BYSTANDER RESPONSES IN THREE-DIMENSIONAL CULTURES CONTAINING RADIONLABELLED AND UNLABELLED HUMAN CELLS

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Research on the radiation-induced bystander effect has been carried out mainly in 2-D tissue culture systems. This study uses a 3-D model, wherein apparently normal human diploid fibroblasts (AG1522) are grown in a carbon scaffold, to investigate the induction of a G1 checkpoint in bystander cells present alongside radiolabelled cells. Cultures were simultaneously pulse-labelled with 3H-deoxycytidine (3HdC) to selectively irradiate a minor fraction of cells, and bromodeoxyuridine (BrdU) to identify the radiolabelled cells. After thorough washing of cultures, iododeoxyuridine (IdU) was administered to detect proliferating bystander cells. The cultures were harvested at various times thereafter, and cells were reacted with two monoclonal antibodies specific to IdU/BrdU or BrdU, respectively, stained with propidium iodide, and subjected to multi-parameter flow cytometry. Cell-cycle progression was followed in radiolabelled cells (BrdU+) that were chronically irradiated by low energy beta particles emitted by DNA-incorporated 3H, and in unlabelled bystander cells (BrdU−) by a flow cytometry based cumulative labelling index assay. As expected, radiolabelled cells were delayed, in a dose-dependent manner, in G2 and subsequently G1. No delay occurred in progression of bystander cells through G1, when the labelled cells were irradiated at dose rates up to 0.32 Gy h−1.

INTRODUCTION

The radiation-induced bystander effect has been defined as the occurrence of biological effects in unirradiated cells, as a result of exposure of other cells to ionising radiation(1). Most research in this field has been carried out in two-dimensional (2-D) tissue culture systems, and mostly using alpha particles(2). Intercellular communication, via gap junctions or secreted diffusible factors, has been shown to be a major mediator of such non-targeted effects. With relevance to human radiation protection, diagnostics and therapy, it would be of particular significance to determine the existence and underlying mechanisms of radiation-induced bystander responses at the multicellular, tissue and organ levels. Human cells maintained in a three-dimensional (3-D) architecture, which mimics the way cells grow in vivo, offer an ideal study system, where experimental variables can be tightly controlled to investigate bystander effects. The use of normal human cell strains is encouraged, as it permits investigation of mechanisms in a phenotype, where signalling pathways are presumably normal. Bystander responses, including DNA damage, gene expression and alterations in cell cycle progression have been measured in AG1522 cells in 2-D culture(3). Bystander effects were also observed with V79 cells maintained in multicellular 3-D clusters consisting of cells that incorporated 3H-thymidine and unlabelled cells(4). Here, we build on those initial observations and use apparently normal human skin fibroblasts (AG1522) maintained in vitro in 2-D and in a 3-D growth model to investigate induction of cell cycle checkpoints in bystander cells present alongside cells that harbour 3H-deoxycytidine (3HdC) in their DNA. For these studies, the well-established cumulative labelling index (CLI) assay(5) was modified to distinguish cells labelled with 3HdC from proliferating bystander cells.

METHODS

Cell culture

Parental AG1522 were routinely cultured in 2-D in Eagle’s MEM supplemented with 2 mM L-glutamine, 12.5% foetal bovine serum, 100 U ml−1 penicillin and 100 ng ml−1 streptomycin. This growth medium is designated cMEM. For 3-D culture, 105 cells were seeded on fibronectin-coated Cytomatrix units (Cytomatrix, USA) in 1 ml cMEM, incubated at 37°C in 24 well plates and moved gently on a nutator in humified atmosphere of 5% CO2 in air. Cells formed 3-D interactions among each other, and cultures were fed with fresh cMEM on alternate days. Experiments involving 2-D monolayer cultures were fed with cMEM on the same schedule.

Micro-irradiation and bystander environment

Bromodeoxyuridine (BrdU) and 3HdC are non-competitively incorporated into the DNA of actively advancing S-phase cells. Therefore, co-pulse-labelling cell cultures with 10 μM BrdU and 0–740 kBq ml−1 3HdC enables identification of 3H-labelled cells by anti-BrdU immunodetection.

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methods. Hence, BrdU\(^+\) cells are \(^3\)HdC labelled, while BrdU\(^-\) carry no radioactivity and may be considered bystanders. Cultures are pulse-labelled for 3 h, washed extensively in cMEM and incubated for various times. DNA-incorporated \(^3\)H is capable of delivering a high radiation absorbed dose to the cell nuclei of the labelled cells while imparting a negligible dose to the nuclei of the surrounding cells\(^4,6\). During the pulse-label, all cells in the culture are exposed to radiation from decays occurring in the culture medium. However, the dose delivered to all cells during the pulse due to extra-cellular beta decays (<2 mGy in 3 h) is not expected to interfere with effects induced in unlabelled cells. Pulse-labelling was done 24 h after the last cMEM feeding, where the percentage of labelled cells was 3–16\%. These cells incorporate \(^3\)HdC in their nuclei, although the complementary fraction constitutes unlabelled, bystander cells. Thus shortly after pulse-labelling, bystander cells are distributed among G\(_0\), G\(_1\), M and G\(_2\), and labelled cells are in S or early G\(_2\).

**Cellular dosimetry**

A known number of cells were subjected to liquid scintillation counting for determination of average cellular activity. As only a minor fraction of the cultured cells contain radioactivity, the measured average mBq per cell was converted to average mBq per labelled cell, upon dividing the former by the fraction of BrdU\(^+\) cells. The BrdU\(^+\) fraction was measured using flow cytometry (FCM) on parallel cultures. Assuming spherical nuclei of 3.5 \(\mu\)m radius, cellular doses were calculated using the cellular S-value \(4.09 \times 10^{-3}\) Gy (Bq·s)\(^{-1}\)\(^6\).

**Multi-parameter flow cytometry-based CLI assay**

A modified version of the CLI assay has been developed. Briefly, the assay allows measuring the fraction of proliferating bystander cells, via immunodetection of DNA-incorporated iododeoxyuridine (IdU), specifically in BrdU\(^-\) cells co-cultured with BrdU\(^+\) cells. This is facilitated by concurrently using a BrdU-specific antibody and an anti-BrdU antibody that also reacts with IdU. After pulse-labelling with \(^3\)HdC/BrdU, cultures are incubated in cMEM supplemented with 1 \(\mu\)M IdU and harvested with trypsin 15–40 h later. BrdU specific incorporation is associated with high levels of Alexa 633 fluorescence, while high levels of Alexa 488 fluorescence indicate incorporation of BrdU and/or IdU. Low fluorescence intensity of Alexa 633–BrdU concomitant with high levels of Alexa 488 fluorescence indicates IdU incorporation in BrdU\(^-\) cells (Figure 1). Under the culture conditions used, the majority of BrdU\(^+\) cells on the Cytomatrix units or monolayers were in G\(_0\) phase, due to contact growth inhibition. Thus, the procedure outlined above measures the G\(_1\) checkpoint in proliferating bystander cells. For assessment of a G\(_1\) checkpoint in, otherwise excluded, G\(_0\)-phase bystander cells, 3-D-Cytomatrix cultures were pulse-labelled, held for 2, 8, or 48 h, dismantled and seeded (\(\leq 1.5 \times 10^4\) cells cm\(^{-2}\)) in P60 dishes in cMEM medium containing 1 \(\mu\)M IdU. Cells were then fixed, permeabilised

![Figure 1](https://example.com/figure1.png)
RESULTS

FCM–CLI assay validation

Preliminary tests were run to validate the FCM-based CLI assay. Cell cultures in 3-D, fed 24 h earlier, were pulse-labelled with $^3$HdC and BrdU, washed, harvested and subjected to FCM or autoradiography. For both techniques, the measured fraction of labelled cells was $\sim 7\%$, indicating that BrdU uptake, measured by FCM, is a suitable marker of DNA-incorporated radioactivity. FCM analyses were also used to ascertain the specificity of anti-BrdU and anti-BrdU/IdU antibodies in multi-parameter FCM-CLI assay.

Cell cycle progression of $^3$HdC-labelled cells

Relative to sham labelled (BrdU$^+$), cells labelled with $^3$HdC/BrDU experienced a dose-dependent G$_2$ checkpoint (Figure 2). Dose rates as small as 0.02 Gy h$^{-1}$ resulted in measurable G$_2$ arrest. Moreover, cells which overcame the G$_2$ block, to progress through M were subjected to a G$_1$ checkpoint at the subsequent cell cycle.

Cell cycle progression of G$_1$/G$_2$/M bystanders

G$_1$$\rightarrow$S transition was measured using the FCM–CLI assay in cycling unlabelled bystander cells (BrdU$^-$) (i.e. excluding G$_0$ cells which are confluence growth inhibited). Four experiments indicated no appreciable cell cycle progression delays, relative to sham bystander cells, when labelled cells were irradiated at dose rates up to 0.11 Gy h$^{-1}$.

DISCUSSION

Repeat experiments indicated that no significant G$_1$ checkpoint is induced in bystander cells cultured in 2-D or 3-D when the dose rate in $^3$HdC-labelled cells ranged from 0.02 to 0.32 Gy h$^{-1}$ (1.5–22 mBq per labelled cell). This was the case regardless of their cell cycle position at the beginning of irradiation. It is worth pointing out that, in this study, cells are labelled in situ. This means that the cellular activity (and dose rate) builds up slowly during pulse-labelling. Thus, any early radiation-induced intercellular signalling events are accounted for in the measured responses.
Interestingly, other 2-D co-culture studies with AG1522 cells indicate that DNA-incorporated ³H can elicit a G₁ checkpoint in bystander cells only when the dose rate to the labelled cells (10% labelled, 90% bystanders) exceeds ~1 Gy h⁻¹ (Azzam et al., manuscript in preparation). This may explain the lack of effect in the present 2-D work at lower dose rates. In other 2-D co-culture studies with WB-F344 rat liver epithelial cells, DNA-incorporated ³H was found to accelerate proliferation of co-cultured bystander cells when the dose rates to labelled cells ranged from 0.004 to 0.05 Gy h⁻¹ (⁸). The percentage of labelled cells had to be at least 50% to observe a proliferative response⁹. In another 3-D culture model using the V79 cell line, cells were held at 10.5°C for 72 h while accumulating beta decays from DNA-incorporated ³H (<250 mBq per cell). In these studies, pronounced bystander killing was observed⁴ and its intensity also appeared to be modulated by the fraction of cells labelled⁵. When CHO and A₁L cells were 3-D co-cultured at 11°C in a ratio of 1:5, ³H-thymidine (12 h labelling, 100 µCi) incorporation in CHO cells yielded a significant dose-dependent increase in A₁L bystander killing and mutagenesis¹⁰. It appears that ³H-induced bystander responses, within a given model, may be a function of both the fraction of labelled cells and dose or dose rate to the latter. In fact, responses may even change from pro-survival to cytotoxic depending on the concentration of the bystander signalling factors². Whether the propagated effect is inhibitory or proliferative may also be determined by the radionuclide, as suggested by studies with ¹²⁵/¹²³Iododeoxyuridine (presented by A. I. Kassis at this Microdosimetry Symposium).

It is apparent that comparing results from different laboratories is complicated by the diversity of experimental models, since the microenvironment may regulate intercellular signalling¹¹. Nevertheless, a variety of bystander responses are possible depending upon many variables, among them the radionuclide, percentage of cells labelled, dose and experimental conditions. Studies on radiation-induced bystander responses in 3-D models are emerging. In the 3-D model used in this work, dose rates up to 0.32 Gy h⁻¹ from DNA-incorporated ³H do not induce significant alterations in the G₁→S transition in bystander cells. Absence of cell cycle arrest in bystander cells is suggestive of absence of stress, including DNA damage.

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