeled cells died (Figures 3B, 4B and 5).

Radiation dosimetry plays a very important role in the effort to discern whether a radiation-induced effect in a tissue system is due simply to direct irradiation of the affected cells and/or to a radiation-induced bystander effect. To discern whether the data presented herein can definitively be construed to be due, at least in part, to a bystander effect, it was necessary to determine if the absorbed dose received by cells that adjudged as unlabeled could lead to levels of toxicity that would be anticipated based on known dose-responses to ionizing radiation. To achieve this, the self-dose to labeled $D_{\text{self}}^{\text{labeled}}$ and unlabeled $D_{\text{self}}^{\text{unlabeled}}$ cells, and the cross-dose to labeled and unlabeled cells $D_{\text{self}}^{\text{labeled+unlabeled}}$, were determined using well established macroscopic and cellular dosimetry procedures (Table I). The need for this level of detailed analysis arose, in part, because of the unanticipated substantial positive shift in the peaks of the fluorescence intensity histograms of EdU⁺ subpopulations (Figures 2A and 2B). The shift is likely attributable to EdU/ ^{125}I IdU incorporation into the mitochondrial DNA of unlabeled cells. This is a process that occurs throughout the cell cycle (Magnusson et al. 2003, Lentz et al. 2010). It can be expected then, that all cells, even those that are not in S-phase during the 3 h pulse-labeling period, will incorporate EdU/ ^{125}I IdU into their mitochondrial DNA *albeit* to a much lower extent than the amount incorporated into nuclear DNA of S-phase cells. Irradiation of the nucleus by electrons and photons emitted by ^{125}I localized in mitochondria via the radiochemical ^{125}I -dihydrorhodamine (i.e., $D_{\text{self}}^{\text{unlabeled}}(\text{N} \leftarrow \text{Cy})$) has been shown to cause a low-LET type dose response (Kassis et al. 1987a). Therefore, it is necessary to ascertain what level of cell killing can be expected when the cells used in the present work are irradiated with low-LET ionizing radiations. As shown by the dashed lines in Figure 6 which represent published low-LET dose-response studies for these cells (Lehnert et al. 1990, Hahnel et al. 2010, Weigel et al. 2010, Jain et al. 2011), the small absorbed doses received by the nucleus of the unlabeled cells (Table I and Figure 6) from electrons and photons emitted by cytoplasmic ^{125}I should only cause a very small reduction in surviving fraction. Another possible explanation is that the incorporation of ^{125}I IdU specifically into mitochondrial DNA causes damage beyond what is expected based on the low-LET type response to ^{125}I -dihydrorhodamine in the mitochondria. However, this possibility is refuted by work that has demonstrated that incorporation of ^{125}I IdU into mitochondrial DNA does not contribute significantly to the high-LET type radiotoxicity observed for ^{125}I IdU labeled cells (Yasui and Hofer 1986). Therefore, the enhanced killing of unlabeled cells beyond what is expected based on dose response to low-LET radiation, depicted in Figure 6, appears to be due to a radiation-induced bystander effect.

In the present experiments, it is also possible that ^{125}I is released by dead or dying cells and reutilized by neighboring cells. In fact, there is evidence that tritiated thymidine ($^3\text{HTdR}$), and its nucleoside analog ^{125}I IdU, can be released from dead cells and incorporated into the DNA of neighboring cells (Feinendegen 1967). Cell culture studies with L strain fibroblasts

indicated that phagocytized nuclei or DNA remnants were hydrolyzed to small nucleotides before being reutilized (Horikawa et al. 1963, Feinendegen 1967). The efficiency of this process for ^{125}IdU is very low compared to $^3\text{HTdR}$ (Feinendegen 1967, Myers and Feinendegen 1975). Nevertheless, there is evidence that MCF-7 cells undergoing autophagy are phagocytized by neighboring live MCF-7 cells (Petrovski et al. 2007). Therefore, it is possible that the apparent bystander effect observed in the present work may be due, at least in part, to high-LET type radiotoxicity in ‘unlabeled’ cells whose DNA was unexpectedly labeled via reutilization of ^{125}IdU . However, Urashima et al. (2006) have shown that MCF-7 cells are relatively resistant to nuclear DNA-incorporated ^{125}IdU compared to other tumor cell lines. They obtained a mean lethal activity of 2.98 mBq/cell when a 48 h labeling period was used, followed by washes and seeding for colony formation. Assuming that the average ^{125}I activity per cell increases linearly during the labeling period and decreases exponentially during the colony forming period which is typical for ^{125}IdU (Kassis et al. 1987b, Howell et al. 1991), then their 2.98 mBq/cell corresponds to a cumulated activity of about 622 Bq s. Applying the same S value used in the present work for intranuclear localization of ^{125}I in MCF-7 cells, the D_{37} in the MCF-7 experiments of Urashima et al. (2006) can be calculated to be about 3.2 Gy. Absorbed doses of this magnitude, with a corresponding drop in surviving fraction, cannot be achieved in our unlabeled cells even if all of the activity in the labeled cells is released into the loosely packed cell matrix and then entirely incorporated into the DNA of the unlabeled cells. Therefore, the apparent bystander effect cannot be due simply to reutilization of ^{125}IdU .

Based on the above arguments, the killing of unlabeled cells (Figure 6) appears to be caused, at least in part, by a ^{125}IdU radiation-induced bystander effect. The observation of this lethal ^{125}IdU -induced bystander response is consistent with earlier findings with cultured Chinese hamster cells (Howell and Bishayee 2002); however, the cell packing density in the present work is considerably lower due in part to limitations of growing the very large number of cells that are required to densely pack a CytomatrixTM. The ^{125}I -induced bystander response is also consistent with those observed with human colon LS174T adenocarcinoma cells in a mouse tumor model (Xue et al. 2002, Kishikawa et al. 2006).

The presence, absence, and nature of radiation-induced bystander effects caused by incorporated radioactivity appear to be dependent on many variables such as cell type, radionuclide (types of radiations emitted), and radiochemical (molecular location of radiation emitter). DNA-incorporation of ^3H -thymidine and ^{125}IdU resulted in increased bystander cell killing in Chinese hamster cells assembled into tightly packed multicellular clusters (Bishayee et al. 1999, 2001, Howell and Bishayee 2002, Persaud et al. 2005). ^3H -thymidine also induced mutations in the same model (Persaud et al. 2007). However, ^3H -thymidine also induced proliferative bystander effects in monolayer cultures of liver epithelial cells (Gerashchenko and Howell 2004), and the magnitude of the effect was strongly dependent on the number of adjacent irradiated (labeled) cells (Gerashchenko and Howell 2005). A similar proliferative bystander effect was induced in vivo by ^{123}IdU (Kishikawa et al. 2006). Bystander effects were neither imparted significantly when ^3H -deoxycytidine was incorporated by a small fraction of normal AG1522 human fibroblasts growing in 3-dimensional in a CytomatrixTM carbon scaffold (Pinto and Howell 2007) nor when a small fraction of cells in the spleen were labeled with $^3\text{HTdR}$ (Blyth et al. 2010).

Yet, bystander effects have been demonstrated for radiochemicals that emit low-LET beta particles (^{131}I) and high-LET α -particles (Boyd et al. 2006, 2008). In the midst of the myriad of bystander responses observed, the present study brings the importance of cell phenotype to the fore. Here, the radiation-induced bystander effect is studied in two different human breast cancer cell lines, MDA-MB-231 and MCF-7, and it appears that the latter are considerably more sensitive to a ^{125}IdU -induced bystander effect. Interestingly, formation of MCF-7 tumor xenografts in mice requires the presence of estrogen, whereas, estrogen is not required for MDA-MB-231. This raises interesting questions regarding the possibility that hormone responsiveness could play some role in bystander responses in vivo, and perhaps could be manipulated for therapeutic purposes.

Conclusions

This study evaluates DNA-incorporated ^{125}IdU -induced bystander cell killing in human breast cancer cells, using clonogenic cell survival as a biologic endpoint. A significant level of bystander cell killing was demonstrated in MCF-7 cells, whereas only minimal bystander killing was observed in MDA-MB-231 cells. These findings demonstrate the importance of cancer cell phenotype in supporting ^{125}IdU -induced bystander responses.

Acknowledgments

The authors thank Sukwinder Singh and Dana Stein for their assistance in the New Jersey Medical School Flow Cytometry Core Facility. They also thank Drs Ketai Wang and Amin Kassis of the Harvard Medical School for generously providing kits for preparing ^{125}IdU .

This work was supported in part by National Institutes of Health (NIH) Grant Number R01 CA083838 - 09. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the NIH.

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Figure 1.

Representative cellular fluorescence intensity histograms from *Protocol B* following treatment of MDA-MB-231 cells with (A) 0 mM and (B) 0.04 mM 5-ethynyl-2'-deoxyuridine (EdU); and of MCF-7 cells with (C) 0 mM and (D) 0.04 mM of EdU for 3 h in suspension. Samples corresponding to panels A and C were not exposed to ¹²⁵IIdU, while those corresponding to panels B and D were concomitantly treated with a concentration 0.5 MBq/ml of ¹²⁵IIdU. Gates represent the percentages of cells in each culture that had incorporated EdU (EdU⁺ or ¹²⁵IIdU/EdU⁺).

Figure 2.

Representative cellular fluorescence intensity histograms following treatment of: (A) MDA-MB-231 cells and (B) MCF-7 cells with 0 mM (black) or with 0.04 mM 5-ethynyl-2'-deoxyuridine (EdU) and ^{125}I IdU at a concentration of 1.85 MBq/ml (blue). Cellular fluorescence intensity histograms for cells treated with 0.04 mM EdU and 1.85 MBq/ml of ^{125}I IdU, sorted, and then analyzed by flow cytometry into EdU⁻ (blue) and EdU⁺ (brown) populations: (C) MDA-MB-231, (D) MCF-7. Black histograms are for control samples (0 mM EdU).

Figure 3.

Surviving fraction of MDA-MB-231 cells as a function of mean absorbed dose to the labeled cell nuclei after treatment with graded amounts of ^{125}IdU using: (A) *Protocol A* or (B) *Protocol B*. Solid curves represent least-squares fits of data (filled symbols) to 2-component exponential functions. Solid squares and diamonds represent independent experiments. Dashed lines represent the 95% confidence bands of linear regression fits to the anticipated surviving fraction ($1-f^{\text{labeled}}$, open symbols). Error bars represent SEM of triplicate measurements.

Figure 4.

Surviving fraction of MCF-7 cells as a function of mean absorbed dose to the labeled cell nuclei after treatment with varying concentrations of ^{125}I dU using: (A) *Protocol A* or (B) *Protocol B*. Solid curves represent least-squares fits of data (filled symbols) to 2-component exponential functions. Solid squares and diamonds represent independent experiments. Dashed lines represent the 95% confidence bands of linear regression fits to the anticipated surviving fraction ($1-f^{\text{labeled}}$, open symbols). Error bars represent SEM of triplicate measurements.

Figure 5.

Comparison of measured surviving fractions (colony) with the anticipated surviving fractions for MDA-MB-231 and MCF-7 cells treated with 0.04 mM EdU and varying concentrations of ^{125}I dU, using the Mann-Whitney rank sum test. Only data for absorbed doses wherein the fitted curve for the measured survival was less than or equal to the anticipated survival curve ($1-f_{\text{labeled}}$) were included in the analysis. The horizontal lines within the boxes represent the medians (solid) and means (dashed) of the corresponding data sets, and the error bars represent the standard deviation ($n = 7, 8$ or 12). A P value of less than 0.05 indicates the difference between the medians of the compared data sets is statistically significant.

Figure 6.

Surviving fractions of unlabeled cells as a function of the mean absorbed dose to nuclei of unlabeled cells for: (A) MDA-MB-231 and (B) MCF-7 cells. Solid lines represent two-component exponential fits to the experimental data (filled symbols). Error bars represent propagated standard errors of the mean for triplicate measurements of clonogenic cell survival. These responses are compared to those that would be expected, based on linear quadratic fits of data in the literature, if the unlabeled cells were irradiated acutely with external beams of low linear-energy-transfer ionizing radiation. The dash-dot lines represent published responses for MDA-MB-231: 15 MV photons ($\alpha = 0.083 \text{ Gy}^{-1}$, $\beta = 0.0059 \text{ Gy}^{-2}$, [Jain et al. 2011]) and MCF-7 wild type: ^{60}Co γ -rays ($\alpha = 0.59 \text{ Gy}^{-1}$, $\beta = 0.0031 \text{ Gy}^{-2}$, [Lehnert et al. 1990]).

Table I

Mean absorbed doses to labeled and unlabeled cells from different ¹²⁵I source volumes encountered in the experimental protocol. The absorbed doses correspond to those delivered *per* mBq of mean cellular activity *A*₀ for each combination of cell line and protocol.

	f^{labeled}	$D^{\text{labeled}}_{\text{self}}$ (N←N) (Gy)	$D^{\text{labeled+unlabeled}}_{\text{self}}$ (N←Cy) (Gy)	$D^{\text{labeled+unlabeled}}_{\text{cross}}$ (N←N) (Gy)	$D^{\text{labeled+unlabeled}}_{\text{cross}}$ (N←MC) (Gy)	D^{labeled} (N) (Gy)	$D^{\text{unlabeled}}$ (N) (Gy)
MDA-MB-231							
<i>Protocol A</i>	0.10	10.60	0.008	0.042	0.011	10.65	0.06
<i>Protocol B</i>	0.22	5.29	0.004	0.021	0.011	5.32	0.04
MCF-7							
<i>Protocol A</i>	0.15	3.15	0.006	0.016	0.011	3.18	0.03
<i>Protocol B</i>	0.35	1.62	0.003	0.008	0.011	1.64	0.02

N, cell nucleus; Cy, cytoplasm; MC, MatrigelTM/CytomatrixTM cell culture unit; labelled, EdU⁺ cell that has incorporated ¹²⁵I into its nuclear DNA while in S-phase during pulse-labeling; unlabeled, cell that did not incorporate ¹²⁵I into its nuclear DNA (i.e., was not in S-phase during pulse-labeling); self, received from radiations emitted from within the cell for which the absorbed dose is calculated; cross, received from radiations emitted by other cells in the population.