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Ionizing radiation induces DNA double-strand breaks in bystander primary human fibroblasts

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That irradiated cells affect their unirradiated 'bystander' neighbors is evidenced by reports of increased clonogenic mortality, genomic instability, and expression of DNA-repair genes in the bystander cell populations. The mechanisms underlying the bystander effect are obscure, but genomic instability suggests DNA double-strand breaks (DSBs) may be involved. Formation of DSBs induces the phosphorylation of the tumor suppressor protein, histone H2AX and this phosphorylated form, named γ-H2AX, forms foci at DSB sites. Here we report that irradiation of target cells induces γ-H2AX focus formation in bystander cell populations. The effect is manifested by increases in the fraction of cells in a population that contains multiple γ-H2AX foci. After 18 h coculture with cells irradiated with 20 α-particles, the fraction of bystander cells with multiple foci increased 3.7-fold. Similar changes occurred in bystander populations mixed and grown with cells irradiated with γ-rays, and in cultures containing media conditioned on γ-irradiated cells. DNA DSB repair proteins accumulated at γ-H2AX foci, indicating that they are sites of DNA DSB repair. Lindane, which blocks gap-junctions, prevented the bystander effect in mixing but not in media transfer protocols, while c-PTIO and aminoguanidine, which lower nitric oxide levels, prevented the bystander effect in both protocols. Thus, multiple mechanisms may be involved in transmitting bystander effects. These studies show that H2AX phosphorylation is an early step in the bystander effect and that the DNA DSBs underlying γ-H2AX focus formation may be responsible for its downstream manifestations.
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Introduction

The radiation-induced 'bystander effect' refers to the induction of biological effects in cell populations which have not been exposed to ionizing radiation (IR) but which have come into direct or indirect contact with irradiated cell populations. The existence of the bystander effect is a source of considerable controversy in understanding the biological effects of IR, since the traditional paradigm that only direct IR damage to DNA could produce an effect is no longer valid. The bystander effect appears to amplify the effects of low doses of IR by transferring signals from irradiated to unirradiated cells (reviewed by Brenner *et al*., 2001; Hall and Hei, 2003; Bonner, 2004; Mothersill and Seymour, 2004).

End points commonly assessed in bystander effect studies include clonogenic survival, expression of stress-related genes, malignant transformation *in vitro*, and alterations to the genome, such as induction of sister chromatid exchanges (SCE) (Nagasawa and Little, 1992; Wu *et al*., 1999; Zhou *et al*., 2000; Mothersill *et al*., 2002; Mitchell *et al*., 2004; Ponnaiya *et al*., 2004; reviewed by Azzam *et al*., 2004). In experiments with a microbeam α-particle irradiator, where it is possible to irradiate known fractions of a cell population, the transformation frequency was indistinguishable whether 10 of 100% of the cells had been irradiated. These results show that bystander cells as well as the target cells are subject to oncogenic transformation (Sawant *et al*., 2001). These and other end points suggest that DNA damage, including DNA double-strand breaks (DSBs), may be a causal agent in the downstream effects (Kashino *et al*., 2004; Hill *et al*., 2005; Yang *et al*., 2005).

DNA DSBs are detectable with an antibody to H2AX phosphorylated on serine 139. The antibody detects the massive H2AX phosphorylation that occurs in the chromatin adjacent to DSB sites (Rogakou *et al*., 1998, 1999; Redon *et al*., 2002; Fernandez-Capetillo *et al*., 2004). The phosphorylated H2AX species, named γ-H2AX, appears as individual punctate foci at DNA breakage sites in cell nuclei. These individual foci may be counted, making this technique more sensitive than other known DSB-detection methods

(Sedelnikova *et al*., 2002, 2004a, b; Rothkamm and Lobrich, 2003).

H2AX is a tumor suppressor. Mice null for both H2AX and p53 or heterozygous for H2AX and null for p53 rapidly develop immature T and B lymphomas and solid tumors. H2AX helps to prevent aberrant repair of both programmed and general DNA breakage and, thereby, functions as a dosage-dependent suppressor of genomic instability and tumors in mice (Bassing *et al*., 2003). H2AX is also known to facilitate error-free homologous recombination of sister chromatids (Xie *et al*., 2004) and to be essential for class-switch recombination and male sex body formation (Chen *et al*., 2000; Mahadevaian *et al*., 2001; Fernandez-Capetillo *et al*., 2003). γ-H2AX facilitates DNA DSB repair and rejoining by accumulating DNA-repair proteins at DSB sites (Paull *et al*., 2000; Celeste *et al*., 2002, 2003). γ-H2AX foci increase in number during cellular senescence in culture and during aging in mice (Sedelnikova *et al*., 2004a).

Three distinct methodologies have been used to demonstrate the bystander effect. In the first and most direct, individual cells are targeted with defined numbers of charged particles (usually α-particles), and the biological effects recorded both in the irradiated target cells and their unirradiated bystander neighbors, which depending on the cell density, may or may not be in gap-junction communication with the targeted cells. This methodology is possible through the use of a single particle microbeam accelerator, which can target specific cells in a culture with individual ionizing particles, leaving the other cells unirradiated. In the second methodology, cell populations are irradiated and immediately mixed with unirradiated cell populations. In the third, media from irradiated cell cultures are transferred to unirradiated cultures. The biological effects observed in the unirradiated cultures are presumably due to as yet uncharacterized mechanisms. Each methodology has its own unique features, which permit different experimental parameters to be examined.

We have employed all three methodologies for investigating the bystander effect. We show using all three that similar increases in the incidence of γ-H2AX foci were induced in bystander cells. Primary human fibroblasts were used to more closely model normal biological systems. This study is the first direct demonstration that DSB induction is an early step in the bystander response, suggesting that DSBs may be the initial lesions responsible for many of the downstream effects reported by other researchers. It is also the first demonstration of the temporal relationship between events expressed in the directly irradiated and bystander cells.

Results

Coculture

The Columbia University microbeam system can deliver individual α-particles to selected cell nuclei. The nuclei of the cells to be irradiated were stained with Hoechst 33342 (H in Figure 1a) and their location was automatically recorded using an imaging system for computer-driven irradiation with two or 20 α-particles. The bystander cells were stained with the vital dye CellTracker CMRA (S in Figure 1a). In the cocultures prior to α-particle IR, 7% of the CMRA-stained bystander cells contained four or more foci per cell (hereafter $\geqslant$4 fpc) (Figure 2). In contrast, 18 h after IR exposure of the targeted cells with 20 α-particles, 26% of

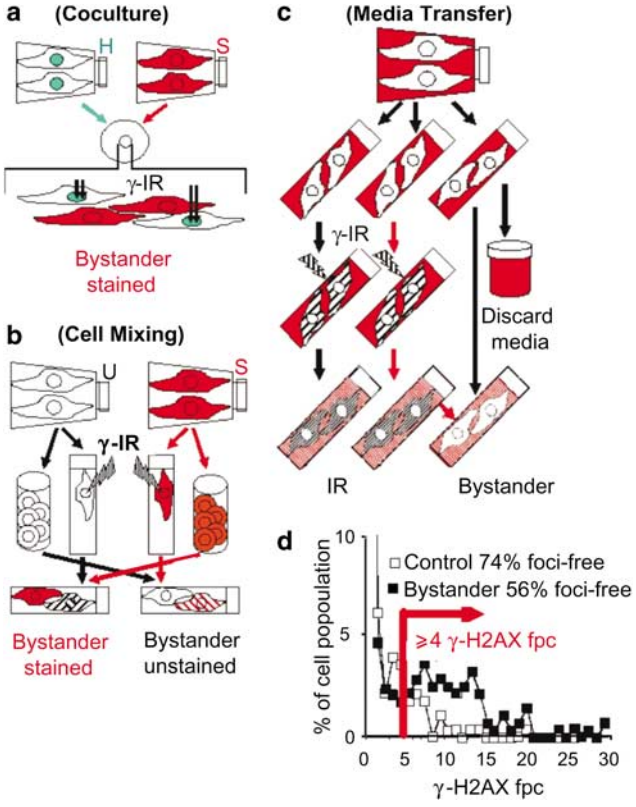

Figure 1 Experimental protocols. (**a**) Coculture. WI38 cells were split into two batches. One was incubated in the presence of the vital dye Hoechst 33342 (H) to mark the nuclei for the microbeam irradiation and the other with the vital dye CMRA (S, stained). The two cell batches were mixed and plated in the well of a microbeam dish. Cell nuclei containing Hoechst 33342 were automatically located and irradiated with two or 20 α-particles. Following incubation, the dishes were processed for microscopy. (**b**) Cell mixing. WI38 cells were split into two batches with one incubated in the presence of CMRA to stain the cells. The cells were transferred to the multiwell slides, grown overnight, then either stained (S) or unstained (U) attached cultures were exposed to IR. Immediately following IR exposure, the unirradiated WI38 cells were added to the multiwell slides, stained to unstained and *vice versa*. After incubation, the slides were processed for microscopy. (**c**) Media transfer. WI38 cells were split into three multiwell slides and grown overnight. Two slides were exposed to IR and incubated for various lengths of time. The media from the unirradiated slide was discarded and replaced with media from one of the irradiated slides. After incubation for various times, the slides were processed for microscopy. (**d**) Bystander cells mixed and incubated with mock (empty squares) or 0.2 Gy-irradiated cells (filled squares). Notice that coculturing with irradiated cells lead to a decrease of γ-H2AX foci-free cells in the bystander population from 74 to 56%

the bystander population contained $\geqslant 4$ fpc, a 3.7-fold increase. The extent of the bystander effect was slightly higher for 20 than for two α-particles per cell, indicating that above a certain threshold, the dose of radiation was not a critical parameter. This lack of a linear dose response for the bystander cells agrees with previous reports (Seymour and Mothersill, 2000).

The incidence of γ-H2AX foci in the targeted cells followed kinetics similar to those previously reported (Rogakou *et al*., 1999; Sedelnikova *et al*., 2004a). It increased at 30 m post-IR, returned to control values by 18 h and remained at initial levels at 48 h (Figure 2). In marked contrast, the focal incidence in bystander cells remained at control values 30 m post-IR, increased by 18 h and remained elevated at 48 h.

Cell mixing

The α-IR methodology did not allow staining-reversal experiments to exclude the potential influence of a dye on DNA repair and cellular response to IR-induced damage. In particular, the Hoechst 33342 dye is needed for the computer system to recognize cell nuclei. However, the effect of dyes on DNA damage responses in cell populations can be addressed with the cell mixing technique (Figure 1b). Reciprocal experiments can be carried out in which the target and bystander cells can be either stained or unstained. This methodology yielded results similar to those of the coculture (Figure 3). In control mixed cell populations, fewer than 11% of the unstained and stained cells contained $\geqslant 4$ fpc (panels Aa and B). However, by 18 h post-IR, unstained bystander cell populations contained 32% cells with $\geqslant 4$ fpc, a 2.9-fold increase (panels Ab and B, right). Similarly, when the bystander cells were stained, the populations contained 28% cells with $\geqslant 4$ fpc at 18 h post-IR, a 2.5-increase (panels Ac and B, left). The focal incidence

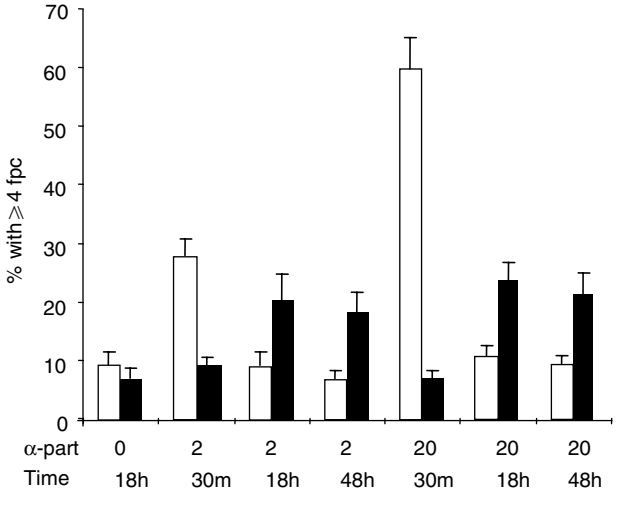

Figure 2 Coculture studies: Target cells (white bars) received two or 20 α-particles. Black bars denote bystander cells. The bystander effect was evident at 18 and 48 h with two or 20 α-particles, but not at 30 min

of the irradiated cells returned to control levels by 18 h. As with α-cocultures, the bystander effect persisted at 48 h post-IR with 23% of the bystander cell population containing $\geqslant 4$ fpc (panel B, right). This methodology demonstrates that staining to distinguish targeted and bystander cell populations had minimal effect on the results obtained.

During these studies, we observed that greater numbers of bystander cells with $\geqslant 4$ fpc were found next to targeted cells (compare Figure 3, panels c–e). If gap-junctions are involved in the transmission of bystander signals, then bystander cells in contact with irradiated cells would be expected to be more affected. In contrast, small molecules released from irradiated cells are able to diffuse through a volume of media to affect distant bystander cells. However, if small molecules are released by targeted cells over a period of time, then gradients may be set up in undisturbed cultures that lead to greater numbers of bystander cells adjacent to target cells containing $\geqslant 4$ fpc. Thus, either of these two transmission mechanisms, previously postulated to be involved in the bystander effect (Azzam *et al*., 2001; Mothersill and Seymour, 2001), can explain the recorded observations.

Media transfer

Many of the bystander effects can be replicated by transferring media conditioned on irradiated cultures to unirradiated cultures (Lyng *et al*., 2002; Suzuki *et al*., 2004; Yang *et al*., 2005). Since the irradiated and bystander cells are physically separate in this methodology, further questions concerning the times of media contact with the targeted cells and with the bystander cells in order to manifest the bystander affect can be addressed. The duration of media conditioning necessary to give the largest bystander effect was found to depend on the radiation dose and was 1, 2, and 4 h for 0.2, 0.6, and 2 Gy, respectively (Figure 4a). Media conditioned on cultures irradiated with 2 Gy was less potent than the lower two doses in inducing the bystander effect. Media harvested from cells irradiated with 0.2 Gy and conditioned for 1 h produced the largest bystander effect, increasing from 4% of cells with $\geqslant 4$ fpc in the controls to 10% at 18 h (2.5-fold increase) and 7% at 48 h post-IR (Figure 4b). Thus, the bystander effect is manifested considerably more slowly and persists much longer than the interval for γ-H2AX focal formation and disappearance in irradiated cells. As in the α-IR experiments, the observed lack of a linear dose response for the bystander agrees with previous reports (Seymour and Mothersill, 2000).

Thus, the results obtained from all three protocols agree that the γ-H2AX foci in bystander normal human fibroblasts are formed in response to a long-lasting change in the status of a subpopulation of bystander cells. In addition, the magnitudes of the effects observed in the different protocols are similar, indicating that the results are not dependent on the radiation type and methodology used.

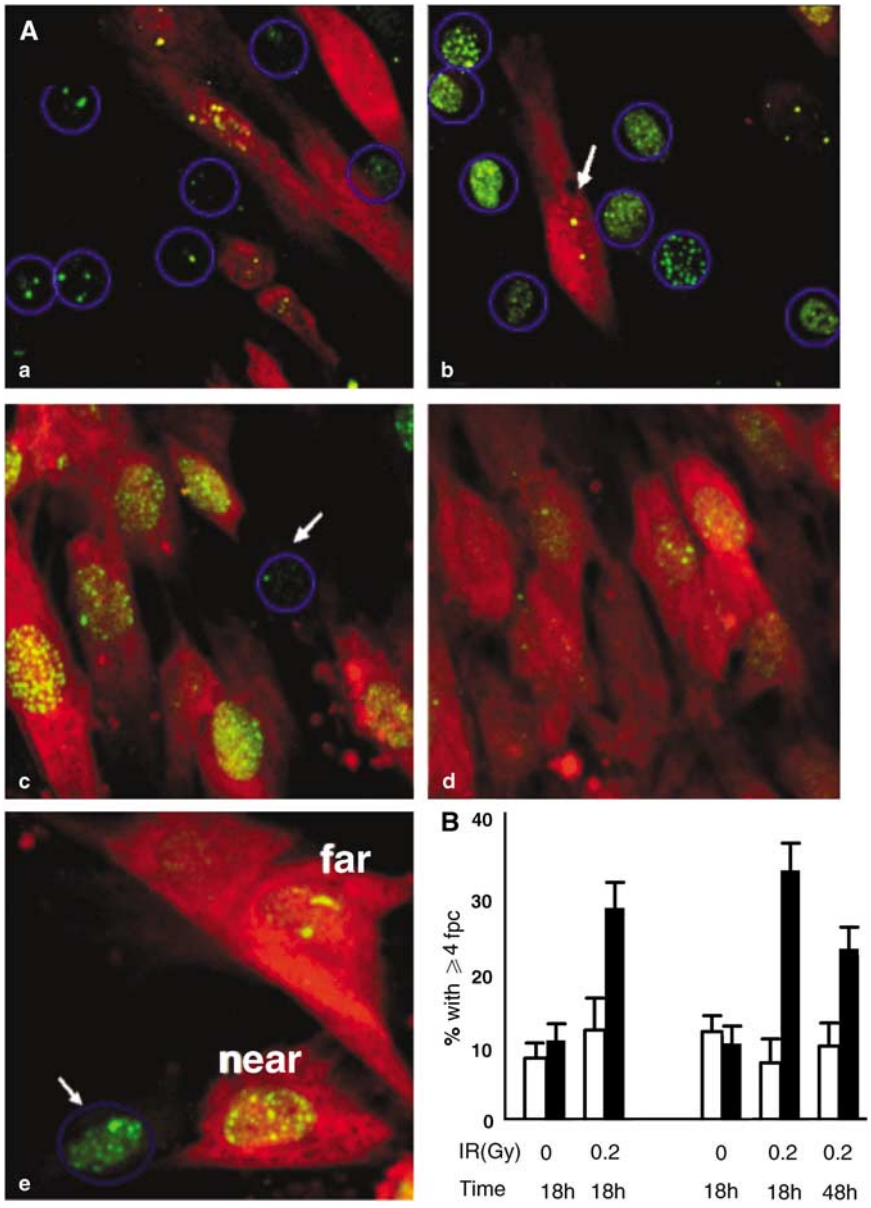

Figure 3 Cell mixing studies: (**A**) Presence of γ-H2AX foci in mixed and grown together γ-irradiated and bystander cell populations 18 h post-IR. (a–d) Representational fields of mixed cell populations. The CMRA-stained cells appear red. The nuclei of the unstained cells are outlined in blue. Arrows mark irradiated cells. (a) Control. Mixed unirradiated stained and unstained cell populations. The focal incidence was similar indicating that the presence of the stain does not affect foci yield. (b) Unstained unirradiated cells close to stained irradiated cells. (c, d) Stained unirradiated red cells close to (c) and distant from (d) unstained irradiated cells. (e) Panel showing both near and far bystander cells. (**B**) Bystander cells after either 18 or 48 h incubation with 0.2 Gy-irradiated cells; staining-reversal experiment. White bars, irradiated cells; black bars, bystander cells. The bystander effect was evident at 18 h in stained cells (left) and at 18 and 48 h in unstained cells (right)

Characteristics of the bystander effect

The γ-H2AX foci observed in previous studies were found to serve as sites for the accumulation of DNA DSB repair proteins, indicating that these sites harbor DSBs (Paull *et al*., 2000; Celeste *et al*., 2002, 2003; Sedelnikova *et al*., 2004a). When bystander cells were examined, the proteins 53bp1, ATM (phospho S1981), Mre11, Rad50 and Nbs1 were all found to accumulate at γ-H2AX foci in both the targeted and bystander cells in both the coculture and cell mixing methodologies (Figure 5a–c). DNA DSB lesions in targeted cells may be part of local multiple damaged sites containing other DNA strand, base, and backbone damage (Ward, 2000), while those formed in bystander cells are likely to be different. However, in both irradiated and bystander cells, the DNA lesions are recognized as DSBs by the cell, and DSB repair is initiated in both situations.

Previous reports have shown that the IR-induced bystander effect can be prevented by lindane

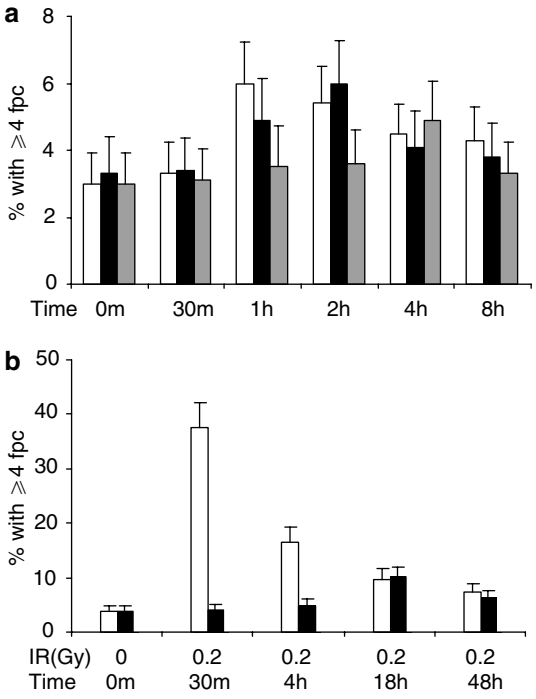

Figure 4 Media transfer. (**a**) Media were conditioned on irradiated cells for the indicated times and transferred to bystander cultures for 18 h. The bystander effect was maximal when media were conditioned on irradiated cells for 1 h with 0.2 Gy (white), 2 h with 0.6 Gy (black), and 4 h with 2 Gy (gray). (**b**) Media from mock or 0.2 Gy irradiated cultures (white bars) were transferred at 1 h post-IR to identical bystander cultures (black bars). Both a directly irradiated culture (white bars, IR in Figure 1c) and the bystander culture were incubated for the indicated times. The bystander effect was maximal at 18 h

(γ-1,2,3,4,5,6-hexachlorocyclohexane), which inhibits gap-junction communication (Azzam *et al*., 2001, 2003), by c-PTIO (2(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1oxyl-3-oxide), which scavenges nitric oxide (NO) (Shao *et al*., 2004), and by AG (aminoguanidine), which inhibits NO synthase (Shao *et al*., 2003). The effect of these inhibitors on the bystander effect in our studies was examined using two methodologies, cell mixing and media transfer. Lindane was found to prevent the γ-H2AX focus formation in bystander cells in cell mixing experiments (Figure 6). In marked contrast, lindane did not prevent the γ-H2AX focus formation in media transfer experiments either when it was added to the media at the time of transfer or when added to the irradiated cells 2 h pre-IR (Figure 6 and data not presented). These results suggest that irradiated cells release messenger molecules into the media when gap-junction transmission is not available. In contrast, AG and c-PTIO, which act to decrease NO levels, are effective even when added only to the bystander cells, suggesting that they may be acting on the bystander and not the irradiated cells (Figure 6). These observations serve as further substantiation that increased γ-H2AX focus formation is part of the bystander effects as reported by other researchers for different end points (Azzam *et al*., 2001; Shao *et al*., 2004).

Discussion

It is well established that increased clonogenic mortality, genomic instability, and expression of DNA-repair

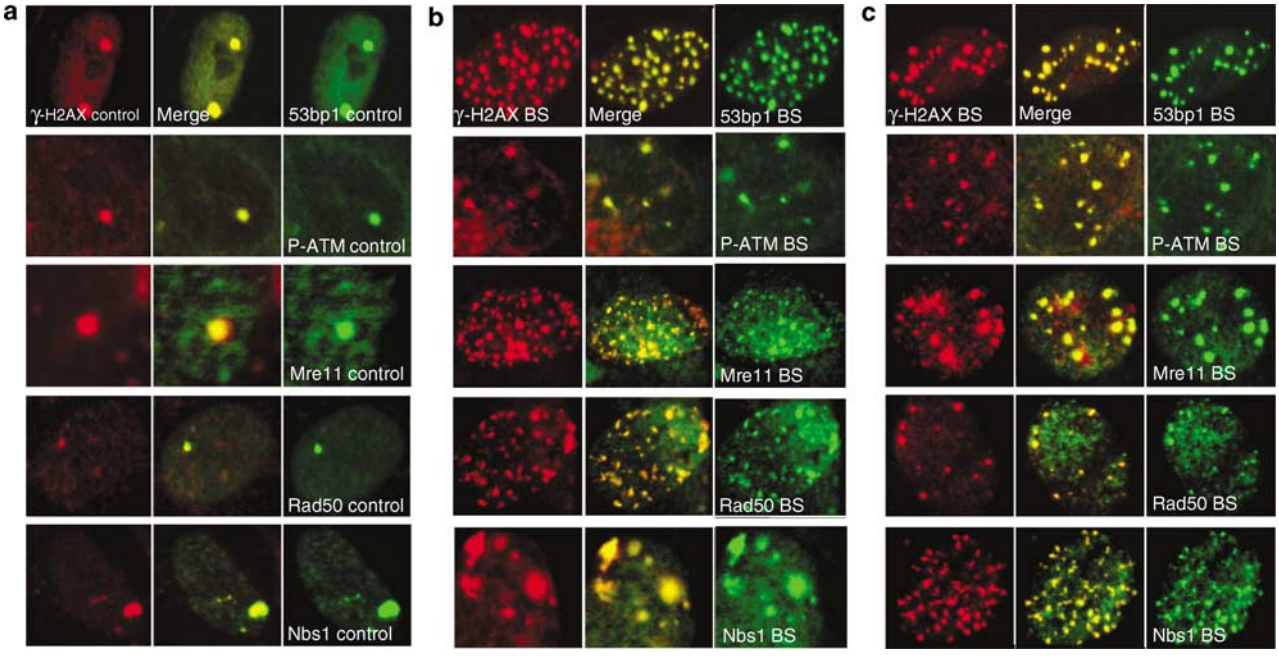

Figure 5 Colocalization of the noted DNA DSB repair proteins with γ-H2AX foci. (**a**) Unirradiated control cells, (**b**) bystander cells (BS) from α-IR experiment, and (**c**) bystander cells from γ-IR experiment

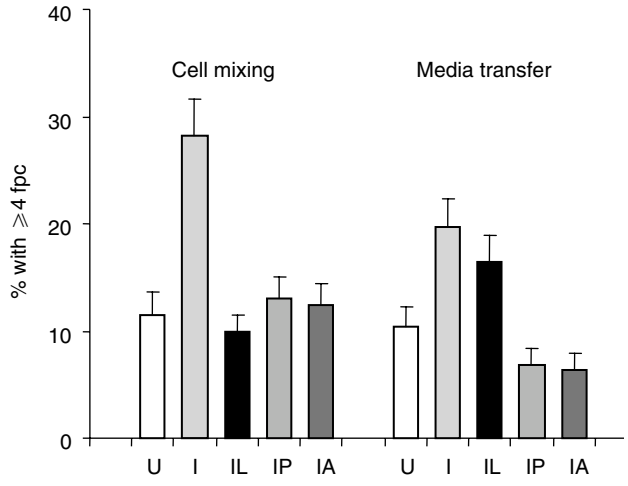

Figure 6 Effect of various inhibitors on the bystander effect. The cell mixing and media transfer protocols were carried out as described in Figure 1. Inhibitors were added 2 h prior to IR of attached cells in cell mixing experiments. Inhibitors were added to the media after removal from the irradiated cultures and before addition to the bystander cultures in media transfer experiments. Bars represent the bystander cells. The target cells were mock (U) or 0.2 Gy irradiated (I). Lindane (IL) appeared to be effective in the cell mixing but not the media transfer protocol. C-PTIO (IP) and AG (IA) appeared to be effective in both protocols

genes occur not only in cells targeted with IR but also in neighboring unirradiated cells. This effect is known as the 'bystander effect'. We have shown here that γ-H2AX foci appear not only in cells targeted with IR but also in unirradiated cells. In addition, as in targeted cells, the γ-H2AX foci in bystander cells are sites of DNA DSB repair protein accumulation. These findings indicate that DNA DSB formation may be a major event in the manifestation of the bystander phenotype. However, DNA DSBs may also be a result of genomic rearrangements induced by factors other than IR. Small numbers of γ-H2AX foci are present in normal human cells in culture during early passages, and the focal incidence increases with cell senescence. A similar phenomenon is observed in mice of different ages (Sedelnikova *et al*., 2004a). In addition, tumor cells in culture have varied numbers of γ-H2AX foci per cell depending on the cell line (MacPhail *et al*., 2003; Banath *et al*., 2004). These foci have no known causal agent, but they may result from a decreased ability to maintain genome integrity as a result of aging, senescence, or oncogenic transformation. Mechanisms behind the formation of these γ-H2AX foci are obscure, but similar processes may be occurring in bystander cells.

If γ-H2AX foci have different origins in targeted and bystander cells, their formation may be expected to follow distinctly different kinetics, as we observed. In numerous studies including this one, γ-H2AX focus formation in targeted cells is maximal at approximately 30 m post-IR and returns to near pre-IR values by 18 h (Rogakou *et al*., 1999; Banath *et al*., 2004). On the other hand, we found that γ-H2AX focus formation in bystander cells is low at 30 min post-IR, elevated by 18 h and remains so at 48 h. Later time points were not examined in this study, but there is precedence for long-term increases in γ-H2AX focus formation occurring during premature senescence induced in normal human fibroblast cultures by exposure to low levels of H_2O_2 and bleomycin (Sedelnikova *et al*., 2004a).

The clonogenic survival of bystander cells after the IR exposure is also distinct from that observed in directly irradiated cells (Mothersill *et al*., 2002). One major distinction is that above a certain threshold, higher IR doses do not induce a greater bystander response so its contribution to clonogenic death becomes smaller with increasing doses (reviewed by Mothersill and Seymour, 2001). We observed a similar effect for γ-H2AX focal incidence as well. With α-particles, two particles per target cell were almost as effective as 20 particles per target cell in inducing γ-H2AX focus formation in the bystander cells at 18 h (Figure 2). With media transfer, 0.2 and 0.6 Gy exposure of the target cells resulted in even greater γ-H2AX foci formation in bystander cells than for 2 Gy (Figure 5). In addition, exposures of the target cultures to higher doses of IR resulted in delayed ability of the transferred media to induce bystander effects. Media harvested from cells exposed to 0.2 Gy were most potent in inducing the bystander effect with a 1 h pretransfer incubation period, and less potent at 2 h and later times. With 0.6 Gy, the greatest potency was observed with a 2 h pretransfer incubation period, and with 2 Gy, the greatest potency was observed at 4 h, decreasing at 8 h. However, even at its peak, media collected from cells exposed to 2 Gy were less potent in inducing γ-H2AX foci in the bystander cultures. The threshold dose and the lack of further increase in the magnitude of the bystander effect suggest that target cells switch to an altered metabolic state in which specific signals are transmitted to other cells either through gap-junctions or through the medium.

Yang *et al*. (2005), using a different methodology, recently reported increases in the incidence of p21-positive cells, of cells containing micronuclei, and of cells with γ-H2AX foci. They also reported that the increased γ-H2AX formation was independent of radiation dose in the range of 0.1–1.0 Gy, and that the increase was apparent at 2 h post-IR. In contrast, we did not observe significant increases in γ-H2AX focus formation at 4 h post-IR. This apparent discrepancy might be explained by the fact that different cell lines and/or different media transfer methodologies were used. However, in general, there is a consistency between their and our observations of γ-H2AX focus formation in bystander cells.

It has been reported that several inhibitors such as lindane, c-PTIO, and AG abolish the bystander effect (Azzam *et al*., 2001, 2003; Shao *et al*., 2003, 2004). Our results extended that finding to γ-H2AX focus formation and suggested the involvement of gap-junction communication and NO-related processes in DNA damage response in the bystander effect. Lindane is an inhibitor of gap-junction information transfer, while c-PTIO and AG affect NO levels. While gap-junctions

could not exist between targeted and bystander cells in the media transfer methodology, the existence of unopposed gap-junction hemichannels has been reported (Ye *et al*., 2003). These hemichannels serve as routes of molecule efflux, and are reportedly inhibited by gap-junction inhibitors with similar affinities to their effects on gap-junction transmission. However, even when lindane was present in the targeted cell culture, it was ineffective, suggesting that molecules are not passing through hemichannels into the medium. Thus, unless lindane inhibits other processes, these results suggest that gap-junction communication serves as the primary mode of bystander signal transmission in cell mixing methodologies, whereas secretion of molecules into the medium serves as the primary mode in media transfer methodologies. Clarification of the complex relationships of these two modes of bystander signal transmission awaits further studies. Likewise, unless c-PTIO and AG affect other processes, the results indicate the involvement of NO in the bystander effect.

Our results show that in bystander populations, the increased γ-H2AX focus formation is limited to a subset of cells. Such a subset is also present in smaller proportions in control cultures. Therefore, our findings suggest that a fraction of the cell population is vulnerable even in control unperturbed cultures, and signals transmitted from irradiated cells increase the vulnerable fraction. Since we utilized proliferating cell cultures for these studies, it is possible that certain phases of the cell cycle are more sensitive to the transmitted bystander signals. Marples *et al*. (2003), have shown that the bystander effect is limited to G2 cell populations. It is known that when cell cultures are irradiated, that portion of the cell population within about 30 min of mitosis does not become arrested and continues through mitosis, in spite of the DNA damage (Highfield and Dewey, 1975). Perhaps the fraction of G2 cells approaching mitosis exhibits greater susceptibility to genotoxic agents and manifests this susceptibility with increased levels of postmitotic γ-H2AX foci and decreased clonogenic survival.

Further studies are needed to elucidate many factors involved in signal generation in the targeted cells, signal transmission between targeted and bystander cells, and the characteristics that make some bystander cells vulnerable to these signals.

Materials and methods

Cells and culture

WI38 normal human lung fibroblasts were purchased from Coriell Cell Repositories (Camden, NJ, USA) and maintained according to recommended protocols. The cells were grown for 3–7 days prior to the experiments.

α-IR studies

In this methodology ('Coculture' in Figure 1a), WI38 cultures were split 1 : 2 and reseeded into two T75 flasks. After overnight growth, the cells in one flask were stained with 5 μM CellTracker CMRA dye (Molecular Probes, Eugene, OR, USA) for 30 min. Cells in the other flask were stained with 1 μM Hoechst 33342 for 30 min, as done routinely for α-coculture studies (Mitchell *et al*., 2004; Ponnaiya *et al*., 2004). After incubation in fresh media for 30 min, both cultures were trypsinized and counted. The two cell batches were mixed together in a 1 : 1 ratio and 500 cells of the mixture were seeded in a 2 μl drop in a 60 mm microbeam dish with a 3.8 μm thick polypropylene film covering a 0.25-in hole drilled in the center of the dishes to create a miniwell. The Columbia University microbeam system was used for irradiation. It allows individual α-particles to be delivered to cells with high reproducibility and accuracy (Randers-Pehrson *et al*., 2001). After cell attachment (up to 4 h), the coordinates of each Hoechst-stained nucleus were automatically recorded using an imaging system for computer-driven irradiation with two or 20 90 keV/μm α-particles per cell. Thus, 50% of plated cells were hit with α-particles. The CMRA dye identified the bystander cells. When double-immunostaining for γ-H2AX and DNA DSB repair proteins was performed, the CMRA dye was not used. The cultures were fixed in 2% paraformaldehyde at 30 min, 18 or 48 h post-IR.

γ-IR studies

In this methodology ('Cell mixing' in Figure 1b), WI38 cells were seeded in Labtek II two-well glass slides (Nalge Nunc International, Naperville, IL, USA) at 7.5×10^4 cells per well and in two T75 flasks at 5×10^6 cells per flask. After overnight growth, 5 μM CMRA was added to one set of multiwell slides and one T75 flask for 30 min according to the company's protocol. The cells were then incubated for 30 min in fresh media. The multiwell slides were either exposed to mock irradiation or 0.2 Gy delivered at a rate of 1.3 Gy/min from a ^{60}Co source at ambient temperature. The T75 cultures were trypsinized and added to the irradiated cultures immediately after IR (stained to unstained and *vice versa*) to a total cell count of 1.5×10^5 per well, $\sim$70% confluency. In all γ-IR studies, only attached cells were irradiated. The mixed cell cultures were incubated for either 18 or 48 h before fixation in 2% paraformaldehyde. In some cases, 50 μM lindane, 20 μM c-PTIO, or 20 μM AG (all from Sigma, St Louis, MO, USA) were added 2 h before irradiation.

Media transfer studies

In this methodology (Figure 1c), WI38 cultures were seeded onto Labtek II two-well glass slides at a density of 3×10^5 cells per well and irradiated with 0.2, 0.6 and 2 Gy at a rate of 0.6 Gy/min from a ^{137}Cs source. At various times after irradiation, the media were removed from the irradiated cultures, filtered through 0.22 μm MILLEX$^{@}$GP filters (Millipore, Bedford, MA, USA), and added to the chamber slides containing the unirradiated bystander cells. The cultures containing the conditioned media were incubated for various times (30 min–48 h). Media transferred from unirradiated cultures and media irradiated in the absence of cells were used as controls, and both showed no bystander effect. In some cases, 50 μM lindane, 20 μM c-PTIO, or 20 μM AG were added to the media transferred from irradiated cultures.

Immunocytochemistry

γ-H2AX foci numbers were assessed by microscopy. Cultures on polypropylene films in microbeam dishes or on Labtek II 2-well slides were processed for immunofluorescent microscopy as previously described (Rogakou *et al*., 1999). For double-labeling, γ-H2AX primary antibody from either rabbit or mouse (Upstate BioTech, Lake Placid, NY, USA) was used

depending on the origin of the other antibodies, anti-Mre11, anti-Rad50, anti-Nbs1, and anti-ATM (phospho S1981) (Novus Biologicals, Inc., Littleton, CO, USA), or anti-53bp1 (generously provided by Dr J Chen, Department of Oncology, Mayo Clinic and Foundation). Following antibody staining, the polypropylene films were transferred from the microbeam dishes onto microscopic slides. After mounting in antifade media, the samples were examined by fluorescent and confocal microscopy with a Nikon PCM 2000 (Nikon Inc., Augusta, GA, USA). The γ-H2AX foci were counted by eye in a blinded fashion in at least 200 randomly chosen cells. Bystander cells were assessed regardless of their distance from irradiated cells. Since WI38 primary human fibroblasts were subcultured during these experiments, the numbers of population doublings changed and correspondingly the background level of γ-H2AX foci varied slightly among experiments (Sedelnikova *et al*., 2004a). Therefore, the incidence of background γ-H2AX foci was counted in each independent experiment.

Threshold determination for scoring affected bystander cells

Preliminary γ-IR cell mixing experiments revealed that the increase in γ-H2AX focal incidence was not uniform in the bystander cell population, but rather limited to a fraction of the cell population (Figure 1d). We found that using a threshold of $\geqslant 4$ γ-H2AX fpc was optimal for determining the fraction of affected bystander cells. This value is high enough to exclude cells with foci numbers following the Poisson distribution. With an average of <1 fpc in primary fibroblast cultures (Sedelnikova *et al*., 2004a), fewer than 0.3% of the cells will contain $\geqslant 4$ fpc by random Poisson distribution. The results were qualitatively similar if thresholds of $\geqslant 1$ or $\geqslant 10$ fpc were used. The error bars signify standard deviations expressed in percents and calculated as $100 \times$ square-root ((number of cells with $\geqslant 4$ foci)/(total number of cells counted)).

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