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# A MICROBEAM STUDY OF DNA DOUBLE-STRAND BREAKS IN BYSTANDER PRIMARY HUMAN FIBROBLASTS

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**Radiation-induced bystander effect has been well documented. However, the mechanisms are poorly understood. How we incorporate this effect into the classical models of risk assessment remains an open question. Here, the induction of bystander effect was studied by assessing DNA double-strand break (DSB) formation *in situ* with the rapid and sensitive γ-H2AX focus formation assay. Utilising the Columbia University single-cell microbeam system to deliver 2 or 20 individual alpha particles to selected cell nuclei in a precisely known proportion of cells in a population, the induced DNA DSB incidences were quantified 30 min and 18 h post-IR. The increase in DNA DSB incidence in bystander cells lacked of a linear dose response indicating that neither the dose of irradiation nor proportion of irradiated cells in a population, is a critical parameter. This study confirms a binary all-or-nothing model of triggering the bystander response. The delay and persistence of the bystander response suggests a different mechanism of DSB induction in bystander cells than in directly irradiated cells.**

## INTRODUCTION

Classical dogma for assessment of radiation risk mostly accepts a linear, no-threshold model obtained by epidemiological data from high-dose exposure. However, a number of reports have described experiments in which cells in the same culture with cells that had been hit with alpha particles, but which had not been hit themselves, still exhibited signs of biological damage[1,2]. This phenomenon, called 'radiation-induced bystander effect' has become a focus of studies in radiation biology during the last decade[3–6]. Recent studies of cell survival after X-ray microbeam irradiation (IR) demonstrated that the probability of triggering a bystander response increases linearly with the dose delivered to the single cell reaching 100% above ~0.3 Gy. These findings suggest that the bystander response is triggered in an all-or-nothing manner[7].

Bystander cells exhibit a variety of characteristics of IR-induced genomic instability. Recently DNA double-strand breaks (DSBs) have been shown to be involved in bystander response[8–11]. Using the γ-H2AX focus formation assay as an indicator of DNA DSBs[12–14], the authors have shown an increase in γ-H2AX focal incidence in bystander cell populations cultured in media conditioned on gamma irradiated cells or cocultured with microbeam- or gamma-irradiated cells[8]. In alpha particle IR experiments, γ-H2AX focus formation has been distinguished in irradiated and bystander cell populations stained with different dyes. Directly irradiated cells have exhibited the expected early and linear dose-dependent γ-H2AX focus formation and most of the foci have been repaired by 18 h post-IR. In contrast, the increased γ-H2AX focal incidence in bystander cells has been detected at that time point and stayed elevated up to 48 h post-IR. The bystander response has been found to be similar for different doses of IR. The DSB nature of γ-H2AX foci has been confirmed by their colocalization with DNA DSB-repair proteins. It has been suggested that H2AX phosphorylation is an early step in the bystander effect and that DNA DSBs underlying γ-H2AX foci may be responsible for its downstream manifestations.

This report extends our previous microbeam experiments. The Columbia University single-cell microbeam system was utilised to deliver 2 or 20 individual alpha particles to selected cell nuclei in a precisely known proportion of cells in a population (10, 20 and 50%). The increase in DNA DSB incidence in bystander cells lacked a linear dose response indicating that neither the dose of IR nor proportion of irradiated cells in a population is a critical parameter.

## MATERIALS AND METHODS

### Cell culture and alpha particle irradiation

WI38 normal human diploid lung fibroblasts were obtained from Coriell Cell Repositories (Camden, NJ) and maintained according to recommended protocols. The Columbia University microbeam system was used for alpha particle IR. The cells were stained with 1 μM Hoechst 33342 for 30 min, trypsinised, counted, and 500 cells were seeded on a polypropylene film, covering a 0.25 inch hole drilled in the centre of a 60 mm microbeam dish. After further

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2 h, the microbeam dishes were placed in a humidified chamber at room temperature under the microscope and the coordinates of each Hoechst-stained nucleus were recorded by a computerised imaging system. The cell populations of 10, 20 or 50% were randomly picked for irradiation with 2 or 20 90 keV $\mu m^{-1}$ alpha particles per cell. Control dishes (mock IR) went through the same procedure without being irradiated. The cultures were fixed in 2% paraformaldehyde at 30 min or 18 h post-IR.

### Immunocytochemistry and DSB measurement

The cells in microbeam dishes were processed for immunofluorescence as previously described[12]. Custom-made anti-γ-H2AX rabbit antibody and secondary anti-rabbit Alexa-488-labelled antibody (Molecular Probes, Eugene, OR) were used. Following antibody staining, the polypropylene films were transferred from the microbeam dishes onto microscopic slides. After mounting in anti-fade media, the samples were examined by fluorescent and confocal microscopy with a Nikon PCM 2000 (Nikon Inc, Augusta, GA). The γ-H2AX foci were counted by eye in 100–200 randomly chosen cells. Previous experiments revealed that the increase in γ-H2AX focal incidence in bystander cell populations was not uniform, but limited to a fraction of the population[8]. A threshold of 4 γ-H2AX foci per cell (≥4 fpc) was optimal for determining the fraction of affected bystander cells. The error bars signify standard deviations expressed in percentage and calculated as 100 × square-root [(number of cells with ≥4 foci) / (total number of cells counted)].

## RESULTS

γ-H2AX has been shown to be detectable within 3 min after IR and to rapidly increase until a plateau is reached 10–30 min post-IR, the level of plateau being proportional to dose[15]. γ-H2AX foci also appear at the DNA break sites immediately after IR and the number of foci is proportional to the number of induced DSBs[12,14].

To find out how the extent of the bystander response is affected by the proportion of hit cells in a culture, different fractions of cell populations were irradiated. Hoechst 33342-stained nuclei of 10, 20 or 50% were randomly picked for IR with 2 or 20 alpha particles per cell. The cultures were fixed at 30 min and 18 h post-IR, and γ-H2AX foci were counted in randomly picked cells from a total cell population.

When 10% of the cells were irradiated, the fraction of cells with ≥4 fpc increased at 30 min post-IR from 12% pre-IR level (Figure 1A, image a), to 14% for two alpha particles per cell and 45% for 20 alpha-particles per cell [Figure 1A (images b and c) and B]. Likewise, these values increased to 30 and 76% when 20% of the cell population was irradiated and to 34 and 84% at 50%.

After 18 h, independent on the dose of IR and the percentage of irradiated fraction, the number of cells with ≥4 fpc averaged ~32% (28–35% range), a 2.7-fold increase from the pre-IR level, values in agreement with our previously published data[8] [Figure 1A (images d and e) and C]. Our results as well as results published by other researchers[16,17] indicate that almost all IR-induced γ-H2AX foci return to near background values by 18 h post-IR, but in the bystander cells they stay at the elevated level up to 48 h post-IR[8]. Therefore, the observed increase of the focal incidence 18 h post-IR is a result of DSB formation in the bystander cells. Additionally, the lack of linear response (same number of foci after 2 and 20 alpha particle IR) is the characteristic of focal induction in the bystander cell population.

## DISCUSSION

The existence of bystander effects after low dose IR is well documented, but the factors and mechanisms involved are still obscure. Most of the evidence supporting the bystander effect phenomena is based on directly measured endpoints or partial molecular analysis of radiation response pathways in the bystander cells. These evidences show that several aspects of the induced response in the bystander cells are similar to the ones in directly hit cells. One question is whether the process in bystander cells recapitulates the pathways activated in directly hit cells, or does it bypass the initial events to activate molecules and pathways involved in the intermediate and later phases of the radiation response? Another question is how to quantify the bystander effect as intensity and time-dependent event. Our results show that the bystander effect factors induce DNA DSBs in the bystander cells, indicating that this aspect of the overall response is similar to that in directly hit cells. However, the kinetics of the DSB induction in directly hit and bystander cells shows different patterns. In the directly hit cells, DSBs appear almost immediately, reaching their maximum abundance at 10–30 min post-IR and mostly disappear by 18 h, while in contrast, in the bystander cells DSBs are detected substantially later and persist substantially longer than in the hit cells. Hu *et al.*[9] reported the fast initial DSB formation in bystander cells. A fast response might have been detected in this study as well, considering a higher than expected fraction of cells with ≥4 fpc 30 min post-IR. However, our previous study[8] and recent unpublished results on the γ-H2AX focus formation in 3-D human tissue models with a thin single plane irradiated with alpha particles, indicate that the maximum of DSB formation in the bystander cells is detected 1–2 d post-IR and they remain at the elevated level

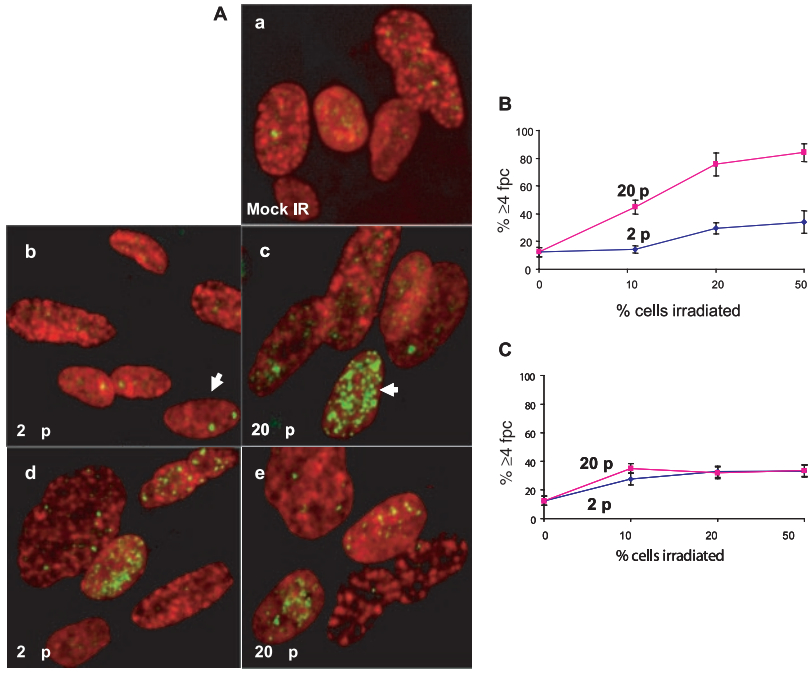


Figure 1. Presence of $\gamma$-H2AX foci in Hoechst 33342-stained cells at different time post-IR. Hoechst 33342-stained WI38 cell populations of 10, 20 or 50% were selected randomly and irradiated with 2 or 20 alpha particles per cell. The bystander effect was evident at 18 h post-IR. (A) Images are the maximum projections of representational fields showing all recorded foci (experiment with 10% cells irradiated). Nuclei were counterstained with propidium iodide. Magnification, 60×. (a) Mock-IR control. 30 min post-IR: 2 (b) or 20 (c) alpha particle-irradiated cell populations. Arrows mark cells which were presumably irradiated and visually distinct from the majority of the cell populations. 18 h post-IR: 2 (d) or 20 (e) alpha particle-irradiated cell populations. (B) Focal incidences in the cell populations received 2 (blue) or 20 (pink) alpha particles, 30 min post-IR. (C) Focal incidences in the cell populations received 2 (blue) or 20 (pink) alpha particles, 18 h post-IR. The bystander response at 18 h post-IR was similar regardless of the dose of IR or proportion of irradiated cells.

for several days. Our present report is in agreement with these data. The different dynamics of the bystander response points out to a different mechanism of induction of DSBs in bystander cells indicating the existence of a response is based on internal pathways, activated by factors released by the hit cells.

The induction of DSBs in bystander cells is a surprising result especially keeping in mind how tightly cells maintain DNA integrity. Spontaneous mutation frequencies are very low for human cells and are in the range of 1–2 mutant genes per cell per individual life time[18]. On the other hand, the induced mutation frequencies by different factors can be considerably higher. A normal cell encounters almost 200,000 DNA mutations daily induced by reactive oxygen species and DNA depurination[19]. Assuming that the directly hit cells secrete factors inducing stress in the neighbouring cells, one conclusion might be that the stress response makes the DNA repair pathways in these cells less efficient, leading to higher rates of mutation accumulation and to the formation of DSBs.

## CONCLUSION

The appearance of DNA DSBs in bystander cells confirms that the effects of ionising radiation are intense, complex and difficult to measure, and that the effects of radiation at low doses cannot be extrapolated from high-dose effects.

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